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UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

Treatment of Experimental Asthma Using a Novel Peptide Inhibitor of the Inducible T Cell Kinase

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

David M. Guimond

Committee in charge:

University of California, San Diego

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Professor Tom Huxford
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2012

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Chair

University of California, San Diego

San Diego State University

2012

DEDICATION

This work is dedicated to my mother whom I look forward to visiting upon the successful completion of my current toils. “It’s all for you...”

EPIGRAPH

"If you can keep your head when all about you are losing theirs,
it's just possible you haven't grasped the situation." Jean Kerr

"I can calculate the motion of heavenly bodies,
but not the madness of people." Sir Isaac Newton

"What we anticipate seldom occurs;
what we least expect generally happens." Benjamin Disraeli

"Minds are like parachutes;
they work best when open." Thomas Dewar

"So it shall be written,
so it shall be done." King Ramses II

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LIST OF ABBREVIATIONS

1°	primary stimulation
2°	secondary stimulation
7-AAD	7-aminoactinomycin D
AA channel	acceptor excitation / acceptor emission channel (acceptor channel)
AC	adenylyl cyclase
ACh	acetylcholine
ACK lysis buffer	ammonium, chloride, potassium lysis buffer
AD	acidic domain (of SLP-76)
AEC	3-amino-9-ethyl-carbazole
AHR	airway hyperresponsiveness
alum	aluminum hydroxide (adjuvant)
AP-1	activator protein 1
APC	antigen presenting cell
APS	ammonium persulfate
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BFA	brefeldin A
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
CBA assay	cytometric bead array assay
CD	cluster of differentiation
Cdc42	cell division control protein 42
CFP	cyan fluorescence protein
CLB	complete lysis buffer
CST beads	cytometer setup and tracking beads
CTL	cytotoxic T lymphocyte
CypA	cyclophilin A
CysLT	cysteinyl leukotrienes
DA channel	donor excitation / acceptor emission channel (FRET channel)
DAG	diacylglycerol
DC	dendritic cell
DD channel	donor excitation / donor emission channel (donor channel)
dH_2O, ddH_2O, dI H_2O	deionized water
DIC	differential interference contrast microscopy

DIUF	deionized, ultrafiltered water
DLN	draining lymph node
DMF	N, N, -dimethyl formamide
DN	double negative (CD4- CD8-) thymocyte
DP	double positive (CD4+ CD8+) thymocyte
E-FRET	FRET efficiency
ECL substrate	enhanced chemiluminescent substrate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISpot	ELISA spot assay
eos	eosinophil
Et-OH	ethanol
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
F_C	corrected FRET
FITC	fluorescein isothiocyanate
Foxp3	forkhead box P3 (transcription factor)
FRET	fluorescence resonance energy transfer
G parameter	Gordon parameter
GDP	guanosine 5'-diphosphate
GEF	guanine nucleotide exchange factor
GRα	glucocorticoid receptor α
G_s	stimulatory G proteins
GTP	guanosine 5'-triphosphate
GαAH	goat anti-armenian hamster antibody
H&E	hematoxylin and eosin stain
HEK cells	human embryonic kidney cell line
HRP	horseradish peroxidase enzyme
HTS	high throughput screening
I	fluorescence intensity
i.n.	intranasal
i.p.	intraperitoneal
IB	immunoblot
ICCS	intracellular cytokine staining
ICS	inhaled corticosteroids
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin

IM	ionomycin
IP	immunoprecipitation
IP_3	inositol triphosphate
IP_4	inositol 1,3,4,5-tetralusphosphate
IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
Itk	inducible T cell kinase
ItpkB	inositol triphosphate 3-kinase B
iTregs	inducible regulatory T cells
KAc	potassium acetate
KCl	potassium chloride
kDa	kilodalton (1 kDa = 1,000 g/mol)
KH_2PO_4	potassium phosphate monobasic
$KHCO_3$	potassium bicarbonate
KO	knockout
LABA	long-acting β_2 agonists
LAT	linker of activated T cells
LB	laemlis buffer
Lck	lymphocyte-specific protein tyrosine kinase
LPS	lipopolysaccharide
LUT	lookup table
MCh	methacholine
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
Mø	macrophage
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
NaN_3	sodium azide
NaOH	sodium hydroxide
NF-κB	nuclear factor-kappa B
NFAT	nuclear factor of activated T cells
NH_4Cl	ammonium chloride
NHS ester	N-hydroxysuccinimide ester
NP-40	Nonidet P-40
OVA	ovalbumin

PAGE	polyacrylamide gel electrophoresis
PAS	protein A sepharose
PB	Pacific Blue
PBS	phosphate buffered saline
PGS	protein G sepharose
PH domain	pleckstrin homology domain
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PKC	protein kinase C
PLC-γ	phospholipase C-γ
pLL	poly-L-lysine
PMA	phorbol 12-myristate 13-acetate
PPXPP	polyproline ligand (of SLP-76)
PRD	proline rich domain (of SLP-76)
PRR	proline rich region
PS	polystyrene
PVDF	polyvinylidene fluoride
pY	phosphotyrosine
R9	polyarginine tag
R9-PQM	RRRRRRRRRPQMPAPQRPQPV
R9-QQA	RRRRRRRRRQQAAVAAQRAMA
R9-QQP	RRRRRRRRRQQPPVPPQRPMA
RBCs	red blood cells
RGB	red, green, blue
RNAse A	ribonuclease A
ROI	region of interest
RαM	rabbit anti-mouse antibody
SABA	short-acting β_2 agonists
SDS	sodium dodecyl sulfate (laurel sulfate)
SEE	staphylococcal enterotoxin E
SH1/2/3 domain	Src homology 1/2/3 domain
SIT	allergen-specific immunotherapy
SLP-76	SH2 domain containing leukocyte protein of 76 kilodaltons
SP	single positive (CD4+ / CD8+) thymocyte
STAT	signal transducer and activator of transcription

T-bet	T-box expressed in T cells (transcription factor)
TBST	Tris buffered saline and Tween 20
TCM	T cell medium
TCR	T cell receptor
TE	Tris EDTA
TEMED	tetramethylethylenediamine
TGF-β	transforming growth factor β
TH domain	Tec homology domain
Th1	type 1 helper T cell
Th2	type 2 helper T cell
TLR	toll-like receptor
TR	Texas red
Treg	regulatory T cell
VCA	verpolin homology, cofilin homology and acidic region (of WASp)
WASp	Wiskott-Aldrich Syndrome protein
WBCs	white blood cells
WT	wild-type
YFP	yellow fluorescence protein
Zap-70	zeta-chain-associated protein kinase 70
β-ME	beta mercapto-ethanol
β_2AR	β_2-adrenergic receptor

LIST OF FIGURES

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I would also like to thank the many lab members that I have had the privilege of working with throughout the years. In particular, I would like to thank Dr. Juris Grasis for providing the solid foundation upon which the current work was built. Particularly relevant now, I am both amused by and value your unconventional advice to view the dissertation defense as an offense rather than a defense. Also, I would like to thank Nicholas Cam and Nupur Hirve for their hands-on contributions to this work. Oftentimes, my days were full with several cages of mice to process within a single day and having this support was critical for the project to move forward.

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Guimond, D. M., Cam, N. R., Hirve, N., Duan, W., Lambris, J.D., Croft, M., & Tsoukas, C.D. (2012). Treatment of Experimental Asthma Using a Novel Peptide Inhibitor of the Inducible T Cell Kinase. Manuscript in preparation.

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Binding and TCR Signaling but Dispensable for Adaptor Protein Interactions. Manuscript accepted for publication in PLoS One.

ABSTRACT OF THE DISSERTATION

Treatment of Experimental Asthma
Using a Novel Peptide Inhibitor of the Inducible T Cell Kinase

by

David M. Guimond

Doctor of Philosophy in Biology

University of California, San Diego, 2012

San Diego State University, 2012

Professor Constantine D. Tsoukas, Chair

As central regulators of asthma pathogenesis, allergen-specific CD4+ T cells are recruited to the lungs and secrete sustained levels of Th2 cytokines upon reencounter with inhaled allergen leading to the development of the

hallmark indicators of asthma including airway hyperresponsiveness, mucus production and the recruitment of eosinophils to the affected lung tissue. Selectively upregulated in Th2 cells, Itk is a member of the Tec familiy of non-receptor tyrosine kinases and is required for Th2 cell development and effector function. Upon stimulation through the TCR, Itk associates with the adaptor protein SLP-76 and is recruited to the immunological synapse as part of a stable signaling complex leading to its subsequent enzymatic activation.

As previously discovered using purified domain fragments, the Itk SH3 domain binds to its specific polyproline ligand represented by the amino acid sequence QQPPVPPQRPMA located on SLP-76 with a reported affinity of 0.77 mM (1). By modifying this sequence with the addition of a polyarginine cell penetration motif, Grasis et al. recently demonstrated that the engineered peptide designated R9-QQP was effective as a competitive inhibitor of Itk activation (2). In the current work, R9-QQP was translated to an OVA-inducible model of experimental asthma. Upon delivery *in vivo*, R9-QQP was demonstrated to specifically inhibit physiologically relevant parameters of airway inflammation and was further characterized as affecting the recruitment of eosinophils to the lung mucosa. Consistent with the role of Itk, Th2 cytokine secretion by lung draining lymph node cells was reduced upon restimulation with OVA *in vitro*.

While current treatments are effective for most patients with clinical asthma, undesirable side effects as well as lack of effectiveness for some

patients underscores the need to develop additional therapeutic strategies that target the immunological basis of allergic disease progression. By blocking an inducible and T cell-specific interaction, this study establishes the importance of the Itk–SLP-76 interaction in an allergic disease model as an attractive therapeutic target for the future development of biologically active small molecules inhibitors.

1. INTRODUCTION

1.1. Asthma Pathogenesis and Treatments

The word asthma was first used in Homer's Iliad and derives from the Greek verb *aazein* meaning to exhale with open mouth or to pant (3). Around 450 B.C., the word asthma adopted a clinical meaning in the writings of Hippocrates. In 1892, asthma was first described in the modern medical literature to refer to symptoms including recurrent episodes of breathlessness, wheezing, chest tightness and coughing (4). Currently, clinical asthma is understood to be a recurrent condition characterized by widespread restriction of the respiratory tract resulting from hyperresponsiveness of smooth muscle surrounding bronchioles and excessive secretion of mucus by goblet cells.

Worldwide, asthma is a chronic condition that affects 300 million persons with the prevalence increasing by 50% each decade. Of this global increase, the largest gains in prevalence are disproportionally attributed to developed countries possibly due to increased exposure to environmental pollutants as well as to the Western diet (5, 6). In the US, asthma affects 35.5 million individuals with 23% requiring emergency room treatment each year and with a mortality rate of 5,000 deaths per year. In addition to the physical symptoms, the direct economic impact resulting from asthma in the US has been estimated to be between $300 - $1,300 per patient per year.

Asthma is a heterogeneous condition classified into atopic, non-atopic and intrinsic types of the disease each with a different etiology (7, 8). Although the exact causes remain unknown, a combination of genetic as well as environmental risk factors are associated with the condition. Mediated by an underlying allergic reaction, atopic asthma is the most common form of the disease and is associated with elevated levels of IgE antibodies directed against household or environmental allergens. Common examples of household allergens include food, animal dander and saliva, molds, and feces from dust mites and cockroaches. Common examples of outdoor allergens include mold spores, pollens, grass and trees. Risk factors for atopic / allergic asthma include mutations in genes affecting all aspects of the immune response from the initial uptake of allergen by antigen presenting cells such as macrophages and dendritic cells at the lung mucosa to the release of inflammatory intermediates by granulocytes such as eosinophils and mast cells during an asthma attack (9). As central regulators of the immune response, genes involved in T helper type 2 (Th2) cell development and effector function have been associated with increased susceptibility to allergic asthma (9). In particular, the inducible T cell kinase (ITK) is required for Th2 cell function and its increased expression has been linked to human allergic rhinitis while its lack of expression by knockout mice leads to an attenuated asthma phenotype (10, 11). In addition to the atopic form of the disease, non-atopic asthma can occur without the involvement of IgE antibodies in

response to irritants including tobacco smoke, cold air, perfumes, exercise and upper respiratory tract infections while intrinsic asthma occurs with symptoms occurring spontaneously in susceptible individuals.

1.1.1. Pathogenesis of Allergic Asthma

Mediated by innate and adaptive cells of the immune system, an allergic response requires an initial sensitization to allergen that is latter triggered upon re-exposure to the same allergen. In a typical allergic reaction, an immune response often occurs at a tissue location that is different from the initial site of exposure to allergen. The development of atopic asthma occurs when an allergen is encountered by specialized antigen presenting cells (APC) of the innate immune system such as a macrophages or dendritic cells which reside at the lung mucosa. Once an allergen is recognized and internalized by an APC, it is subsequently processed into small peptide components that are expressed on the surface of the APC in the context of major histocompatibility complex (MHC) class II molecules. Next, an APC expressing allergen-derived peptide encounters a specialized cell of the adaptive immune system called a CD4+ T lymphocyte. If a CD4+ T cell expresses a T cell receptor (TCR) that is specific for the allergen-derived peptide, it becomes activated and proliferates in response to signals received through its TCR. In response to additional cytokine signaling received from the surrounding milieu, an activated T cell differentiates into a cell capable of secreting the type 2 cytokines IL-4, IL-5 and IL-13 named a T helper type 2 (Th2) cell. Once activated, Th2 cells migrate via the lymphatic circulation to mediastinal lymph nodes that drain the lung tissue. Within the lung draining lymph nodes, Th2 cells recognize B lymphocytes which also present allergen-

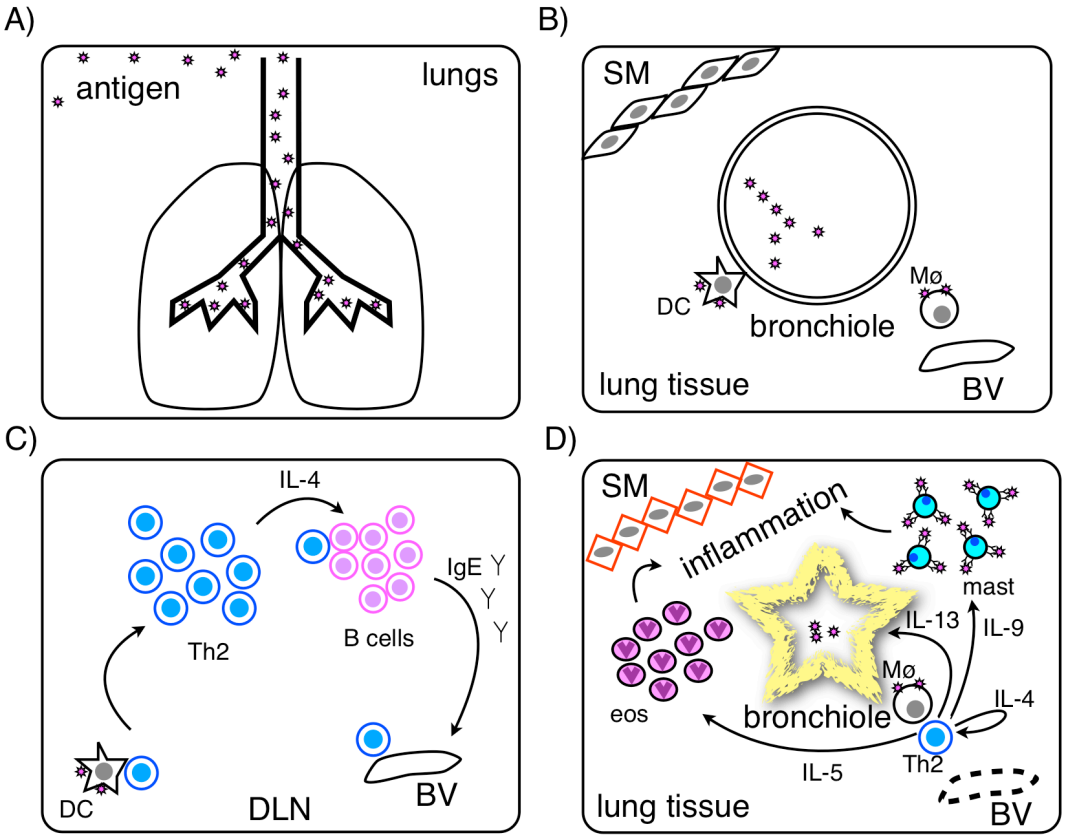
derived peptide in the context of MHC class II molecules. Mediated by secretion of IL-4, a Th2 cell then instructs its cognate B cell to generate and secrete allergen-specific immunoglobulin E (IgE).

IgE produced during the initial sensitization to allergen binds to high affinity FcεRI receptors on the surface of mast cells and dendritic cells which are recruited and maintained by factors secreted by airway epithelial cells (12). During the early allergic response occurring between 1-30 minutes, binding of inhaled allergen by IgE on the surface of mast cells results in cross-linking of FcεRI receptors followed by the immediate release of granules containing preformed histamine (13). Histamine as well as leukotrienes and prostaglandins which are synthesized by mast cells following activation are effectors of the broncho-hyperresponsiveness that is characteristic of an asthmatic attack. During the late allergic response occurring between 6-72 hours, activated mast cells recruit inflammatory cells including macrophages, eosinophils and basophils to the site of allergen exposure mediated by secretion of inflammatory chemokines and cytokines (13). In particular, secreted cytokines induce expression of the adhesion molecules ICAM1 and VCAM1 on the luminal surface of blood vessel endothelial cells leading to recruitment of eosinophils which express VLA4 (14). Although their exact role in asthma pathogenesis is unclear, eosinophils are activated by IL-5 to release granules containing tissue-damaging enzymes and are correlated with disease severity (15-17). In addition, activated mast cells secrete the

chemokines CCL17 and CCL22 that serve to recruit Th2 cells to the site of allergen exposure mediated by binding to chemokine receptor 4 (CCR4). Once recruited to the site of allergen exposure, Th2 cells secrete sustained levels of IL-4, IL-5 and IL-13. The local secretion of IL-13 results in amplification and progression of the immune response including goblet cell hyperplasia, mucus production, bronchiole smooth muscle hyper-reactivity and epithelial wall remodeling. Furthermore, allergen bound by IgE on the surface of mast cells amplifies the immune response through Fcε receptor mediated internalization, antigen presentation and activation of additional T cell clones.

Figure 1: Schematic diagram illustrates sensitization and challenge phases of asthma pathogenesis.

A) First, inhaled antigen enters the lower respiratory tract. B) Next, dendritic cells residing at the lung mucosa take up allergen and migrate to the draining lymph nodes (DLN). C) In the DLN, dendritic cells present allergen-derived peptide to naive CD4+ T cells, which proliferate and differentiation into Th2 effector cells. Mediated by the secretion of IL-4, Th2 effector cells induce cognate B cells to secrete IgE, which circulates in the blood and is captured on the surface of mast cells. D) Upon subsequent challenge, inhaled allergen binds to IgE captured on the surface of mast cells and, mediated by Fc receptor cross-linking, induces the release of preformed inflammatory intermediates, which results in airway hyperresponsiveness and mucus production. In addition, allergen-specific Th2 cells are recruited to the lung tissue and, upon restimulation by macrophages, secrete Th2 cytokines, which sustain the inflammatory response and result in the recruitment and activation of eosinophils.



key:

DC	= dendritic cell
Mø	= macrophage
BV	= blood vessel
SM	= smooth muscle
DLN	= draining lymph node
Th2	= helper T cell type 2
eos	= eosinophil
mast	= mast cell

1.1.2. Immunological Tolerance

Immunological tolerance refers to the development of a protective as opposed to a pathological response to allergens (18, 19). For example, while the development of asthma occurs in susceptible individuals when exposed to environmental allergens, healthy individuals are asymptomatic when exposed to the same levels of allergens (20). Although the mechanism of tolerance to allergens is not completely understood, regulatory cells of the adaptive immune system such as inducible regulatory T cells (iTregs) play an important role. iTregs are antigen-specific and secrete cytokines such as IL-10 and transforming growth factor β (TGF-β) which suppress inflammatory Th2 cells. Development of iTregs occurs in the periphery and requires antigen presentation and co-stimulation provided by dendritic cells along with IL-10- and TGF-β -mediated signaling (21, 22). Consistent with their protective role, Treg function and the levels of the inhibitory cytokine IL-10 are reduced in individuals with asthma (23, 24).

Epidemiological studies have associated an increase in the prevalence of asthma in developed countries with a reduction in the exposure to infectious diseases due to the widespread use of vaccines and antibiotics (25-27). Rephrased, the hygiene hypothesis suggests that the immune response to pathogens encountered during childhood protects against the development of asthma. In support of this concept, several investigators have

suggested that bacterial endotoxin as encountered in manure from farm animals may be important for this protective effect (28).

One mechanism that may explain these observations is that toll-like receptor 4 (TLR4) expressed on dendritic cells in the lungs recognizes inhaled endotoxin and results in the expression of the inducible co-stimulatory molecule (ICOS) which is required for iTreg development. Perhaps the hygiene hypothesis can be explained mechanistically as a lack of TLR-mediated signaling in the absence of endotoxin exposure resulting in anergy as opposed to iTreg development and suppression of a Th2 response to allergen.

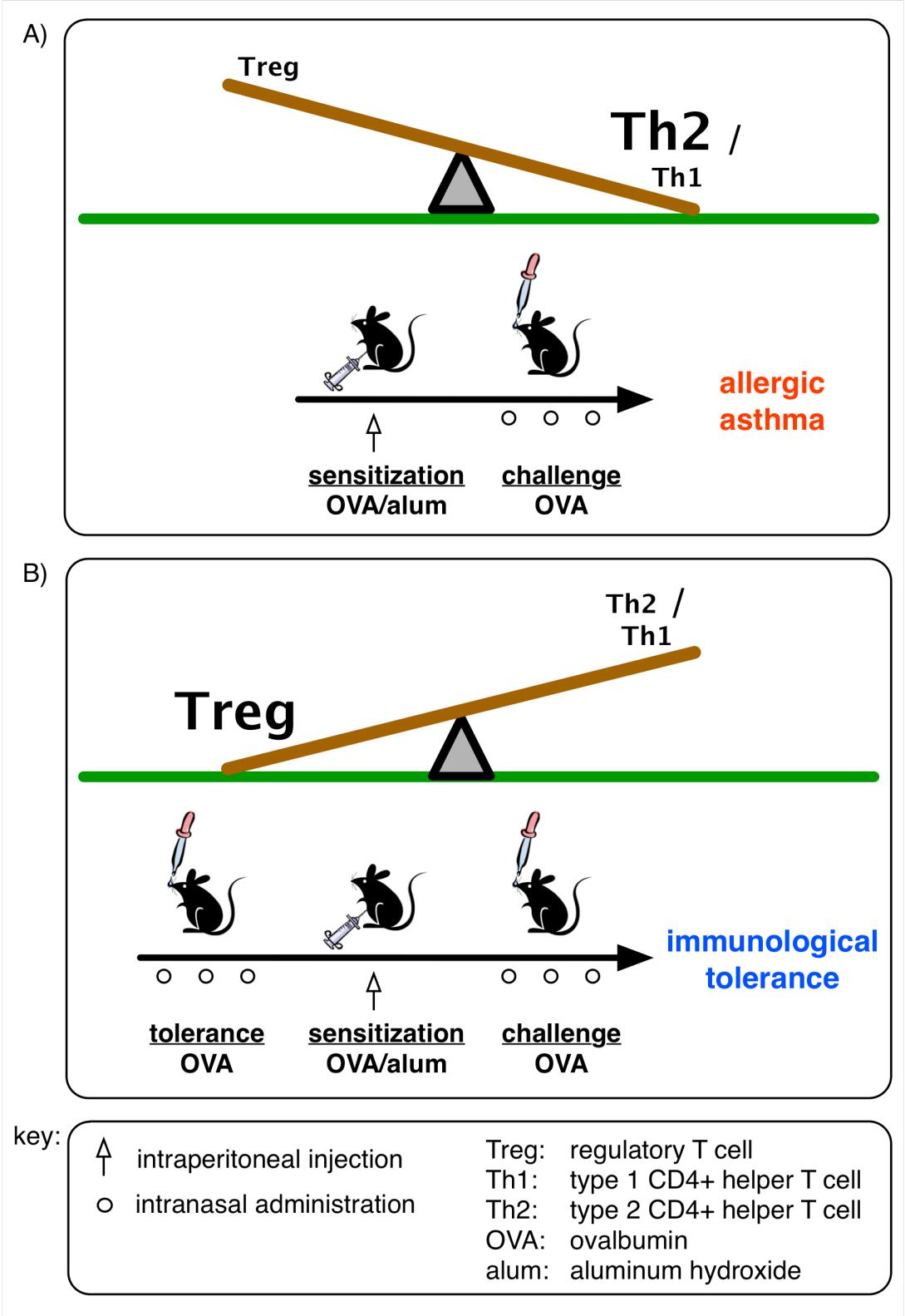
Furthermore, inhaled allergens induce dendritic cells in the lungs to express indoleamine 2,3-dioxygenase (IDO) which metabolizes tryptophan leading to secretion of TGF-β (21). Allergen-induced secretion of TGF-β then results in reduced secretion of the pro-inflammatory cytokine IL-6. Thus, allergen exposure in this pathway promotes iTreg development mediated by inhibition of IL-6.

In a different mechanism of tolerance referred to as immune deviation, non-allergic individuals generate allergen-specific IgG antibodies rather than the IgE isotype that is involved in asthma pathogenesis. While the antibody isotypes produced are different, studies have indicated that the antigenic epitopes and therefore allergen immunogenicity is the same for both healthy and asthmatic individuals that are exposed to the same allergen (29, 30).

Rather than being influenced by the allergen itself, the generation of an IgG versus IgE antibody response appears to depend more on the cytokine milieu that is present during the development of a common progenitor T cell to become a Th1, Treg or Th2 effector, respectively. A developmental fate resulting in the generation of Th1 or Treg effectors leads to the production of non-pathogenic IgG antibodies while the generation of Th2 effectors leads to the production of pathogenic IgE antibodies mediated by B cell helper function.

Figure 2: Schematic diagram of allergic asthma versus immunological tolerance.

A) Imbalance of pathogenic Th2 effector cells leads to development of allergic asthma. B) Increased production of antigen-specific Tregs leads to protective tolerance.


A)
Treg
Th2 /
Th1
allergic
asthma
sensitization
OVA/alum
challenge
OVA
B)
Th2 /
Th1
Treg
immunological
tolerance
tolerance
OVA
sensitization
OVA/alum
challenge
OVA
key:
intraperitoneal injection
intranasal administration
Treg: regulatory T cell
Th1: type 1 CD4+ helper T cell
Th2: type 2 CD4+ helper T cell
OVA: ovalbumin
alum: aluminum hydroxide

1.1.3. Clinical Treatments

Corticosteroids:

Inhaled corticosteroids (ICS) are the most common form of treatment for asthma and affect gene expression through simultaneous trans-activation and trans-repression of target genes resulting in immunosuppression. Corticosteroids diffuse through the plasma membrane and subsequently bind to and activate glucocorticoid receptor α (GRα) in the cytoplasm resulting in receptor acetylation. Activated GRα molecules then homodimerize and translocate to the nucleus. One pathway leading to immunosuppression is mediated by binding of GRα homodimers to glucocorticoid response elements (GRE) located in the cis-regulatory region of several anti-inflammatory genes. Once bound to DNA, GRα associates with transcriptional co-activator molecules such as cAMP responsive element binding protein (CBP) which acetylate histones leading to expression of responsive genes.

For example, MAP kinase phosphatase 1 (MKP-1) is an anti-inflammatory gene that is upregulated in response to corticosteroid treatment. Secretion of IL-4, IL-5 and IL-13 by activated Th2 cells requires phosphorylation of the transcription factor GATA3 by p38 MAP kinase (MAPK). Phosphorylated GATA3 then translocates to the nucleus mediated by importin-α where in binds to DNA and regulates trans-activation of Th2 genes. MKP-1 inhibits trans-activation of Th2 genes by dephosphorylating GATA-3 thus preventing its nuclear import (31). Furthermore, GRα competes

with phosphorylated GATA3 for binding to importin-α thus further reducing its access to the nucleus (31). However, side effects of ICS treatment have been attributed to harmful GRα-mediated repression of beneficial genes. One such example is the GR-α mediated repression of osteocalcin which is involved in bone synthesis.

Another pathway leading to immunosuppression is mediated by binding of GRα to co-activator molecules located in the cis-regulatory region of genes regulated by the pro-inflammatory transcription factors NF-κB and activator protein 1 (AP-1). In this pathway, GRα recruits histone deacetylases such as HDAC2 to the co-activator molecule CBP leading to histone deacetylation and trans-repression of many pro-inflammatory genes such as those coding for cytokines, chemokines, adhesion molecules and inflammatory enzymes.

While corticosteroids are widely effective, patients with severe asthma and people who smoke do not respond well to ICS and some individuals are completely resistant to treatment (32, 33). Corticosteroid insensitivity has been explained biochemically by ubiquitination and degradation of HDAC2 due to oxidative and/or nitrative stress thus preventing its negative regulatory role mediated by GRα (34-38). Other reports have associated corticosteroid insensitivity with phosphorylation of GRα by p38 MAPK and JNK leading to impaired nuclear localization and gene regulatory activity (39, 40).

β_2-Adrenoceptor Agonists:

Short- and long-acting β_2 agonists (SABA, LABA) activate the β_2-adrenergic receptor (β_2AR) which is expressed on smooth muscle cells and triggers a signal transduction cascade leading to bronchodilation and thus opening of the blocked airways of asthma sufferers. Activation of β_2AR results in stimulatory G protein (G_s) -mediated activation of adenylyl cyclase (AC) which results in production of cyclic AMP (cAMP). cAMP then activates Protein Kinase A (PKA) which activates myosin light chain phosphatase. Myosin light chain phosphatase then inhibits myosin light chain kinase resulting in muscle relaxation. In another pathway, activated β_2AR mediates opening of calcium-activated potassium channels resulting in reduced intracellular calcium, inhibition of myosin light chain kinase and bronchodilation. In addition to affecting bronchodilation, cAMP activates exchange protein activated by cAMP (EPAC) which results in decreased smooth muscle cell proliferation (41).

Besides the beneficial effects of β_2 agonists, β_2AR signaling is also associated with bronchoconstriction and activation of inflammatory genes. When coupled with heterotrimeric G_q proteins, activation of β_2AR mediated by acetylcholine (ACh) and histamine leads to activation of phospholipase Cβ1 (PLCβ1) that results in bronchoconstriction (42). Furthermore, phosphorylated β_2AR is down regulated after activation into clathrin-coated pits where it can associate with β-arrestin-2. Association with β-arrestin-2 can lead to ubiquitination of β_2AR followed by receptor degradation (43).

Alternatively, β-arrestin-2 can serve as an adaptor allowing β_2AR to activate MAPK and PI3K signaling pathways leading to activation of inflammatory genes (44, 45).

Anti-Cholinergics:

Anti-cholinergics are antagonists of muscarinic receptors expressed on smooth muscle and other airway cells. Acetylcholine (ACh) released from nerve, epithelial and inflammatory cells binds to G_q-coupled muscarinic receptors resulting in activation of phospholipase C (PLC). PLC then hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5 triphosphate (IP_3) which results in calcium (Ca^{2+}) release from intracellular stores leading to muscle contraction, mucus secretion and structural remodeling (46). Anti-cholinergics are less effective bronchodilators as compared to β2AR agonists and the two drugs combined have been reported to have a synergistic effect.

Leukotriene Modifiers:

Cysteinyl leukotrienes (CysLT) released by mast cells and eosinophils during active asthma bind to the CysLT1 receptor resulting in bronchoconstriction, vascular permeability and mucus production (47). CysLT1 receptor antagonists as well as inhibitors of enzymes involved in CysLT biosynthesis have been developed that are safe and available orally, however, they are less effective than inhaled corticosteroids.

Methylxanthines:

Theophylline is a common and inexpensive xanthine that is chemically related to caffeine and which has been used in the treatment of asthma since 1930. At least three different biochemical pathways are likely involved in the mechanism of action of this class of drugs, however, low efficacy and serious side effects limit their therapeutic usefulness. Mediated by inhibition of cAMP phosphodiesterases (PDE), theophylline increases intracellular cAMP concentration resulting in bronchodilation in a pathway shared with β_2AR agonists. However, side effects related to PDE inhibition include nausea, diarrhea and headaches. Theophylline also functions as an adenosine receptor antagonist resulting in reduced release of inflammatory intermediates by mast cells (48). However, side effects from adenosine receptor antagonism include cardiac arrhythmias and seizures. In a different pathway, theophylline inhibits phosphoinositide 3-kinase (PI3K) which is activated by oxidative stress resulting in degradation of histone deacetylase 2 (HDAC2) (37, 49-51). Inhibition of PI3K by theophylline cooperates with the pharmacological role of corticosteroids in recruiting HDAC to co-repressors resulting in trans-repression of inflammatory genes.

Primary Prophylaxis:

Avoidance of allergens as a means to prevent sensitization early in life has provided contrasting results likely due to the development of inflammation or tolerance as possible outcomes resulting from allergen exposure (52). For example, studies have demonstrated that exposure to house dust mites correlates with the extent of allergic sensitization while reduced exposure to household pets as well as peanuts increases rather than decreases sensitization. In addition, limiting exposure to allergens for previously sensitized individuals is likely undermined by non-allergenic factors that contribute to ongoing disease. For example, a large controlled study involving asthma patients showed no beneficial effect of using impermeable bed covers as means to reduce exposure to house dust mites (53, 54).

Allergen-Specific Immunotherapy (SIT):

Allergen-specific immunotherapy modifies the immune response by repeated subcutaneous or sublingual sensitization to allergens. In asthma patients that are predisposed to generating a Th2 type response to inhaled allergens, SIT redirects the immune response towards the generation of allergen-specific Th1 cells and/or Tregs. The generation of allergen-specific Th1 cells results in immune deviation mediated by production of non-pathogenic IgG and/or IgA blocking antibodies rather than the pathogenic IgE isotype while the generation of Tregs results in tolerance mediated by suppression of Th2 cell effector function (55). In responsive patients, SIT

decreases recruitment of eosinophils and mast cells to the lungs and reduces release of inflammatory mediators leading to an improvement of asthma symptoms. However, low efficacy and the possibility of anaphylactic side effects limit the usefulness of treatment (56).

Anti-IgE Therapeutic Antibody:

Allergen-specific IgE plays an important role in asthma pathogenesis where it functions to cross-link Fc receptors on the surface of mast cells leading to the immediate release of inflammatory mediators. Omalizumab is a humanized IgG1 blocking antibody that targets free IgE thus reducing Fc receptor mediated release of inflammatory mediators by mast cells (57). While effective at controlling asthma symptoms, up to 16 weeks of subcutaneous administration 2-4 times per week is required prior to obtaining clinical benefits. For this reason, omalizumab is used in combination with standard corticosteroid treatment.

Mast Cell Inhibitors:

Mast cells have and continue to be an attractive target in the treatment of allergic asthma owing to their role in both the early release of inflammatory intermediates as well as in the recruitment of inflammatory cells occurring later. The inhaled drugs sodium cromoglicate (SCG) and nedocromil sodium reduce mast cell activation by inhibiting chloride ion flux across the cell membrane thus increasing the threshold required for cell activation (58). Other therapeutics reduce mast cell activation by inhibiting tyrosine kinases

which transduce signaling downstream of the FcεRI and KIT receptors or, conversely, by activating phosphatases which negatively regulate receptor-mediated pathways (59, 60).

Cytokine Immunotherapy:

Cytokine immunotherapy is a treatment approach that attempts to antagonize the inflammatory effects of Th2 cytokines such as IL-4, IL-5 and IL-13 which are elevated in asthmatic patients and are involved in generating and sustaining lung inflammation (52). One approach involves directly blocking Th2 cytokines by inhalation of exogenously produced anti-cytokine monoclonal antibodies or genetically modified soluble cytokine receptors. Alternatively, investigators have attempted studies in mice that involve using vaccines directed against specific Th2 cytokines in order to generate endogenously produced blocking antibodies. Another approach attempts to block cytokine signaling by inhalation of monoclonal antibodies that specifically target the cytokine receptor or by administration of genetically modified dominant negative Th2 cytokines that bind to their target receptor but do not allow downstream signal transduction. Yet another approach attempts to suppress Th2 cytokines by targeting the transcription factors STAT6 and GATA3 which are required for cytokine expression by using dominant negative peptides or RNA interference. In order to achieve a response similar to the goal of allergen-specific immunotherapy (SIT), other investigators have attempted to redirect the immune response by systemic administration of

recombinant Th1 cytokines such as IFN-γ and IL-12. Also, administration of IL-10 which is produced by Tregs has been utilized as a means of suppressing Th2 cells. Clinical trials employing multiple cytokine therapeutic strategies have demonstrated some success and good safety performance, however, low efficacy has limited the usefulness thus far while active research is underway aimed at further development.

1.2. CD4+ Th2 Cells and Experimental Asthma

Several mouse models of asthma exist that reflect the clinical symptoms of human asthma patients including airway hyperresponsiveness (AHR), mucus production, recruitment of eosinophils, elevated IgE and the secretion of Th2 cytokines by effector cells. In particular, the abundant eosinophilia observed in mouse models also occurs in humans and correlates with asthma (61). An extensively studied mouse model employs the immunogenic allergen ovalbumin (OVA) which is a protein derived from chicken egg white. Following adsorption to the adjuvant alum and delivery i.p., CD4+ T cells are generated in the context of a primary immune response to OVA. An inflammatory response that is characteristic of asthma then results when mice are challenged with OVA that is delivered locally to the lungs. While similar in many respects to human asthma, one difference between the murine OVA model commonly used to investigate lung inflammation and human disease is in the involvement of mast cells. Although peribronchial mast cells play a well described role in human asthma patients, few mast cells are detected in the acute mouse model (62). Nevertheless, accumulation of mast cells can be induced in a chronic model that requires repeated exposure to inhaled OVA for several months (62). As discussed in the examples below, many discoveries have been made into the mechanisms of asthma pathogenesis employing the OVA-driven model used in

combination with methods designed to elucidate the role of specific cell types and signaling molecules.

T lymphocytes play a central role in the asthma pathogenesis as demonstrated by the finding that eosinophilia and AHR were prevented upon the specific depletion of CD4+ T cells *in vivo* (63). In support of this finding, OVA-specific Th2 effector cells that were generated *in vitro* and adoptively transferred into unimmunized recipient mice were capable of inducing AHR, mucus production and eosinophilia upon challenge with inhaled OVA (64, 65). Furthermore, IL-4 secretion is required for the recruitment of lymphocytes and eosinophils to the lungs of recipient mice as demonstrated by the adoptive transfer of $IL4^{-/-}$ CD4+ T cells (64-66). However, AHR may occur independently of IL-4 secretion (65). Likewise, mucus production may occur independently of both IL-4 and IL-5 secretion and is instead dependent on IL-4/IL-13 receptor-mediated signaling (66). In another study, eosinophil recruitment and AHR were abolished when asthma was induced in $IL\text{-}5^{-/-}$ mice (67). The subsequent reconstitution of both eosinophilia and AHR upon delivery of recombinant IL-5 to the lungs thus establishes this cytokine as an important mediator of asthma pathogenesis (67).

1.2.1. OX40

OX40 (CD134) is a member of the TNF superfamily of receptors and is inducibly expressed 12-48 hours following antigen-dependent activation of naive T cells. Likewise, OX40 ligand (OX40L) is inducibly expressed by antigen presenting cells such as dendritic cells, B cells and macrophages following toll-like receptor 4 (TLR4) -dependent signaling in response to LPS/ endotoxin. OX40 expressed by CD4+ Th1/2 effector cells functions as a co-stimulatory molecule leading to survival, clonal expansion and memory T cell development after interaction with an antigen presenting cell expressing OX40 ligand (OX40L). The role of OX40 as a co-stimulatory molecule has been demonstrated both in OX40 knockout mice as well as by treatment with anti-OX40L blocking antibody where lung inflammation in response to ovalbumin was reduced in the absence or inhibition of OX40 signaling, respectively (68-70). Specifically, OX40 signaling is required for the clonal expansion and survival of CD4+ T cells during a primary immune response and results in the generation of memory T cells which are activated during a recall response to antigen (70).

In contrast to its co-stimulatory role, OX40 also functions as a suppressor of CD25+ CD4+ regulatory T cell (Treg) development in a pathway that involves downstream inhibition of the transcription factor Foxp3 (71). As discussed in the pathogenesis of asthma above, one mechanism of immunological tolerance involves the generation Tregs that suppress Th2

effector cells mediated by secretion of the inhibitory cytokines IL-10 and TGF-β. In a tolerance model of experimental asthma, ovalbumin is delivered intransally prior to sensitization i.p. with OVA/alum resulting in enhanced Treg development and thus protection from asthma upon i.n. challenge with OVA (22).

However, inhalation of LPS in addition to OVA during the tolerance period results in suppressed Treg development combined with enhanced development of inflammatory Th2 effector cells and thus susceptibility to asthma (72). One mechanism to account for the observed disruption of tolerance is that inhaled LPS is recognized by TLR4 resulting in the inducible expression of OX40L by antigen presenting cells. Next, interaction of OX40L and OX40 expressed by Tregs results downstream in inhibition of Foxp3 and thus suppression of Treg development. In support of this model, treatment of mice with anti-OX40L blocking antibody during the induction phase of tolerance reverses the suppression of Treg development caused by inhalation of LPS thus indicating the requirement for OX40 signaling. To further elucidate the mechanism of inhibition, impaired Treg development of mice treated with an agonist anti-OX40 antibody and LPS delivered i.p. during the induction of tolerance was partially restored by i.p. delivery of anti-IL-6 receptor blocking antibody and was fully restored when combined with i.p. delivery of anti-IL-4 and anti-IFN-γ blocking antibodies. Therefore, IL-6 produced by activated APCs and Th1/2 cytokines secreted by activated T cells

together with OX40 signaling mediates the inhibitory effect on Treg development. Thus, with regard to its co-stimulatory role in Th2 effector cell activation combined with its inhibitory role in Treg development, OX40 is an attractive therapeutic target for the treatment of allergic asthma.

1.2.2. Itk

The inducible T cell kinase (Itk) is a member of the Tec family of non-receptor protein tyrosine kinases that is expressed by mast cells, natural killer (NK) and T cells. As demonstrated in studies using knockout mice, protective Th2 responses to extracellular parasites are impaired leading to increased susceptibility to infection by the protozoa *Leishmania major* and the nematode *Nippostrongylus brasiliensis* (73, 74). Likewise, Itk$^{-/-}$ mice displayed reduced lung inflammation upon exposure to eggs from the helminth *Schistosoma mansoni* (75). Upon challenge with OVA in the experimental asthma model, Itk$^{-/-}$ mice exhibited reduced lung inflammation as compared to WT control mice including reduced AHR, mucus production and recruitment of inflammatory eosinophils and T cells to the lungs (11, 76). Consistent with the reduced asthma phenotype, T cells purified from the spleens of Itk$^{-/-}$ mice displayed reduced proliferation after restimulation with OVA *in vitro* (11). In addition, production of Th2 cytokines was reduced upon restimulation of T cells purified from spleens and DLN of Itk$^{-/-}$ mice (11). Furthermore, defective airway inflammation of Itk$^{-/-}$ mice as measured by AHR was reconstituted by adoptive transfer of purified OVA-specific CD4+ T cells indicating that the observed defects were intrinsic to CD4+ T cells (76). Despite having a reduced asthma phenotype, however, serum from Itk$^{-/-}$ mice had elevated and unaffected levels of total and OVA-specific IgE, respectively (11). In a follow up study, this phenomenon was linked to a unique role for Itk in the negative

regulation γδ NKT cells mediated by the transcription factor PLZF (77). In the absence of Itk, increased numbers of γδ T cells were generated that were capable of producing Th2 cytokines and inducing immunoglobulin class switching of B cells to IgE upon activation (77).

1.3. Itk in T Cell Activation and Development

1.3.1. T Cell Activation

Itk is enzymatically activated downstream of the TCR resulting in phosphorylation of PLC-γ1 followed by the generation of second messengers that regulate T cell function and development (78-80). First, a CD4+ T cell expressing an antigen-specific T cell receptor (TCR) recognizes an APC expressing peptide derived from antigen in the context of major histocompatibility class II molecules (MHC). Upon TCR and CD28 co-receptor engagement, the CD4-associated Src kinase Lck becomes activated and phosphorylates the immunoreceptor tyrosin-based activation motifs (ITAMs) located within the intracytoplasmic domains of the TCR/CD3 complex. Next, the Syk kinase Zap-70 is recruited to the phosphorylated ITAMs where it becomes activated and in turn phosphorylates the adaptor molecules LAT and SLP-76 which together form a stable signaling platform as part of the immunological synapse. LAT is constitutively localized within lipid rafts of the plasma membrane whereas SLP-76 is located in the cytoplasm and is inducibly recruited to LAT upon phosphorylation via the adaptor protein Gads. Also occurring following TCR / CD28 receptor engagement, Lck activates the kinase PI3K which converts the membrane phospholipid PIP_2 to PIP_3. Itk is then recruited to the immunological synapse mediated by interaction of its pleckstrin homology (PH) domain with membrane PIP_3 as well as by interaction of its Src homology 2 and 3 (SH2 and SH3) domains with

phosphorylated SLP-76 (81-83). Specifically, the SH2 and SH3 domains of Itk bind to the phosphotyrosine motif and proline rich region (PRR) of SLP-76, respectively. Once localized properly, Itk is phosphorylated in trans by Lck at tyrosine reside 511 of its Src homology 1 (SH1) domain followed immediately by autophosphorylation in cis at tyrosine reside 180 of its SH3 domain (84-86).

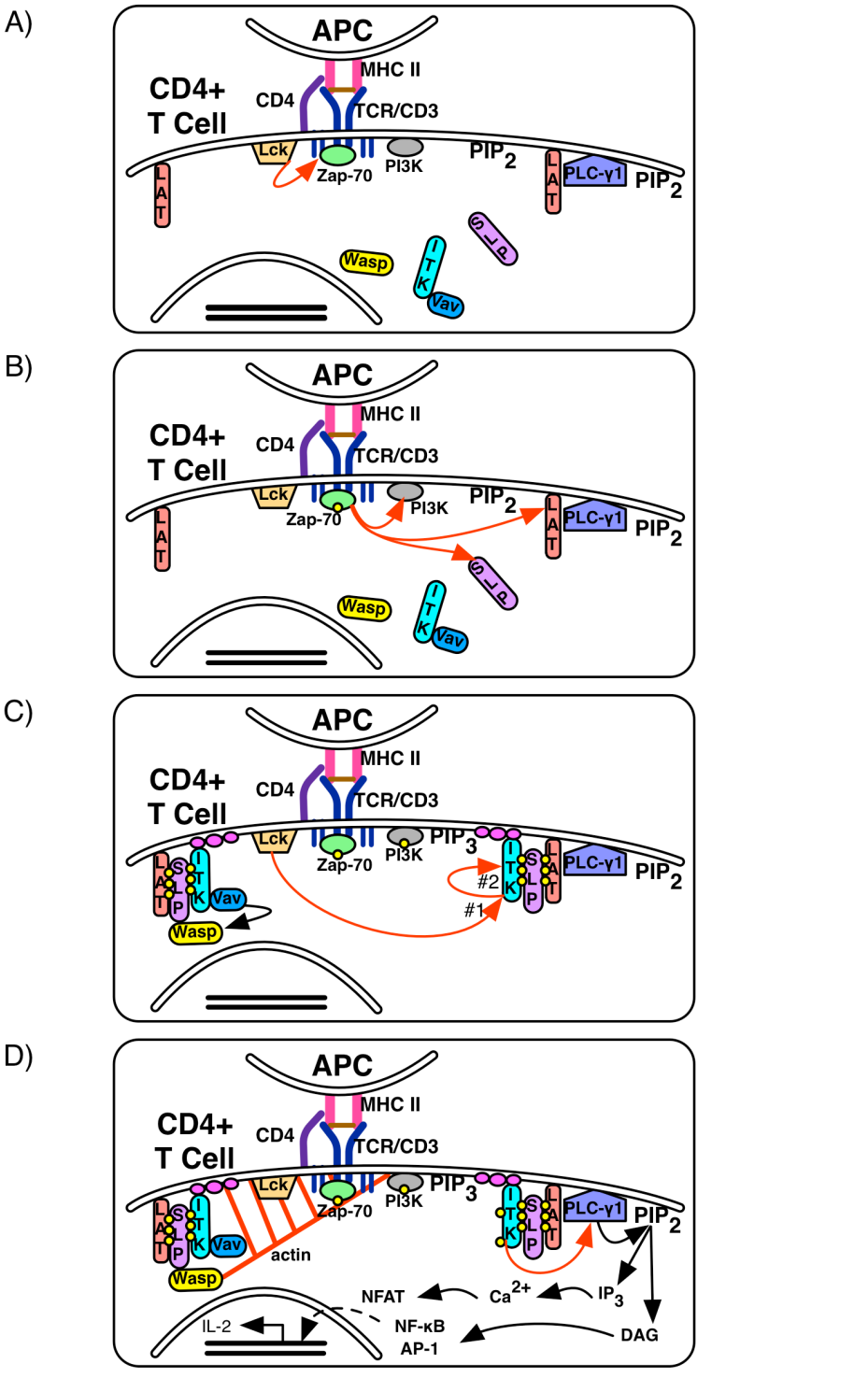
In fulfillment of its enzymatic role, activated Itk phosphorylates phospholipase C-γ1 (PLC-γ1) on tyrosine reside 783 (87, 88). Next, activated PLC-γ1 hydrolyzes membrane PIP_2 to generate the soluble second messengers IP_3 and DAG. In a recently discovered positive feedback loop, IP_4 which is generated by phosphorylation of soluble IP_3 by inositol triphosphate 3-kinase B (ItpkB) binds to the PH domain of Itk and enhances membrane recruitment (89). Following second messenger generation, IP_3 binds to IP_3 receptors on the membrane of the endoplasmic reticulum (ER) leading to intracellular Ca^{2+} release and activation of the transcription factor NFAT while DAG activates protein kinase C (PKC) which leads to activation of the transcription factors NF-κB and AP-1.

In addition to its canonical enzymatic role, Itk also serves as an adaptor protein as part of a signal transduction cascade downstream of the TCR that supports the nucleation of actin filaments mediated by the Wiskott-Aldrich syndrome protein (WASP) (90-94). Independently of Itk, WASP is incucibly recruited to SLP-76 via the adaptor protein Nck (90). In order to become

activated, WASP binds to GTP-bound Cdc42 via its GTPase binding domain (GBD) and undergoes a conformational change that exposes its C-terminal VCA domain (95, 96). Next, the actin nucleating protein Arp2/3 binds to the VCA domain of WASP leading to branching and polymerization of filamentous actin. Local activation of WASP is supported by Itk through its constitutive association with the guanine nucleotide exchange factor Vav (92). As Cdc42 is rapidly inactivated by hydrolysis of GTP, Vav serves to exchange GDP for GTP thus activating Cdc42. WASP- and Itk-dependent rearrangement of the actin cytoskeleton is believed to be important functionally for the formation of stable T cell-APC conjugates and immunological synapse formation resulting in sustained signaling and directed cytokine secretion.

Figure 3: Schematic diagram illustrates the early signaling events leading to T cell activation.

A) First, an antigen-specific T cell receptor (TCR) engages foreign peptide presented in the context of major histocompatibility (MHC) class II molecules expressed by a specialized antigen presenting cell (APC). Upon TCR-mediated activation, the CD4-associated Src kinase Lck phosphorylates the intracytoplasmic domains of the TCR/CD3 complex followed by recruitment and activation of the Syk kinase Zap-70. B) Next, Zap-70 phosphorylates the membrane-associated phospholipid kinase PI3K as well as the adaptor molecules SLP-76 and LAT, which are located in the cytoplasm and within lipid rafts of the plasma membrane, respectively. C) Upon phosphorylation, SLP-76 is inducibly recruited to LAT via the adaptor molecule GADS. In addition, the non-receptor Tec kinase Itk is inducibly recruited to SLP-76 via its SH3 domain. Once assembled into the LAT-nucleated signaling complex, Itk is first phosphorylated in trans by Lck followed by a phosphorylation event in cis leading to full kinase activity. In its separate role as an adaptor for Vav, Itk promotes localized activation of the actin nucleating protein WASp via activation of the GTPase Cdc42 (not shown). D) Next, Itk phosphorylates tyrosine residue 783 on the phospholipase PLC-γ1, which hydrolyzes PIP_2 in the plasma membrane to generate the second messengers IP_3 and DAG. Further downstream, IP_3 activates the calcium-dependent transcription factor NFAT while DAG activates NF-κB and AP-1. After nuclear translocation, binding of transcription factors to the cis regulatory regions of responsive genes leads to expression of inflammatory genes such as IL-2, which leads to proliferation of activated T cell clones. In addition, activation of WASp results in actin polymerization at the immunological synapse.


A)
APC
MHC II
CD4+
T Cell
CD4
TCR/CD3
Lck
Zap-70
PI3K
PIP2
LAT
PLC-γ1
PIP2
SLP
Wasp
ITK
Vav
B)
APC
MHC II
CD4+
T Cell
CD4
TCR/CD3
Lck
Zap-70
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Zap-70
PI3K
PIP3
LAT
SLP
ITK
Vav
Wasp
actin
PLC-γ1
PIP2
NFAT
Ca2+
IP3
IL-2
NF-κB
AP-1
DAG
key:
APC antigen presenting cell
SLP SLP-76
phosphotyrosine
phosphorylation
activity
nuclear translocation

1.3.2. T Cell Development

T cell development occurs in the thymus and, through the stages of positive and negative selection, results in the selection of a population of thymocytes expressing mature T cell receptors (TCR) that are MHC-restricted and self-tolerant (80, 97). First, CD4- CD8- double negative (DN) thymocytes expressing a pre-TCR undergo the process of positive selection that allows the survival of immature T cells based on their ability to recognize MHC molecules expressed by epithelial cells in the cortical region of the thymus. T cells receiving the necessary signals through their pre-TCR proceed to express a mature TCR in addition to the co-receptor molecules CD4 and CD8. Next, CD4+ CD8+ double positive (DP) T cells undergo the process of negative selection that results in the deletion of T cell clones expressing TCRs with high affinity to self-peptides expressed in the context of MHC molecules by medullary epithelial cells. Self-tolerant T cell clones then mature to become either single positive (SP) CD4+ or CD8+ T cells before exiting the thymus.

In the periphery, a mature CD4+ T cell is able to recognize foreign peptide presented by a professional APC in the context of MHC class II molecules (98). Depending on the surrounding cytokine milieu provided by an activated APC, a naive CD4+ helper T cell is capable of differentiating into a T helper type 1 (Th1) or type 2 (Th2) effector cell. Cytokine receptor-mediated signaling coupled with activation through the TCR strongly influences the

developmental fate of a naive CD4+ T cell. In response to surrounding IFN-γ or IL-12, cytokine receptor-mediated signaling proceeds with the activation of STAT1 or STAT4, respectively, leading to induction of the Th1 lineage specific transcription factor T-bet. In contrast, exposure to surrounding IL-4 leads to IL-4 receptor-mediated activation of STAT-6 followed by induction of the Th2 transcription factor GATA3. After differentiation and clonal expansion, Th1/2 competent cells secrete effector cytokines upon antigen-specific restimulation through the TCR. Th1 competent cells secrete the signature cytokines IFN-γ and IL-12 and are able to instruct CD8+ T cells to differentiate into cytotoxic T cells that are capable of lysing infected target cells thus providing immunity to intracellular pathogens. On the other hand, Th2 competent cells secrete the cytokines IL-4, IL-5 and IL-13 and instruct B cells to secrete IgE thus providing protection against extracellular antigens. Alternatively, a naive CD4+ T cell may differentiate into a regulatory T cell (Treg) mediated by signaling in response to TGF-β and induction of the lineage specific transcription factor Foxp3. IL-10 secreted by activated Tregs is important for suppressing Th1 or Th2 effector T cells following the initial inflammatory phase of the immune response.

Studies using knockout mice have revealed that several aspects of T cell development are altered in the absence of Itk. In the initial characterization of the $Itk^{-/-}$ mice, Liao and Littman discovered a reduced ratio of CD4+ to CD8+ thymocytes and a 2-fold decrease in the number of

peripheral CD4+ T cells as compared to wild-type mice indicating a requirement for Itk-dependent signaling during early thymic development (99). In order to investigate the defect further, CD4+ T cells were purified and cultured in the presence of conditions designed to mimic the cytokine milieu provided *in vivo* by an activated APC thus effectively skewing naive T cells along a Th1 or Th2 developmental pathway *in vitro* (100-102). In these studies, TCR stimulation was provided *in vitro* by receptor cross-linking using plate-bound anti-TCR antibody coupled with the addition of exogenous IL-2 to promote proliferation. Specifically, skewing towards the Th1 developmental pathway was achieved by adding exogenous recombinant IL-12 combined with anti-IL-4 blocking antibody while skewing towards the Th2 developmental pathway was achieved by adding exogenous recombinant IL-4 combined with anti-IFN-γ and/or anti-IL-12 blocking antibodies. Th1/2 effector function was then assessed after harvesting and restimulating differentiated cells using plate-bound anti-TCR antibody. These studies revealed that IL-4 secretion by $Itk^{-/-}$ T cells was defective upon restimulation of Th2-skewed cells while IFN-γ secretion by Th1-skewed cells was unaffected in the absence of Itk. In order to determine whether the observed Th2 defect was a result of defective lineage commitment or effector function, Au-Yeung and Fowell crossed knockin mice containing an IL-4-enhanced GFP (eGFP) reporter gene with $Itk^{-/-}$ mice and skewed cells along the Th2 differentiation pathway *in vitro* as described above (101, 102). In summary, these studies identified an essential

role for Itk at the effector stage of Th2 cytokine secretion upon TCR-mediated restimulation while differentiation of Itk$^{-/-}$ CD4+ T cells into Th2 competent cells was not affected as determined by monitoring the expression of the IL-4 reporter gene.

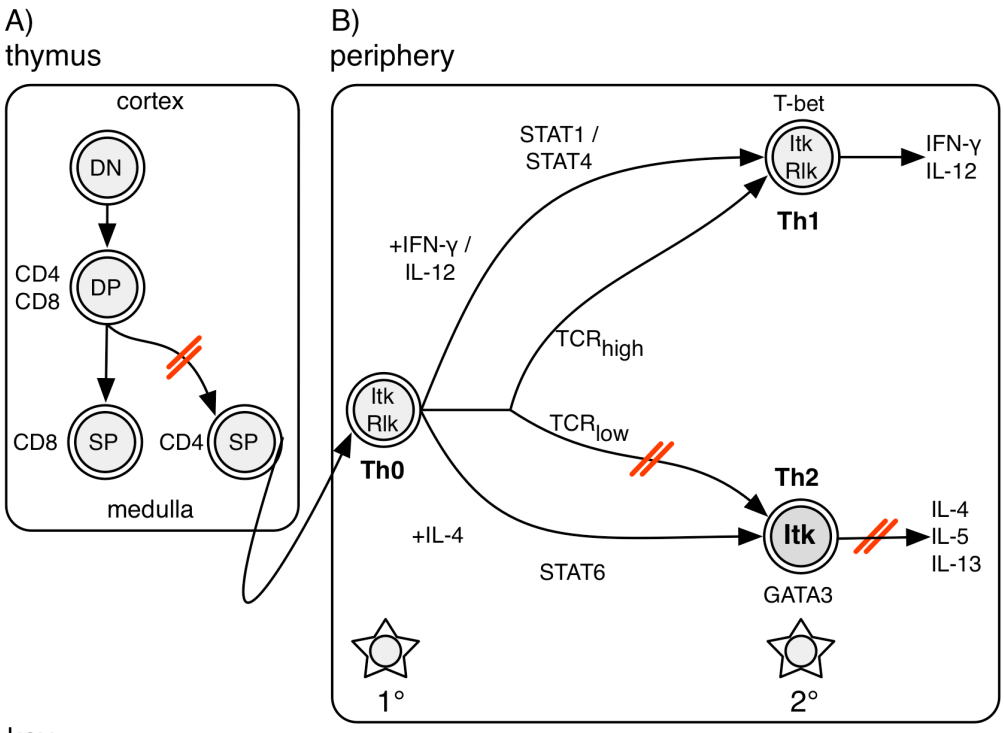
In a separate report, expression of the related Tec kinases Itk and Rlk was compared in Th1 versus Th2-skewed CD4+ T cells. At both the mRNA and protein level, Itk was upregulated in Th2 as compared to Th1-skewed cells (100). Furthermore, Rlk was not expressed in Th2 cells while its expression was selectively induced in Th1-skewed cells. Taken together, the results from the *in vitro* skewing experiments mentioned above are consistent with a model that supports Itk-independent Th2 differentiation driven by cytokine receptor mediated signaling in response to exogenous IL-4 resulting in STAT6 activation and induction of GATA-3. At the effector stage, these observations are also consistent with a dependence on Itk for the expression of Th2 but not Th1 effector function due to the non-redundant expression of the Tec family kinase Itk in Th2-differentiated cells.

Interestingly, studies performed by Miller and Berg using non-skewing culture conditions provided additional insight into the role of Itk during Th cell differentiation (100). In these studies, naive CD4+ T cells were allowed to differentiate in the absence of exogenous skewing cytokines and were directed instead only by the intensity of TCR signaling provided *in vitro*. High intensity TCR signaling resulted in rapid induction of the Th1 lineage specific

transcription factor T-bet and secretion of IFN-γ upon restimulation independently of Itk expression while low intensity TCR signaling resulted in induction of the Th2 lineage transcription factor GATA-3 in both WT and $Itk^{-/-}$ T cells. However, GATA-3 expression was coupled with low T-bet expression in WT T cells resulting in Th2 effector differentiation and secretion of IL-4 upon restimulation whereas $Itk^{-/-}$ T cells unexpectedly had increased T-bet expression resulting in Th1 effector differentiation and secretion of IFN-γ upon restimulation. Separate from the role of Itk in the release of effector function described above, these findings revealed a unique role for Itk in suppressing T-bet expression and thus promoting Th2 lineage commitment under conditions of low intensity TCR stimulation. This function could possibly be achieved by means of a direct effect on T-bet or by transcriptional repression of the T-bet promoter. Thus, Itk serves to regulate early Th2 commitment in terms of its ability to suppress the Th1 transcription factor T-bet as well as to regulate Th2 cytokine secretion upon restimulation with antigen downstream of the TCR.

Figure 4: Schematic diagram illustrates the developmental pathways, including Itk-dependent defects, leading to generation of T helper type 1 (Th1) and type 2 (Th2) effector cells.

A) In the cortical region of the thymus, bone marrow-derived T cells are double negative (DN) for expression of the surface markers CD4 and CD8. During positive selection, signaling through the pre-TCR leads to survival and expression of a mature TCR as well as to double positive (DP) expression of CD4 and CD8. Next, self-reactive T cells clone are removed through the process of negative selection while self-tolerant clones mature into CD4 or CD8 single positive (SP) T cells before exiting the thymus. In the absence of Itk signaling, development of CD4+ T cells is impaired leading to a decreased ratio of CD4+ to CD8+ T cells in the periphery. B) In the periphery, primary stimulation in response to antigen results in T cell activation and development into Th1 or Th2 effector cell populations depending on additional cytokine and/ or T cell receptor-mediated signaling. In the presence of exogenous IL-4, cytokine receptor-mediated signaling induces expression of the transcription factor GATA3, which promotes Th2 differentiation. In contrast, exogenous IFN-γ and/or IL-12 induces expression of the transcription factor T-bet, which promotes Th1 differentiation. In the absence of exogenous cytokines, high affinity TCR signaling induces expression of T-bet resulting in Th1 differentiation while low affinity TCR signaling induces expression of GATA-3 resulting in Th2 differentiation, which is impaired in the absence of Itk. Upon secondary TCR-mediated stimulation, differentiated Th1 and Th2 cells become effector cells, which are capable of secreting IFN-γ and IL-12 or IL-4, IL-5 and IL-13, respectively. Furthermore, Th2 effector function as measured by secretion of IL-4, IL-5 and IL-13 by competent cells is impaired in the absence of Itk.



key:

DN = double negative (CD4- CD8-)
DP = double positive (CD4+ CD8+)
SP = single positive (CD4+ / CD8+)
1° = primary stimulation
2° = secondary stimulation
// = Itk-dependent defect

1.4. Itk Regulation and Association with SLP-76

Itk is structurally organized into modular domains with shared homology to other Tec kinases that contribute to its overall kinase and adaptor functions in T cells. Importantly, negative regulation is a critical feature of all kinases in order to prevent uncontrolled phosphorylation of targets within the cell. Negative regulation of Src kinases such as Lck is well known to be achieved via an autoinhibitory conformation of the molecule that is mediated by the interaction of a phosphotyrosine residue in the kinase domain with the adjacent SH2 domain thus allosterically preventing kinase activity (103, 104). TCR-mediated release of the autoinhibitory conformation may be achieved through multiple mechanisms including dephosphorylation of the inhibitory phosphotyrosine by protein tyrosine phosphatases such as CD45, binding of exogenous ligand to regulatory domain(s) and/or activating phosphorylation events (105, 106). In contrast to Src kinases, Tec kinases do not have an autoinhibitory phosphotyrosine site in their kinase domains and therefore negative regulation is achieved by different means. Using a panel of purified Itk domain fragments, Andreotti et al. demonstrated *in vitro* that binding to exogenous substrates was inhibited for a fragment composed of the Itk SH3 domain and the proline motif KPLPPTP located in the adjacent Tec homology (TH) domain (107). As confirmed by NMR analysis, a model for negative regulation of Itk was proposed that is mediated by intramolecular association of the SH3 domain and the adjacent proline region within the TH domain.

In addition to the intramolecular self-association, Brazin and Andreotti discovered an intermolecular self-association with regulatory implications mediated by non-classical binding of the SH3 domain to the SH2 domain of another Itk molecule (108). This novel association was dependent on the SH3 domain binding pocket as demonstrated by disruption of dimer formation by mutation of the conserved tryptophan residue at position 208 (W208K) and involved binding to a novel surface on the SH2 domain as revealed by NMR (108). Furthermore, binding studies revealed that a proline-rich peptide was able to displace the SH3 domain from the SH2 domain while a phosphotyrosine-containing peptide had no significant effect on dimer formation (108). These results indicated that Itk dimer formation required mutually exclusive binding of the SH3 domain to the novel SH2 ligand while the phophotyrosine binding pocket of the SH2 domain was outside of the binding surface. Importantly, the Itk homodimers identified *in vitro* were demonstrated to be functionally significant in a negative regulatory capacity. As evidence of this, the Itk SH3 domain was replaced with the SH3 domain from the related kinase Btk which does not bind to the SH2 domain thus disrupting dimer formation (109). Upon expressing the $Itk_{Btk\ SH3}$ variant in a heterologous insect cell line, downstream activation of PLC-γ and ERK was enhanced as would be predicted for disruption of an autoinhibitory molecular association (109). However, this effect was not observed using a more physiological T cell expression system perhaps as a consequence of impaired

binding of the full-length Btk SH3 domain to polyproline ligands present on signaling adaptor molecules expressed in T cells such as SLP-76 (110). In a separate report, Severin and Andreotti identified that cis-trans isomerization of the imide bond preceding proline 287 of the SH2 domain regulates the SH2/SH3 mediated dimer formation with the cis isomer favoring association with the SH3 domain (111). Furthermore, the peptidyl-prolyl isomerase cyclophilin A catalyzes this cis-trans isomerization and mice lacking the cyclophilin A gene are susceptible to a spontaneous Th2 allergic condition characterized by swelling of the eyelids (112).

The structural studies described above support a model for negative regulation of the cis Itk isomer mediated by SH2-SH3 dimer formation that is in dynamic equilibrium with an autoinhibitory monomer mediated by SH3-proline intramolecular interactions. Upon transition to the trans isoform, the Itk SH2 domain favors binding to stimulation-dependent phosphotyrosine ligands transiently present on signaling adaptor proteins such as SLP-76 and, stabilized by binding of the SH3 domain to exogenous polyproline ligands, results in disruption of the autoinhibitory conformation. Following activation, Itk rapidly cycles to return to the cis isomer in this model and is again driven towards stable dimer formation. In the absence of cyclophilin A, the trans isomer would be predicted to persist allowing prolonged Itk activation and T cell signal transduction resulting in enhanced susceptibility to allergic conditions.

As revealed by studies using a PH domain deletion mutant (ΔPH-Itk), the N-terminal PH domain of Itk is required for the proper recruitment of Itk to the plasma membrane in addition to its co-localization with the TCR/CD3 signaling complex and enzymatic activation (83). The defects observed in the absence of the PH domain are consistent with the role of its inositide-binding pocket in associating with the membrane lipid PIP_3 which is generated following TCR-dependent activation of PI3K (81). In order to further elucidate its role, Hirve et al. generated point mutations in the PH domain of Itk at the conserved FYF amino acid motif located within the central β-barrel structure (manuscript accepted). Despite the inability to bind to PIP_3, TCR-dependent membrane localization of the FYF-Itk mutant was only partially reduced in contrast to the complete localization defect previously observed for ΔPH-Itk. Furthermore, the inducible association of the FYF-Itk mutant with the adaptor proteins SLP-76 and LAT occurred normally. Taken together, these observations are consistent with a possible regulatory role for the Itk PH domain in promoting association with a cluster of signaling molecules following TCR-dependent stimulation. However, the FYF-Itk mutant was enzymatically inactive suggesting a requirement for stable membrane interaction mediated by binding to PIP_3 in order to induce a conformational change that allows phosphorylation in trans by Lck. Indeed, fluorescence resonance energy transfer (FRET) analysis of the FYF-Itk mutant at the immunological synapse reveals that this molecule is conformationally altered

in comparison to WT Itk. In addition to its PIP_3-binding and possible adaptor roles, the PH domain of Itk has also been identified as a regulator of Itk activity. For example, ΔPH-Itk displayed spontaneous *in vitro* kinase activity suggesting a negative regulatory function in resting T cells. Furthermore, the PH domain has also been implicated in positive regulation of Itk activity mediated by binding to soluble IP_4 which is generated by ItpkB following T cell stimulation (89). Thus, the PH domain likely provides distinct adaptor and regulatory functions in addition to its essential role in binding to membrane PIP_3.

As shown by reconstitution of a deficient Jurkat cell line, the SH2 domain-containing protein and leukocyte protein of 76 kDa (SLP-76) is required for TCR-dependent Itk transphosphorylation and activation (113). SLP-76 is organized into an N-terminal acidic domain, a large proline rich region and a C-terminal SH2 domain. The N-terminal acidic domain contains three tyrosine residues that are phosphorylated upon TCR-dependent stimulation with the tyrosine at position 145 being specifically required for Itk-dependent signaling (114, 115). However, the TCR-inducible biochemical association of Itk and SLP-76 was not affected by mutation of this tyrosine residue despite its importance for Itk activation (115, 116). As previously identified *in vitro*, the purified SH2 domain of Itk specifically binds to phospho-Tyr145 within the acidic domain of SLP-76 while the Itk SH3 domain binds to the central polyproline region of human SLP-76 located at a minimal binding

site from amino acid residues 184 to 195 corresponding to the sequence QQPPVPPQRPMA (81, 117).

In order to further investigate the role of the Itk SH3 domain, Grasis et al. added a cell-penetrating poly-arginine motif to the SH3 domain polyproline ligand identified from the *in vitro* binding studies and treated Jurkat cells and primary T cells with the modified peptide which was referred to as R9-QQP (2). In contrast to the role of the N-terminal Tyr145 in the SLP-76 acidic domain, the polyproline region of SLP-76 was necessary for mediating TCR-dependent Itk–SLP-76 biochemical association as revealed by dose-dependent competition for binding to the Itk SH3 domain using the R9-QQP peptide. Consistent with the adaptor role of SLP-76, the study by Grasis et al. demonstrated that recruitment of Itk to the immunological synapse as well as its TCR-mediated transphosphorylation were inhibited upon treatment of T cells with R9-QQP. Furthermore, Itk-dependent catalytic and adaptor function were also affected as revealed by decreased Th2 cytokine production and reduced actin polymerization at the immunological synapse, respectively.

Importantly, inhibition of binding to SLP-76 as well as the consequent effects on Itk activation and function were specific as determined by treatment of T cells with a control peptide containing a scrambled polyproline motif. For example, the adaptor protein GADS constitutively binds to SLP-76 mediated by its SH3 domain where it serves to provide a link via its SH2 domain to the scaffolding protein LAT which lacks an SH3 domain (118, 119). At the same

time that SH3-mediated binding of Itk at the location of its polyproline ligand on SLP-76 was inhibited by R9-QQP, the SH3-mediated association of GADS at the location of its distinct polyproline ligand on SLP-76 was not affect by R9-QQP thus demonstrating sequence specificity.

In addition to binding to Itk, an identical location within the proline region of SLP-76 is also required for binding to the Src kinase Lck as demonstrated by binding to an Lck SH3 domain fragment *in vitro* (120). Furthermore, this association was demonstrated to be functionally significant by genetic reconstitution of $SLP76^{-/-}$ mice with a version of SLP-76 lacking the specific proline ligand binding region (121). However, treatment of T cells with R9-QQP inhibited SH3-mediated binding of Itk but not Lck to the same polyproline ligand of SLP-76 (2). One explanation for the disparate *in vivo* binding results reported for Lck could involve the strategy used for blocking its interaction to SLP-76. Genetically altering SLP-76 by deletion of a portion of its proline region could induce allosteric changes that disrupt physiological binding to full length Lck. In contrast, the use of a competitive inhibitor to disrupt binding leaves SLP-76 intact and allows additional stabilizing interactions outside of the polyproline ligand binding region to occur unperturbed. In this scenario, association of full length Lck with SLP-76 may be preserved by treatment with R9-QQP but not for the SLP-76 deletion mutant.

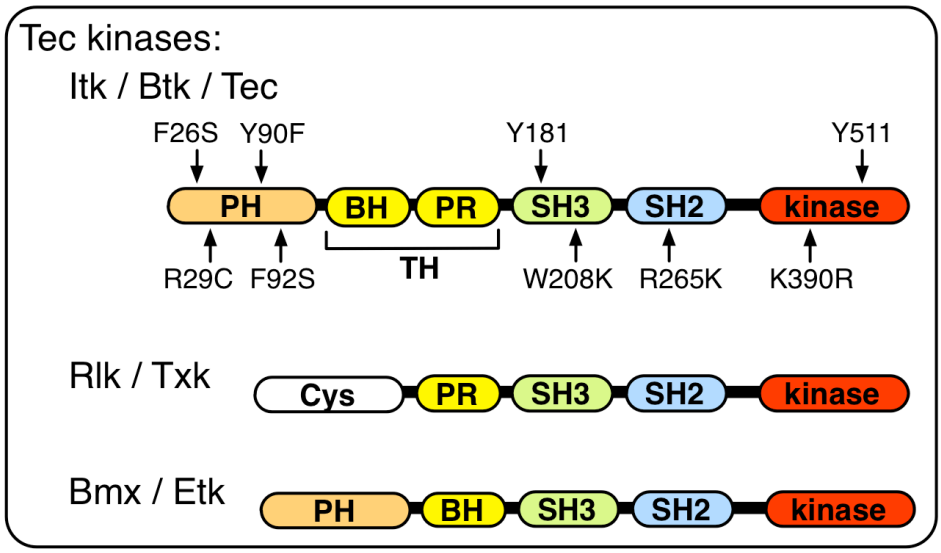
While polyproline regions are functionally important in their ability to serve as SH3 ligands, they may also play an independent structural role. For example, the P-1 section of the SLP-76 proline region was shown to be capable of binding to the PLC-γ1 SH3 domain *in vitro* (122). Furthermore, binding to PLC-γ1 was abolished upon reconstitution of deficient Jurkat cells with SLP-76 mutants containing various partial deletions within the P-1 section (123). However, PLC-γ1 was functionally active despite the lack of binding to SLP-76 for all partial deletion mutants. In contrast, PLC-γ1 function was only impaired upon deletion of the entire P-1 section and this defect was partially restored by replacement with a P-1 section containing a scrambled sequence. Thus, the results of this study are consistent with a structural role for the proline region within the P-1 section of SLP-76. In this context, the ability of the P-1 section of SLP-76 to bind to the SH3 domain of PLC-γ1 appears to be unrelated to the SLP-76 requirement for PLC-γ1 activation. While polyproline regions may play a structural role in certain contexts, this is not inconsistent with the role of polyproline motifs as specific although low affinity ligands for SH3 domain containing signaling proteins that provide important functional roles as demonstrated for the Itk–SLP-76 association. Rather than a direct association, subsequent work indicated that recruitment of PLC-γ1 to SLP-76 was dependent on its association with GADS while activation of PLC-γ1 was dependent on its SLP-76-mediated association with Itk and Vav as part of a LAT-nucleated signaling cluster in proximity to the TCR/CD3 complex (124).

Based on studies of Itk domain function, the cumulative data support a model for autoinhibition of Itk mediated by dimer and folded monomer conformations involving SH2-SH3 domain interaction in trans and SH3-polyproline interaction in cis, respectively (1, 107). Upon isomerization of the imide bond within the SH2 domain to a trans isoform, Itk is then able to bind via its SH3 domain to its polyproline ligand within the proline region of SLP-76 (108, 112). Additionally, interaction of the SH2 domain with phospho-Tyr145 within the acidic domain of SLP-76 is required in order for Itk to become activated enzymatically (115, 116). Combined with the membrane targeting and adaptor roles of the PH domain, the cooperative SH2 and SH3 mediated binding of Itk to SLP-76 is likely required in order to stabilize a conformation of Itk that allows phosphorylation on tyrosine 511 within its SH1 domain by Lck (2, 110). This modification then induces a conformational change that activates the kinase domain of Itk. Furthermore, autophosphorylation of the Itk-SH3 domain on tyrosine 181 is likely important for stabilizing Itk in an active conformation perhaps by preventing intramolecular folding mediated by the inhibitory SH3-proline interaction (86). Once fully activated, Itk is then capable of phosphorylating PLC-γ1 on tyrosine 783 which is required along with its association with Vav for its lipase activity followed downstream by second messenger generation and transcriptional activation resulting in T cell development and effector function (124).

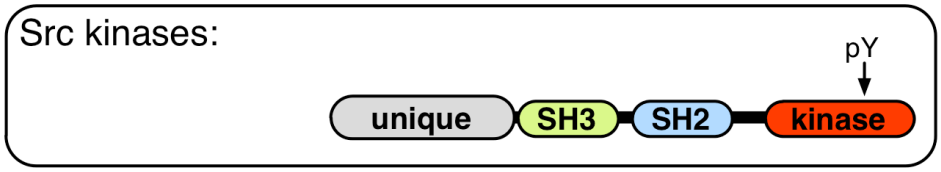
Figure 5: Schematic diagram compares and contrasts the domain structure composition of Tec and Src family kinases.

A-B) In contrast to Src kinases, the Tec family of non-receptor tyrosine kinases have an N-terminal pleckstrin homology (PH) domain, which binds to the membrane phospholipid PIP_3. Furthermore, Tec kinases lack a negative regulatory phosphotyrosine residue in their C-terminal kinase domain, which is present in Src kinases. Similarly, both Tec and Src kinases share SH2 and SH3 domains with adaptor functions in addition to a conserved kinase domains with catalytic function. Itk is phosphorylated in trans on tyrosine 511 within its kinase domain, which results in phosphorylation in cis on tyrosine 181 within its SH3 domain. In addition, several point mutations have been introduced with differential effects on Itk function including R29C and FYF (F26S, Y90F and F92S) in the PH domain, which prevent binding to PIP3, W208K in the SH3 domain, which prevents binding to polyproline ligands, and R265K in the SH2 domain, which prevents binding to phosphotyrosine ligands.

A)



B)

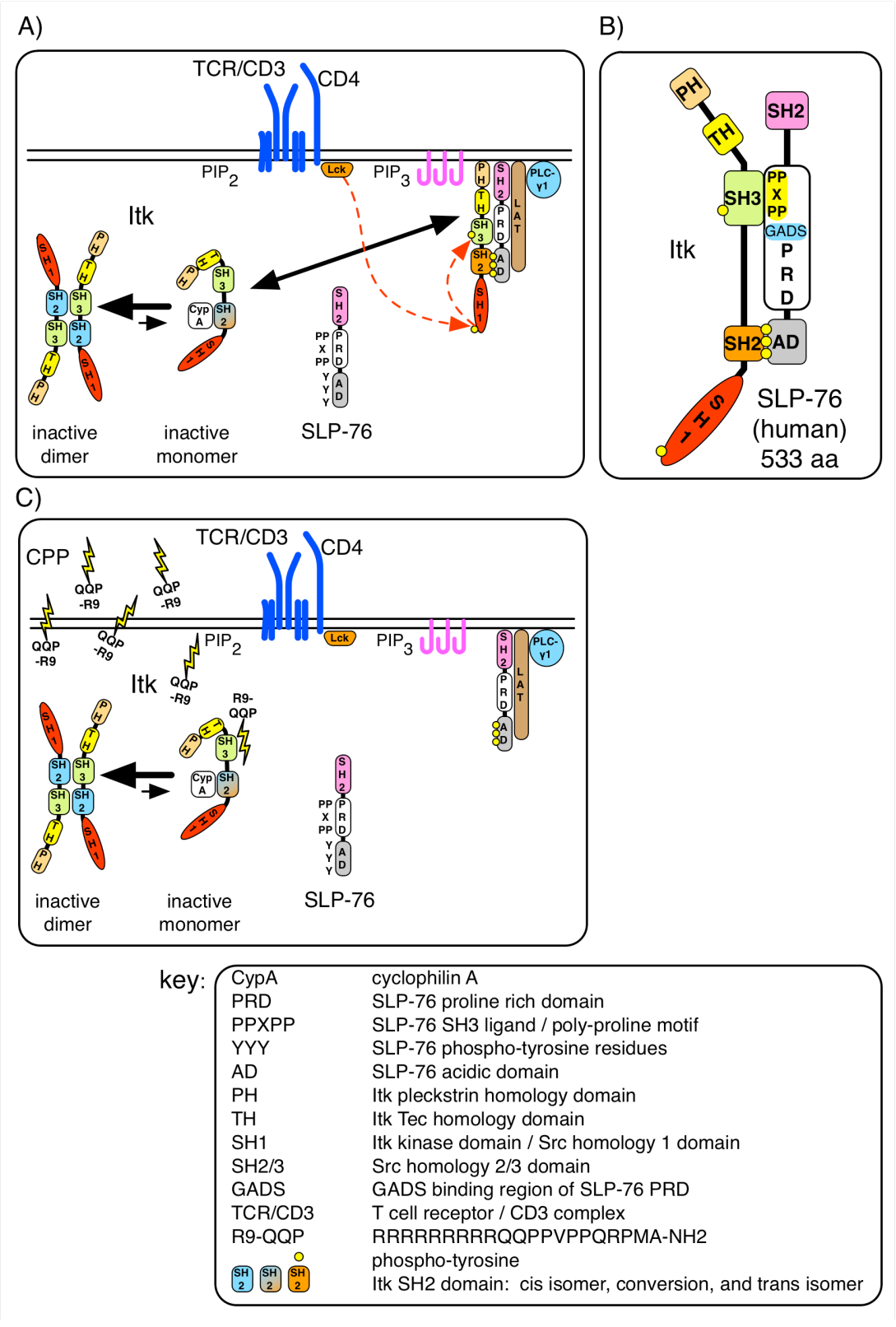


key:

PH	pleckstrin homology domain
BH	Btk homology domain
PR	proline rich region
TH	Tec homology domain
SH3	Src homology 3 domain
SH2	Src homology 2 domain
Cys	cysteine motif
pY	phospho-tyrosine

Figure 6: Schematic diagram illustrates regulatory Itk domain interactions including dominant negative inhibition by R9-QQP.

A) Itk activity is believed to be negatively regulated by stable Itk heterodimers mediated by non-canonical SH2-SH3 domain interactions that are in dynamic equilibrium with folded Itk monomers mediated by SH3-proline interactions. Catalyzed by cyclophilin A (CypA), proline 287 of the Itk SH2 domain converts from the cis to the trans isoform resulting in enhanced affinity for TCR-dependent phosphotyrosine ligands present in the acidic domain (AD) of SLP-76. In this model, cooperative binding of the Itk SH2 and SH3 domains to the acid and proline rich domains (PRD) of SLP-76 induces a conformation of Itk that allows phosphorylation by Lck followed by stabilization of the active conformation by autophosphorylation. After the initial TCR-dependent phosphorylation of SLP-76, dynamic cycling of Itk molecules then returns Itk to an inactive conformation. B) Enlargement of Itk and SLP-76 interaction highlights the domain-specific interactions between that are disrupted the QQP peptide, which is derived from the proline rich domain (PRD) of SLP-76. C) The cell permeable R9-QQP peptide inhibits Itk by binding to the Itk SH3 domain in a competitive manner thus disrupting the association of Itk with SLP-76 which is necessary for its activation.



key:

CypA	cyclophilin A
PRD	SLP-76 proline rich domain
PPXPP	SLP-76 SH3 ligand / poly-proline motif
YYY	SLP-76 phospho-tyrosine residues
AD	SLP-76 acidic domain
PH	Itk pleckstrin homology domain
TH	Itk Tec homology domain
SH1	Itk kinase domain / Src homology 1 domain
SH2/3	Src homology 2/3 domain
GADS	GADS binding region of SLP-76 PRD
TCR/CD3	T cell receptor / CD3 complex
R9-QQP	RRRRRRRRRQQPPVPPQRPMA-NH2
	phospho-tyrosine
	Itk SH2 domain: cis isomer, conversion, and trans isomer

1.5. R9-QQP Characterization and Translation to Asthma Model

As illustrated in the example of the SLP-76 derived R9-QQP peptide, cell penetrating peptides (CPP) are useful tools for studying the function of physiological protein domain-mediated binding events occurring within live cells. Whether mediated by direct penetration of the plasma membrane or by endocytosis, covalent addition of a polyarginine tag to the cargo peptide sequence allows delivery of the polyarginine fusion peptide into the cytoplasmic compartment of the cell. Once inside the cell, free peptide is able to bind to its target protein thus blocking its functional interaction with another endogenously expressed binding partner in a dominant negative manner (125-127). Because the transduction efficiency of polyarginine-conjugated peptides may also depend on the amino acid composition of the peptide cargo, addition of fluorescent tags combined with confocal microscopy and/or detection of fluorescence by flow cytometry are useful methods for monitoring peptide uptake. Particularly at high concentrations, one complication from the use of CPP is the possible disruption of the plasma membrane that may occur during the cellular uptake process. As for treatment with any drug in a cell-based system, determination of cell viability at the effective concentration and for the duration of the assay is a routine but important assessment of any cytotoxic effects that may affect interpretation of peptide sequence specific binding effects.

In the study by Grasis et al., cellular uptake of R9-QQP by Jurkat T cells and primary murine splenocytes was determined by use of FITC which was covalently attached to the N-terminus of the polyarginine cell penetration motif. As determined by FACS analysis, uptake of FITC-R9-QQP by both Jurkat cells and splenocytes was dose dependent following a 30 min. *in vitro* incubation with peptide in concentrations ranging from 0-20 μM. Once taken up by T lymphocytes, the TCR-inducible biochemical association of Itk and SLP-76 was blocked by R9-QQP in a dose-dependent and peptide sequence specific manner as determined by co-immunoprecipitation experiments targeting SLP-76 followed by immunoblotting for Itk. In order to investigate the effect of this biochemical association on TCR-inducible recruitment, Jurkat cells expressing GFP-Itk were treated with R9-QQP and allowed to form conjugates with anti-TCR coated beads which serve as surrogate antigen presenting cells. Consistent with the requirement for interaction with SLP-76, analysis of images acquired by confocal microscopy revealed that recruitment of GFP-Itk to the cell-bead contact site was impaired in cells that had been treated with R9-QQP.

According to its adaptor role, Itk supports localized actin polymerization through its constitutive association with Vav which activates WASp. In order to investigate this Itk-dependent function, Jurkat-bead conjugates were stained with TRITC-conjugated phalloidin to visualize filamentous actin after treatment of cells with R9-QQP. In agreement with the Itk localization defect,

R9-QQP specifically disrupted accumulation of actin at the cell-bead contact site. Furthermore, Itk is enzymatically activated by phosphorylated on tyrosine 511 within its kinase domain by Lck in mechanism that depends on SLP-76. Indeed, Itk activation was also inhibited in R9-QQP treated cells as determined using a phospho-specific antibody combined with detection by both immunoblotting and phospho-flow cytometric methods.

Following TCR and Itk-mediated activation of PLC-γ1, second messengers such as IP_3 and DAG are generated that result in activation of downstream signaling pathways which result in transcriptional activation of Th2 inflammatory cytokine genes. In order to assess the effects of R9-QQP on expression of cytokine genes, CD4+ T cells were purified from WT C57BL/6 mice and treated *in vitro* with R9-QQP or scrambled control peptide. 24 hours after the primary stimulation with anti-TCR and anti-CD28 antibodies, cells were restimulated with anti-TCR antibody and incubated for an additional 6 hours in the presence or absence of the protein transport inhibitor Brefeldin A. Next, cytokine gene expression of Brefeldin A treated cultures was detected by the intracellular cytokine staining method and supernatants from independent cultures were harvested and tested for cytokine secretion by ELISA. Similar to the developmental defects observed for $Itk^{-/-}$ T cells, inhibition of Itk activation by treatment with R9-QQP specifically reduced expression as well as secretion of the Th2 cytokines IL-4, IL-5 and IL-13 while the signature Th1 cytokine IFN-γ was not significantly affected.

In addition to the R9-QQP specific effects described for the treatment of T cells *in vitro*, Grasis et al. extended the biochemical and functional significance of these findings by treating mice with peptide delivered *in vivo*. In these experiments, 20 mg/kg of R9-QQP or control peptide was injected intraperitoneally (i.p.) at 24 and 0.5 hrs. prior to harvesting spleens. After processing into a cell suspension, the biochemical defects affecting the Itk–SLP-76 association as well as Itk enzymatic activation were confirmed using this *in vivo* peptide treatment protocol. Lastly, the specific functional effects of inhibiting the Itk–SLP-76 interaction on Th2 cytokine expression and secretion were also demonstrated by administration of R9-QQP *in vivo*. While not unexpected, the functional effects precipitated by disrupting the Itk–SLP-76 interaction as detailed in this study elegantly refined the precise binding requirements necessary for Itk activation while leaving endogenously expressed signaling proteins untouched by mutational approaches. Namely, interaction of the Itk SH3 domain interaction with the SLP-76 polyproline ligand was demonstrated to be of paramount importance for physiological association of the two full-length molecules. Furthermore, competitive inhibition of this interaction by R9-QQP resulted in a cascading series of biochemical and functional defects resulting in decreased Th2 cytokine production.

In addition to being useful tools for studying biochemical signaling pathways, CPP have also been used to investigate disease pathogenesis with

translational potential for clinical application (128-130). Treatment of many inflammatory skin diseases such as psoriasis and atopic dermatitis is often complicated by the poor epidermal penetration of topically applied immunosuppressive drugs such as cyclosporin A. On the other hand, orally administered immunosuppressive drugs are effective but often have undesirable toxic and carcinogenic side effects. Using a mouse model of contact dermatitis, Rothbard et al. demonstrated that conjugation of a polyarginine tag to the calcineurin inhibitor cyclosporin (CsA) facilitated entry of R7-CsA into T cells located at the site of inflammation within the dermal layer of the skin (130). In order to determine effectiveness, one ear from each mouse in the contact dermatitis model was treated with either R7-CsA or unmodified control CsA. As an indicator of inflammation, ear swelling was measured following treatment. The results of these experiments revealed that R7-CsA significantly reduced ear swelling as compared to the unmodified control. Importantly, treatment with R7-CsA did not affect ear swelling on the untreated ear and R7-CsA was not detected in serum collected from these mice indicating that the effect was occurring locally as opposed to systemically. As demonstrated in this study, polyarginine modification can be useful as a strategy to enhance delivery of biologically active compounds to sites of inflammation within tissues and inside of cells that are otherwise inaccessible to unmodified drugs.

In another study targeting the same pathway, Noguchi et al. used the NFAT inhibitor peptide VIVT to successfully prevent rejection of transplanted pancreatic islets using a mouse model of transplant rejection (129). For patients with autoimmune-based type 1 diabetes, successful transplant therapy requires tolerance to mismatched MHC molecules that can be achieved therapeutically by immunosuppressive drugs such as cyclosporine A and FK506. By inhibiting the calcium-dependent phosphatase calcineurin, these drugs prevent dephosphorylation and nuclear translocation of NFAT which transcriptionally activates many pro-inflammatory genes. While effective immunosuppressors, these drugs also have toxic and carcinogenic side effects associated with their long term use. When modified by the addition of 11 arginines and delivered *in vivo*, 11R-VIVT specifically reduced rejection of islet allografts mediated by inhibition of pro-inflammatory cytokines. In addition, 11R-VIVT had reduced toxic effects as demonstrated by reduced insulin secretion by a β-pancreatic cell line that was exposed to peptide *in vitro*. By directly inhibiting NFAT rather its upstream regulator, the polyarginine-tagged peptide VIVT is an effective and less toxic immunosuppressor as compared to therapeutics that target calcineurin.

In an study demonstrating treatment of lung inflammation, McCusker et al. used a dominant negative peptide inhibitor of STAT-6 that was delivered intranasally to mice during the challenge period of the ovalbumin-induced asthma model (128). During an inflammatory response, OVA-induced IL-4

and IL-13 bind to cytokine receptors which share a common IL-4 receptor alpha subunit and that are expressed on T cells as well as airway structural cells. Upon binding of IL-4/IL-13, cytoplasmic STAT-6 is recruited to the cytokine receptor where it is phosphorylated on tyrosine residue 641 (pY 641). Mediated by a subsequent pY 641-SH2 domain interaction, STAT-6 dimerizes and translocates to the nucleus where it promotes transcriptional activation of pro-inflammatory genes. In order to block STAT-6 dimerization, a dominant negative inhibitor peptide that included pY 641 was tagged with a cell penetration motif derived from the HIV-TAT transcription factor protein transduction domain. Upon intranasal delivery, this peptide was demonstrated to enter airway structural cells which express STAT-6 and respond to IL-4/ IL-13-dependent cytokine signaling. Mice treated intranasally with the STAT-6 inhibitor peptide displayed reduced lung inflammation characterized by reduced eosinophil infiltration, mucus production and airway hyperresponsiveness. In addition, lung cells from peptide-treated mice showed reduced expression of inflammatory genes such as eotaxin which recruits eosinophils as well as genes involved in mucus production. Together, these data establish that a cell permeable peptide can be efficiency delivered to structural cells lining the lung mucosa and can be used to treat lung inflammation associated with experimental asthma.

While current treatments for asthma are effective for most individuals, the lack of responsiveness in some groups of patients as well as the serious

side effects noted in the review above highlight the need to develop additional treatment options. In terms of its requirement for Th2 cell effector function as well as the reduced asthma phenotype of knockout mice, Itk is an attractive therapeutic target for the treatment of allergic diseases such as asthma (11, 100, 101). Previously developed small molecule inhibitors of Itk target its conserved kinase domain which shares homology with other kinases and are likely to affect many unrelated signaling pathways at therapeutically effective concentrations (131, 132). In contrast, the R9-QQP inhibitor peptide characterized in the study by Grasis et al. specifically targets the TCR-inducible interaction of the Itk-SH3 domain with the polyproline ligand of SLP-76 and identifies this association as a necessary condition for the enzymatic and adaptor functions of Itk in T cells. Combined with the feasibility of applying CPP to disease models including experimental asthma as mentioned above, the current study attempts to translate the biochemical and functional characterization of R9-QQP to a mouse model of experimental asthma generated in response to OVA.

Chapter 1, in part, is currently being prepared for submission for publication of the material. Guimond, D.M., Cam, N.R., Hirve, N., Duan, W., Lambris, J.D., Croft, M., & Tsoukas, C.D (2012). Treatment of Experimental Asthma Using a Novel Peptide Inhibitor of the Inducible T Cell Kinase. The dissertation author was the primary author of this material.

2. MATERIALS AND METHODS

2.1. Mice

Female C57BL/6 or BALB/c mice between 6-8 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME). Itk$^{-/-}$ mice were generated as previously described by Dr. D. Littman (New York University School of Medicine; New York, NY) (99). A breeding pair was provided by Dr. T. Kawakami (La Jolla Institute for Allergy and Immunology; La Jolla, CA) for colony maintenance at the San Diego State University Vivarium Facility and genotyping by PCR analysis using tail fragments was performed as described below to confirm the presence of the homozygous knockout alleles. All mice were housed in microisolator cages on a 12 hr. light / dark cycle and were provided with acidified, autoclaved water and an irradiated rodent diet *ad libitum*. Euthanasia was performed by first anesthetizing mice inside of a jar containing cotton saturated with isoflurane (TW Medical Veterinary Supply, Cat. # 21234484; Lago Vista, TX) followed by sacrificing unconscious mice by cervical dislocation. Experiments using mice were approved and conducted following the guidelines of the Institutional Animal Care and Use Committees (IACUC) at San Diego State University and the La Jolla Institute for Allergy and Immunology (for AHR assay), respectively.

2.2. Diagnosis of Itk$^{-/-}$ Mice by PCR Analysis

Itk$^{-/-}$ mice used for characterization of the asthma assay were diagnosed by PCR for the homozygous presence of the mutant Itk allele containing the neomycin cassette followed by a stop codon in place of Itk exon 3, intron 3 and exon 4 as described previously (99). First, 1 cm tail fragments were removed and solubilized overnight at 55°C in the presence of 20 mg/mL proteinase K (Invitrogen / Life Technologies, Cat. # 25530-015; Carlsbad, CA) in tail solubilization buffer (100 mM NaCl, 1% SDS, 5 mM EDTA and 10 mM Tris-HCl, pH 8.0). Next, SDS and proteins were precipitated by the addition of 0.4 volume of tail salts buffer (4.21 M NaCl, 0.63 M KCl and 10 mM Tris-HCl, pH 8.0) and incubation for 4 hrs. on ice followed by centrifugation for 10 min. at 14,000 rpm (4°C). 80 μL of supernatant was then transferred to a microcentrifuge tube and DNA was precipitated by the addition of 2 volumes of ice-cold 100% ethanol and incubation for 4 hrs. at -20°C followed by centrifugation for 10 min. at 14,000 rpm (4°C). After ethanol precipitation, the supernatant was discarded and DNA contained in the pellet was washed with 500 μL ice-cold 80% ethanol followed again by centrifugation as before. Lastly, the supernatant was discarded and purified DNA contained in the pellet was allowed to dry before being dissolved in 50μL of TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20°C.

For each PCR reaction, 3 μL of purified template DNA obtained following mouse tail biopsy as described above was added to a PCR tube in addition to the following components which were purchased from Invitrogen / Life Technologies and adjusted to a final volume of 50 μL using PCR grade water: 0.2 mM dNTP mixture, 2 mM $MgSO_4$, 0.2 μM of each primer, 1x High Fidelity PCR buffer and 1 unit of Platinum Taq DNA Polymerase High Fidelity (Cat. # 11304-011). The following primers were added together to each PCR tube: neomycin forward primer (5' CTT, GAC, GAG, TTC, TTC, TGA, GGG, GA 3'), Itk exon 2 forward primer (5' GGC, TCC, ATT, GAA, CTC, TCC, AG 3') and Itk intron 4 reverse primer (5' GGG, ATG, CCA, GAA, TCA, ACT, TTG, GTA, G 3'). PCR was performed using an Eppendorf Mastercycler thermal cycler that was programmed to repeat 40 cycles of template denaturation for 1 min. at 93°C and primer annealing for 1 min. at 58°C followed by strand extension for 8 min. at 65°C. 5 μL of PCR product was added to each well of a 0.8% agarose gel and bands were resolved by gel electrophoresis. A single 1.9 kb band was predicted to result following PCR amplification of template DNA purified from a homozygous $Itk^{-/-}$ mouse as compared to a single 7.5 kb band from a WT (C57BL/6) mouse and a dual 1.9 kb and 7.5 kb band from a heterozygous $Itk^{+/-}$ mouse, respectively.

2.3. Confirmation of Itk$^{-/-}$ Mice by Immunoblotting

The lack of Itk expression by Itk$^{-/-}$ mice was confirmed by immunoblotting of lysates prepared from spleens of WT (C57BL/6) or Itk$^{-/-}$ mice using anti-Itk antibody. First, spleens were harvested and cell suspensions were prepared as described below for cell culture. Next, 60x10^6 splenocytes from each mouse were resuspended in 0.5 mL of ice-cold NP-40 lysis buffer (20 mM Tris-HCl, 0.4 mM EDTA and 1% NP-40) supplemented with 5 μg/mL of leupeptin (Sigma; St. Louis, MO), 5 μg/mL of pepstatin A (MP Biomedicals; Solon, OH) and 1 mM of PMSF (Calbiochem / EMD Millipore; Billerica, MA). After lysis for 1 hr. on a tube rotator at 4°C, cellular debris was pelleted by centrifugation for 20 min. at 14,000 rpm using a refrigerated microcentrifuge. A 10 μL aliquot of supernatant was then combined with an equal volume of 2x laemmli sample buffer (125 mM Tris-HCl, 20% glycerol, 4% SDS, 0.2 M DTT and 0.02% bromophenol blue) and proteins in lysate samples were denatured by placing tubes in boiling water for 5 min. followed by centrifugation for 30 sec. at maximum speed. Next, 20 μL of lysate sample was added to each well of a 7.5% acrylamide gel (Sigma) and proteins were resolved by SDS-PAGE followed by transfer to a PVDF membrane (Pall Life Sciences, Cat. # 66543; Port Washington, NY). After blocking for 1 hr. with 5% BSA, faction V (Calbiochem / EMD Millipore, Cat. # 2930) in TBST (10 mM Tris, 150 mM NaCl and 0.1% Tween 20, pH 7.4), the membrane was probed with anti-Itk antibody (clone 2F12; Upstate / EMD Millipore, Cat. #

05-476; Waltham, MA) diluted 1:1,000 in 2.5% BSA / TBST for 1 hr. followed by incubation with 0.2 μg/mL of HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, Cat. # 115-035-146; West Grove, PA) for 30 min. while washing 3x with TBST between each step. Lastly, enhanced chemiluminescent substrate (ECL; Pierce / Thermo Fisher Scientific, Inc., Cat. # 34077; Rockford, IL) was added and bands representing Itk were visualized and confirmed by size in reference to the protein standard (Bio-Rad, Cat. # 161-0374; Hercules, CA).

2.4. Cell Culture

Jurkat clone E6.1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cells were maintained in RPMI-1640 medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 100 I.U./mL penicillin, 100 μg/mL streptomycin (Mediatech Cellgro / Corning, Inc.; Manassas, VA) plus 10% heat-inactivated fetal bovine serum (FBS) (SAFC Biosciences; Lenexa, KS). Jurkat cells were seeded into vented flasks at an initial density of $0.1x10^6$ cells/mL and split prior reaching a density of 10^6 cells/mL. Cell concentration and viability was routinely monitored by removing an aliquot of cells from culture, diluting 1:2 in 0.15% erythrosine B and counting live / dead cells using a hemacytometer.

Primary mouse lymphocytes used for *in vitro* cytokine production were obtained from draining lymph nodes (DLN) or spleens. First, lymphoid tissue was mechanically dissociated using the blunt end of a 1 mL syringe plunger against a 70 μm nylon cell strainer. After rinsing each strainer with RPMI, cells were collected in a 50 mL tube and centrifuged for 5 min. at 1,500 rpm (4°C). Next, red blood cells (RBCs) were lysed by resuspending cells in ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA) and incubating for 5-10 min. at room temperature. Cells were then washed and resuspended in RPMI-1640 medium supplemented as described above in addition to 0.05 mM 2-mercaptoethanol (2-ME; Sigma) plus 10% heat-inactivated FBS. After counting cells using a hemacytometer, cell

concentrations were adjusted and 10^5-10^6 cells in 0.1 mL were seeded to each of 3-5 replicate wells of round-bottom 96-well tissue culture treated plates (Sarstedt, Cat. # 83.1837; Nümbrecht, Germany). Next, cells were stimulated by addition of an equal volume (0.1 mL) of culture medium containing 0.1-0.2 mg/mL of OVA for a final concentration of 50-100 μg/mL of OVA. After incubation for 4 days, cell culture supernatants were harvested and cytokine secretion was analyzed by ELISA as described below.

In order to reduce the potential for contamination, all cell culture work was performed in a laminar flow biological safety cabinet (Baker Company, Inc.; Sanford, ME) using aseptic technique and sterile, pyrogen-free instruments / consumable reagents. After counting cells using a hemacytometer, tissue culture vessels were seeded at the appropriate densities and placed inside of a humidified, 5% CO_2, 37°C incubator.

2.5. Cell Penetrating Peptides

RRRRRRRRRQQPPVPPQRPMA (R9-QQP), RRRRRRRRRPQMPAPQRPQPV (R9-PQM) and RRRRRRRRRQQAAVAAQRAMA (R9-QQA) peptides were custom synthesized by GL Biochem, Ltd. (Shanghai, China) using L-enantiomer amino acids. Peptides were amidated at the carboxy (C)-terminus and FITC was conjugated at the amino (N)-terminus for uptake studies. All peptides were delivered > 95% pure by reverse-phase high-performance liquid chromatography (HPLC) and were analyzed by mass spectroscopy. Lyophilized peptide stocks were stored inside desiccators at -20°C. Prior to use, peptides were reconstituted at 20 mg/mL by nominal mass in cell culture grade PBS (Mediatech) for *in vivo* experiments or in cell culture grade water (HyClone / Thermo Fisher Scientific, Inc.; South Logan, UT) for *in vitro* experiments. In order to avoid repeated freeze-thaw cycles, aliquots of peptide stock solutions were prepared in microcentrifuge tubes and were stored at -20°C for ≤ 4 weeks.

2.6. Peptide Uptake Studies

For *in vitro* uptake studies, Jurkat cells were resuspended in supplemented RPMI-1640 without FBS and aliquots of $5x10^6$ cells per mL were dispensed to FACS tubes for each peptide treatment condition. Next, FITC-conjugated R9-peptides were added and tubes were placed inside of a humidified, 5% CO_2, 37°C incubator for 30 min. Following peptide treatment, cells used for uptake studies were washed with ice-cold PBS and resuspended in 0.05% trypsin - 0.53 mM EDTA in HBSS (Mediatech, Cat. # 25-051-CI). In a separate control experiment, Jurkat cells were treated with trypsin and enzyme kinetics were determined by staining cells for surface expression of the trypsin-sensitive transferrin receptor (CD71) with anti-CD71 antibody (clone OKT9, purified from ascites) followed by detection with FITC-conjugated goat anti-mouse antibody (FITC-GαM; Upstate / EMD Millipore, Cat. # 12-506). After incubation for 10 min. at 37°C, trypsin was quenched by addition of FACS buffer (0.5% BSA/PBS + 0.2% NaN_3) and cells were washed for an additional 3 times while being kept on ice throughout. Next, cells were fixed with 2% paraformaldehyde (Fisher Scientific; Waltham, MA) / PBS for 10 min. on ice, washed, and resuspended in 300 μL of FACS buffer prior to acquiring FACS data for FSC, SSC and FITC channels. Jurkat cells were gated based on FSC and SSC and the mean FITC fluorescence intensity (MFI) was recorded for each peptide treatment condition.

For investigation of systemic uptake in mice, 1 mg of FITC-conjugated R9-QQP was delivered i.p. at -24 and/or -0.5 hrs. prior to harvesting spleens and processing splenocytes as described above. In addition, mice were sensitized with OVA/alum on day 0 and local uptake at the lung mucosa was investigated by i.n. delivery of 200 μg of FITC-conjugated R9-QQP 2 hrs. prior to each challenge with OVA on days 7-9 as described below for induction of allergic asthma. On day 10, bronchoalveolar lavage was performed and BAL cells were processed as described below. Next, mouse cells were treated with trypsin and washed as described above for Jurkat uptake studies.

For identification of CD4+ T cells, ~10^6 primary cells (100 μL) in FACS buffer were transferred to labeled FACS tubes and 1 μg of Fc blocking antibody (BD Biosciences, Cat. # 553141; San Jose, CA) was added to each tube followed 5 min. later by the addition of 1 μg each of Pacific Blue-conjugated anti-mouse CD4 (clone MR4-5; BioLegend; San Diego, CA) and APC-conjugated anti-TCRβ (clone H57-597; BioLegend) antibodies. After a 45 min. incubation on ice, cells were fixed with 2% paraformaldehyde in PBS and resuspended in 300 μL of FACS buffer prior to acquiring FACS data for FSC, SSC, APC, PB and FITC channels. FACS analysis was performed by gating lymphocytes based on FSC and SSC followed by gating TCR+ cells as defined by APC fluorescence. Next, TCR+ lymphocytes were further characterized by CD4 expression as defined by Pacific Blue fluorescence versus FITC fluorescence representing internalized peptide. Peptide uptake

was quantified by determining the MFI of gated CD4+ T cell in addition to determining the percentage of CD4+ FITC+ T cells based on the background FITC fluorescence of mice treated with vehicle.

2.7. In Vitro Peptide Treatment and Restimulation of Splenocytes with OVA

C57BL/6 or BALB/c mice were sensitized i.p. with 20 μg OVA, grade V (Sigma, Cat. # A-5503) / 2 mg alum (Pierce / Thermo Scientific, Inc., Cat. # 77161) and mice were sacrificed 7 days later. Spleens from each mouse were then harvested and dissociated into separate cell suspensions as described above. After RBC lysis, splenocytes were resuspended at $10x10^6$ cells/mL in supplemented RPMI without FBS. Next, splenocytes were dispensed to labeled FACS tubes, peptides were added at the indicated μM concentrations and tubes were placed inside of a humidified, 5% CO_2, 37°C incubator for 30 min.

For analysis of cytokine secretion by ELISA, 10^6 splenocytes in 0.1 mL were added to round-bottom 96-well plates and cultured as described above. For quantification of the number of cytokine producing cells, splenocytes were added in a similar manner to 96-well filter plates that were previously coated with anti-cytokine specific capture antibody and blocked as described below for the ELISpot assay. After seeding plates, peptide-treated splenocytes were stimulated by addition of an equal volume (0.1 mL) of supplemented RPMI containing 0.1-0.2 mg/mL OVA and 20% FBS for a final concentration of 50-100 μg/mL OVA and 10% FBS. After incubation for 4 days, cell culture supernatants were harvested and analyzed by ELISA or filter plates were developed as described below for the ELISpot assay.

2.8. In Vivo Peptide Treatment and Induction of Allergic Asthma in Response to OVA

For characterization of the Itk KO asthma phenotype, mice were sensitized and challenged similar to the protocol described by Mueller and August (11). First, mice were sensitized with 20 μg OVA, grade V / 2 mg alum delivered i.p. in 150 μL on days 0 and 5. On days 12-15, mice were anesthetized in the presence of 4.5% isoflurane mixed with oxygen (Airgas West; Long Beach, CA) at a flow rate of 1 L/min. and challenged daily with various amounts of OVA in 20 μL of PBS delivered intranasally (i.n.).

For peptide studies, C57BL/6 mice were injected i.p. with 25 mg/kg (~0.5 mg) of R9-QQP or R9-QQA (control) peptide at -24 and -0.5 hr. prior to sensitization with 20 μg of OVA / 2 mg alum delivered i.p. as described above on day 0. Next, peptide was again delivered i.p. 24 hours prior to the first challenge with OVA on day 7. In addition, mice were treated with peptide i.p. as before and 200 μg of peptide was delivered intranasally (i.n.) to anesthetized mice 2 hours prior to each challenge with 10 μg of OVA in 20 μL of PBS delivered i.n. on days 7-9 for a total exposure of 30 μg of OVA. Total peptide delivery was ~3.0 mg i.p. and ~0.6 mg i.n. for a total *in vivo* exposure of ~3.6 mg per mouse.

For each asthma experiment, groups of 2-4 mice were sensitized on the same date using commonly prepared OVA/alum and challenged using a common OVA/PBS solution except for OVA titration studies. Similarly, a

common peptide stock solution was prepared prior to each experiment and aliquots were used throughout the experiment for *in vivo* delivery to each mouse belonging to the same group. In addition, each experiment investigating the effect of peptides delivered *in vivo* also included a positive control group that received vehicle for comparison.

2.9. Airway Hyperresponsiveness (AHR)

Asthma was induced as described above and invasive airway hyperresponsiveness (AHR) was measured 24 hours after the final challenge with OVA using the FlexiVent small rodent ventilator system (Scireq, Inc.; Montreal, QC, Canada). Calibration of the FlexiVent system was performed prior to acquiring data for each subject according to the manufacturer's instructions.

First, mice were anesthetized by i.p. injection of a cocktail containing 80 mg/kg of ketamine (~2 mg; Fort Dodge Animal Health; Overland Park, KS) and 8 mg/kg of xylazine (~0.2 mg; Lloyd, Inc.; Shenandoah, IA). Next, tracheotomy was performed on anesthetized mice and an 18 gauge cannula (BD Medical, Cat. # 408208; Franklin Lakes, NJ) was inserted into the trachea and secured using surgical suture as described above. Then, the endotracheal cannula was connected via the adaptor end to the FlexiVent system and ventilation was initiated. Once mice were passively breathing, airway resistance was measured in response to increasing doses of aerosolized methacholine (0, 3, 6, 12 and 24 mg/mL; Sigma, Cat. # A2251) that were delivered to the lungs through the endotracheal cannula. The maximal airway resistance values for each dose of methacholine were recorded for each mouse and averaged (+/- SEM) for all mice belonging to the same treatment group.

2.10. Lung Histological Analysis

Following asthma induction and collection of BALF as described above, a single lung lobe from each mouse was extracted, rinsed with PBS and stored in a vial containing 10% zinc formalin (Protocol / Fisher HealthCare, Cat. # 313-095; Houston, TX). Preserved lung tissue was submitted to Pacific Pathology (San Diego, CA) for further processing. First, tissue was embedded in paraffin, cut into 5 μm sections around the middle of the specimen and at least four sections were mounted onto each microscopy slide. Next, separate slides from each specimen were stained as described below for detection of mucus-producing goblet cells or inflammatory cells. All slides were coded with randomly assigned numbers prior to visualization by light microscopy using a 40x objective.

Mucus was detected by staining slides with periodic acid schiff (PAS) reagent for 5 min. and rinsing with cold water followed by counter-staining with hematoxylin. Goblet cell hyperplasia and mucus production were evaluated by assigning a mucus severity score according to the percentage of each bronchiole cross section staining fuchsia as follows: a value of 0 indicates no detectable mucus, a value of 1 indicates ≤ 10% staining positive for mucus, a value of 2 indicates > 10% and ≤ 25% staining positive, a value of 3 indicates > 25% and ≤ 50% staining positive, a value of 4 indicates > 50% and ≤ 75% staining positive and a value of 5 indicates > 75% and ≤ 100% staining positive.

Recruitment of inflammatory cells to the lungs was visualized by staining slides with hematoxylin and eosin (H&E). The degree of inflammation was evaluated by assigning a score according to the depth of infiltrating cells surrounding each blood vessel or bronchiole as follows: a value of 0 indicates no infiltrating cells, a value of 1 indicates a single layer of cells, a value of 2 indicates a layer of 2-4 cells, a value of 3 indicates a layer of 5-7 cells and a value of 4 indicates a layer of ≥ 7 cells.

2.11. IgE Assay

Prior to performing bronchoalveolar lavage, mice were anesthetized using isoflurane and 0.3-0.5 mL of retroorbital blood was collected in separate microcentrifuge tubes for each mouse. After allowing blood to clot for ≥ 2 hrs. at room temperature, tubes were centrifuged for 15 min. at 3,000 rpm (4°). Sera were then transferred to a new set of tubes and stored at -20°C prior to analysis. Total serum IgE was detected by the sandwich ELISA method described below. Antibodies used for the IgE-specific ELISA were purchased from Southern Biotech (Birmingham, AL). First, 5 μg/mL of goat anti-mouse IgE (Cat. # 1110-01) in PBS and was added to individual wells of 96-well ELISA plates. After overnight incubation at 4°C, plates were blocked and either serum samples or dilutions of purified mouse IgE standard (clone 15.3; Cat. # 0114-01) ranging from 200 to 3.1 ng/mL were added in duplicate to designated wells while washing plates between each step. Next, 0.1 μg/mL of biotin-conjugated anti-mouse IgE (clone 23G3; Cat. # 1130-08) followed by 1x streptavidin-HRP diluted in 0.1 μg/mL BSA/PBS were serially added to wells and plates were processed from addition of substrate to measurement of absorbance as described below.

2.12. Bronchoalveolar Lavage and Lung Tissue Dissociation

24 hours after the final challenge with OVA, mice were sacrificed and dissected to reveal the chest cavity and trachea. Tracheotomy was performed by creating a small incision in the trachea at the location of the cartilage ring. Next, an endotracheal cannula consisting of polyethylene tubing connected to the end of an 18 gauge needle was inserted via the tubing into the trachea to a depth of 1 cm posterior to the incision. After securing the tubing using surgical suture, 1 mL of ice-cold PBS was slowly dispensed to the lungs using a syringe attached to the adaptor end of the 18 gauge needle. Next, bronchoalveolar lavage fluid (BALF) was aspirated and dispensed to a labeled FACS tube and kept on ice throughout. After repeating for a total of 5 times for each mouse, BALF was centrifuged for 5 min. at 1,500 rpm (4°C). RBCs were then lysed by resuspending the pellet in ACK lysis buffer and incubating for 5 min. at room temperature. Next, BALF cells were washed, resuspended in 300 μL of FACS buffer and counted using a hemacytometer to determine total cell number.

Following bronchoalveolar lavage, individual lung lobes were excised, rinsed in a petri dish filled with PBS and minced using forceps into 1-2 mm pieces. Next, tissue was transferred to a FACS tube containing 1 mL of culture medium supplemented with 3 mg/mL of collagenase (Worthington Biochemical Corp., Cat. # LS004186; Lakewood, NJ) and 100 μg/mL of DNAse I (Sigma, Cat. # DN25). After enzymatic digestion for 1 hr. at 37°C,

tissue was transferred onto 70 μm cell stainers and mechanically dissociated followed by rinsing with FACS buffer and recovery of cells. Next, lung cells were centrifuged for 5 min. at 1,500 rpm (4°C), resuspended in 4 mL of FACS buffer and counted using a hemacytometer.

2.13. Cytospin Preparation and Staining for Quantification of Inflammatory Cells by FACS

For preparation of cytospin slides, ~10^5 BAL cells in 400 μL of FACS buffer were added to cytology funnel / filter card / slide assemblies (Biomedical Polymers, Inc., Cat. # BMP-CYTO-S50; Gardner, MA) and were attached to glass slides after centrifugation for 5 min. at 600 rpm using a Shandon Cytospin 2 centrifuge (Thermo Scientific, Inc.; Waltham, MA). Next, slides were stained using a modified Wright-Giemsa dye according to the manufacturer's instructions (Protocol / Fisher HealthCare, Cat., # 122-911) and coverslips were adhered following application of 10 μL of Permount mounting medium (Fisher Scientific, Cat. # SP15-100). Alevolar macrophages were identified by light microscopy as large cells with atopic nuclei and cytoplasm staining purple while eosinophils were identified as small cells with bilobed nuclei and cytoplasmic granules staining pink.

Quantification of inflammatory cells by FACS was performed using ~10^5 BAL cells or ~10^6 lung cells in 100 μL of FACS buffer obtained as described above. Staining was performed in FACS tubes using 0.5 μg of all antibodies which were purchased from BD Biosciences. 5 min. prior to the addition of fluorophore-conjugated antibodies, Fc receptors expressed on cells were blocked by the addition of rat anti-mouse CD16/CD32 (BD Biosciences, Cat. # 553141).

Staining for eosinophils and macrophages was performed according to the method described by Stevens and Braciale (133). First, a cocktail containing 0.5 μg each of PE-conjugated rat anti-Siglec-F (BD Biosciences, Cat. # 552126), PerCP-Cy5.5 rat anti-CD45 (BD Biosciences, Cat. # 550994) and FITC-conjugated hamster anti-mouse Cd11c (BD Biosciences, Cat. # 557400) was directly added to tubes containing BALF or lung cells. After incubation for 45 min. at 4°C, cells were washed with FACS buffer and fixed for 10 min. with 2% paraformaldehyde on ice. Next, cells were washed again and resuspended in 300 μL of FACS buffer prior to acquisition of FACS data for FSC, SSC, PE, PerCP-Cy5.5 and FITC channels. A minimum of 10,000 or 20,000 events were collected for BAL or lung cell samples, respectively. FACS analysis was performed by first generating a histogram displaying PerCP-Cy5.5 fluorescence and gating total leukocytes as defined by CD45 expression. Next, CD45+ events were further analyzed by generating a two-color plot displaying PE fluorescence on the y-axis versus FITC fluorescence on the x-axis. Eosinophils and macrophages were both positively defined by expression of Siglec-F and were differentially gated based on low versus high expression of Cd11c, respectively. Eosinophil and macrophage percentages of CD45+ cells were recorded for each sample.

For quantification of T cells, a cocktail containing 0.5 μg each of PerCP-Cy5.5 rat anti-CD45 and PE-Cy7 hamster anti-CD3ε antibodies (BD Biosciences, Cat. # 552774) was added to a separate aliquot of BAL cells.

After incubation on ice, stained cells were fixed and resuspended in 300 μL of FACS buffer while washing between each step as described above. Next, a minimum of 20,000 events were acquired for FSC, SSC, PerCP-Cy5.5 and PE-Cy7 channels. FACS analysis was then performed by gating CD45+ leukocytes as described above followed by further defining lymphocytes based on FSC versus SSC. Lastly, CD45+ lymphocytes were further analyzed by generating a plot displaying FSC on the y-axis versus PE-Cy7 fluorescence on the x-axis and gating TCR+ lymphocytes based on the background PE-Cy7 fluorescence of BAL cells from negative control mice. T cell percentages of CD45+ cells (grandparent population) were recorded for each sample.

2.14. Flow Cytometry

Cells used for flow cytometry were stained with fluorophore-conjugated antibodies, fixed with 2% paraformaldehyde / PBS and resuspended in FACS buffer inside 5 mL round-bottom FACS tubes (BD Biosciences, Cat. # 352054) while being protected from light and on ice throughout. FACS data was acquired within 72 hours after fixing cells and 10,000 - 50,000 events were acquired for each sample. Prior to acquiring FACS data, voltages for all channels were calibrated using CST beads (BD Biosciences) and compensation was performed based on single-color control samples to correct for spectral overlap among fluorophores as needed. FACS analysis and graphs created for figures were created using FlowJo software (Tree Star, Inc., Ashland, OR). Tick marks displayed on FACS plots represent a linear scale for FSC and SSC channels and a logarithmic scale for fluorescence channels.

Acquisition of FACS data for peptide uptake studies using mouse primary cells was performed using a BD FACSAria flow cytometer. Each fluorophore was excited using a different laser as follows: Pacific Blue (PB) was excited using a 405 nm laser and was detected using a 450/40 emission filter; FITC was excited using a 488 nm laser and was detected using a 530/30 nm emission filter; and, APC was excited using a 633 nm laser and detected using a 660/20 emission filter. For peptide uptake studies using

Jurkat cells, FACS data was acquired using the 488 nm excitation laser in combination with the 530/30 nm emission filter.

Acquisition of FACS data for quantification of inflammatory cells from BALF and lung cell samples was performed using a BD FACSCanto flow cytometer using the 488 nm laser. Samples stained for quantification of eosinophils and macrophages were excited using the 488 nm laser with detection of PE using the 585/42 nm filter, of PerCP-Cy5.5 using the 670 nm LP filter and of FITC using the 530/30 nm filter. Independent samples were stained for quantification of T cells and were excited using the 488 nm laser with detection of PerCP-Cy5.5 using the 670 nm LP filter and of PE-Cy7 using the 780/60 nm filter.

2.15. DLN Cell Restimulation with OVA

For identification of DLN, the chest cavity was expanded by pinning the left and right sides of the rib cage to the dissection board. Assisted by a dissection microscope and light source, individual DLN were excised using fine point forceps and transferred to a 15 mL tube containing culture medium on ice. In order to increase cell yield, DLN from groups of 2-3 mice were pooled prior to dissociation into a cell suspension. Next, RBCs were lysed, cells were counted and 10^5 or 10^6 cells in 0.1 mL were seeded to round-bottom 96-well tissue culture treated plates. Next, cells were stimulated by addition of an equal volume (0.1 mL) of culture medium containing 0.1 mg/mL OVA for a final concentration of 50 μg/mL of OVA. After incubation for 4 days, cell culture supernatants were harvested and cytokine secretion was analyzed by ELISA as described below.

2.16. ELISA

Cytokines were quantitated from cell-free samples by sandwich ELISA for IL-4, IL-5, IL-13 and IFN-γ. The detection limit was 4 pg/mL for IL-4, IL-5 and IL-13 and 31.3 pg/mL for IFN-γ. Anti-mouse IL-4 capture antibody (clone 11B11), recombinant standard and biotin-conjugated detection antibody (clone BVD6-24G2) were purchased from eBioscience, Inc. (San Diego, CA). Anti-mouse IL-5 capture antibody (clone TRFK5), recombinant standard and biotin-conjugated detection antibody (clone TRFK4) were purchased from eBioscience, Inc. Anti-mouse IL-13 capture antibody (clone eBio13A), recombinant standard and biotin-conjugated detection antibody (clone eBio1316H) were purchased from eBioscience, Inc. Anti-mouse IFN-γ capture antibody (clone R4-6A2) and biotin-conjugated detection antibody (clone XMG1.2) were purchased from BD Biosciences and recombinant standard was purchased from eBioscience, Inc.

Capture antibodies were diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) at 2 μg/mL for IL-4, IL-5 and IL-13 or at 4 μg/mL for IFN- γ and were added to individual wells of designated 96-well ELISA plates (BD Biosciences, Cat. # 353279). After overnight incubation at 4°C, plates were washed (0.05% Tween-20 / PBS), blocked for 1.5 hrs. with 1% BSA (Fisher Scientific, Inc., Cat. # BP1600-100) in PBS and washed again prior to addition of 50 μL volume per well in duplicate of samples or recombinant standards diluted in 0.1% BSA/PBS. After incubation with samples for 1.5 hrs., plates

were washed between each step and serially incubated with biotin-conjugated detection antibodies at 2 μg/mL and with 1x streptavidin-HRP (BD Biosciences, Cat. # 51-9002812) both diluted in 0.1% BSA/PBS for 1.5 hrs. and 45 mins., respectively. Next, 1x TMB substrate (eBioscience, Inc., Cat. # 00-4201-56) was added to each well followed directly by addition of stop solution (1 M H_3PO_4) within 30 min. Finally, absorbance was measured at 450 nm with subtraction at 650 nm using a filter-based Emax Endpoint ELISA Microplate Reader (Molecular Devices, Sunnyvale, CA). Based on cytokine-specific standard curves generated independently for each plate, absorbance values for each sample were reported as cytokine concentrations in pg/mL or ng/mL as determined using SoftMax Pro software (Molecular Devices).

2.17. ELISpot Assay

For quantification of the number of cytokine producing cells, anti-mouse IL-4 capture antibody (eBioscience, Inc., clone 11B11) was diluted to 4 μg/mL in PBS and added to 96-well filter plates (EMD Millipore, Cat. # MSIPS4510; Billerica, MA). After overnight incubation at 4°C, plates were washed (0.05% Tween-20 / PBS) and blocked with 1% BSA/PBS + 0.05% Tween-20 for 2 hrs. at 37°C. Next, plates were seeded and peptide-treated splenocytes were restimulated with OVA as described above. After incubation for 4 days, filter plates were washed between each step and serially incubated with biotin-conjugated anti-IL-4 (clone BVD6-24G2) detection antibody at 0.5 μg/mL and with 1x streptavidin-HRP (BD Biosciences) both diluted in 1% BSA/PBS + 0.05% Tween-20 for 2 hrs. and 45 min., respectively. Next, AEC (3-amino-9-ethyl-carbazole; Sigma, Cat. # A6926-50TAB) substrate solution was added to each well and plates were washed with tap water within 30 min. Plates were then allowed to dry completely and individual spots representing cytokine-producing cells were quantitated using an automated Zeiss ELISpot reader and KS ELISpot software (Carl Zeiss AG; Oberkochen, Germany).

2.18. Statistical Analysis

Graphs were created using Prism software (GraphPad Software; La Jolla, CA) and display the average and SEM of cumulative data that were obtained from individual mice or groups of mice as stated in the figure legends. Statistical significance for OVA-dependent cytokine production following *in vitro* peptide treatment of splenocytes was calculated using a paired student's t test comparing cytokine amounts from peptide treated culture supernatants to untreated, positive control (PC) samples that were obtained from the same mouse. An unpaired student's t test was used for all other statistical comparisons and each peptide treatment group was compared to vehicle for calculation of statistical significance. For all p values reported, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Guimond, D.M., Cam, N.R., Hirve, N., Duan, W., Lambris, J.D., Croft, M., & Tsoukas, C.D (2012). Treatment of Experimental Asthma Using a Novel Peptide Inhibitor of the Inducible T Cell Kinase. The dissertation author was the primary author of this material.

3. RESULTS

3.1. R9-QQP and control peptides delivered *in vitro* efficiently enter Jurkat T cells

In order to determine the ability of R9-QQP to enter cells, FITC was covalently tagged at the N-terminus and FITC fluorescence was measured as a reporter of cellular uptake following treatment of Jurkat T cells with FITC-R9-QQP *in vitro*. For assessment of specificity, the SLP-76 polyproline ligand represented by the R9-QQP peptide sequence was scrambled to generate the control peptide named R9-PQM. As an additional control for specificity used in this study, each proline in the sequence of the R9-QQP peptide was substituted with an alanine to generate the peptide named R9-QQA. For evaluation of peptide uptake, aliquots of Jurkat cells were incubated with 1 μM, 5 μM and 10 μM of FITC-R9 peptides for 30 min. and cells were washed with ice-cold PBS to remove free peptide immediately following the incubation period as described in Materials and Methods. In order to enhance detection of internalized peptide, peptide-treated cells were then incubated with trypsin prior to acquisition of FITC fluorescence by FACS.

In a separate control experiment, the efficacy and kinetics of the trypsin treatment protocol were first established by incubating aliquots of Jurkat cells with trypsin for various time intervals followed by staining for endogenous expression of the trypsin-sensitive transferrin receptor (CD71). After

treatment with trypsin, Jurkat cells were stained with anti-CD71 (clone OTK9) antibody followed by secondary detection using FITC-conjugated goat anti-mouse antibody. Next, stained cells were fixed with paraformaldehyde and FITC fluorescence was measured by FACS. As displayed in Figure 7A, mean FITC fluorescence for each sample was inversely correlated with trypsin incubation time suggesting that trypsin was effective in enzymatically digesting the transferrin receptor that was accessible at the cell surface. Specifically, incubation with trypsin for 10 min. resulted in a 80.4% reduction in the ability to detect surface expression of CD71 (i.e., from 20,915 relative fluorescence units for untreated cells as compared to 4,092 units) and this incubation time was used for subsequent peptide uptake studies.

In continuation, peptide-treated cells were incubated with trypsin for 10 min. as established above to enhance detection of internalized rather than membrane-associated peptide. Next, trypsin was quenched by addition of medium containing 10% FBS and cells were again washed for three additional times with ice-cold PBS. Lastly, cells were fixed with paraformaldehyde and FITC fluorescence was acquired by FACS. As displayed in overlaid histograms (Figure 7B) and in bar graphs (Figure 7C), incubation of Jurkat cells with increasing concentrations of FITC-R9 peptides correlated with increasing FITC fluorescence indicating that all peptides efficiently enter cells. Furthermore, comparison of fluorescence of cells treated with different peptides at the same nominal concentration suggests that the R9-PQM and

R9-QQA control peptides are no less efficiently taken up by cells as compared to the R9-QQP inhibitor peptide.

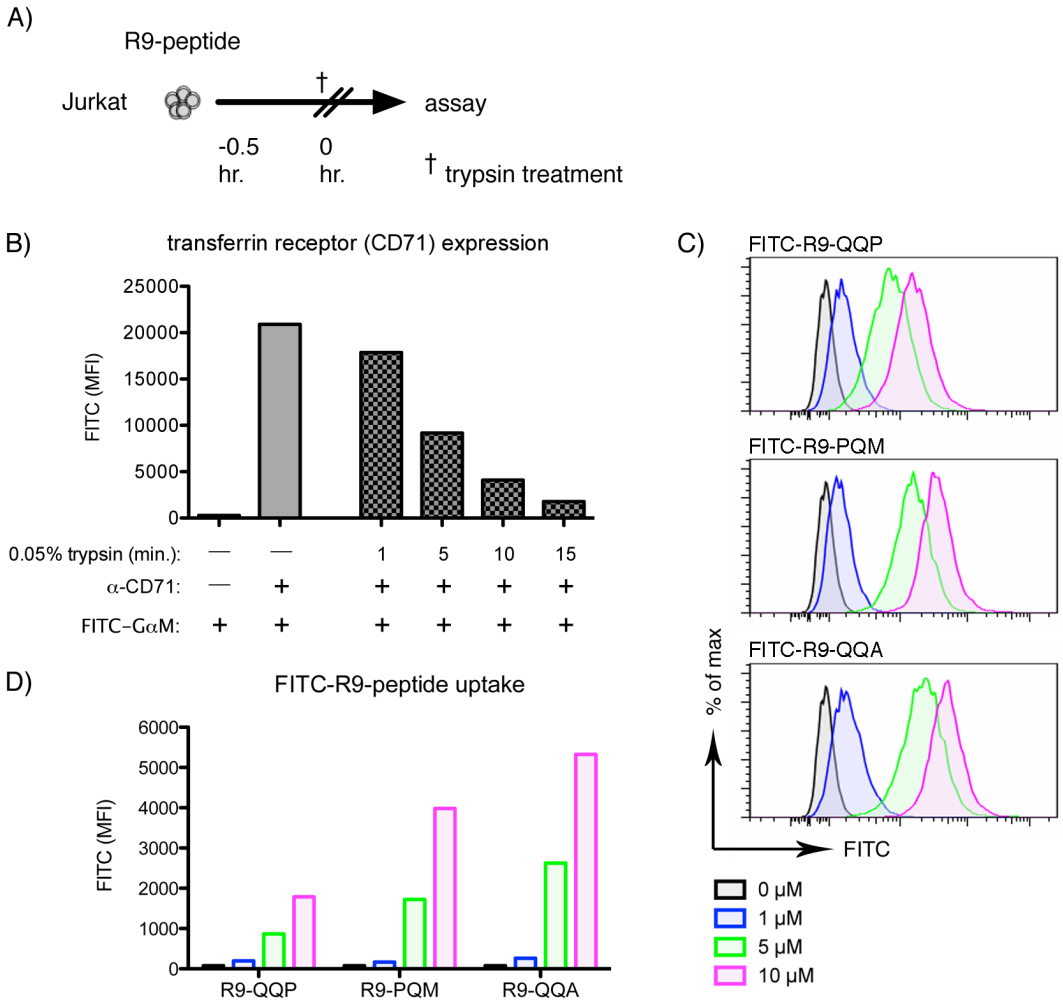


Figure 7: FITC-conjugated R9-peptides are efficiently taken up by Jurkat T cells.

A) Schematic diagram of *in vitro* peptide treatment protocol. B) In order to determine the effectiveness and kinetics of trypsin used for peptide uptake studies, Jurkat cells were treated with 0.05% trypsin for various amounts of time as described in Materials and Methods. Next, trypsin-treated Jurkat cells were stained with anti-CD71 (OKT9) antibody followed by FITC-conjugated goat anti-mouse (GαM) secondary antibody and surface expression of the trypsin-sensitive transferrin receptor was evaluated by FACS. C) Jurkat cells were treated with various μM concentrations of FITC-conjugated R9-peptides for 30 min. followed by treatment with trypsin for 10 min. Overlaid histograms display FITC fluorescence of Jurkat cells after incubation with various concentrations of each peptide. D) Bar graph compares mean FITC fluorescence intensity (MFI) of FACS data in panel B.

3.2. R9-QQP added to splenocytes *in vitro* inhibits OVA-dependent cytokine secretion in a dose-dependent and peptide-specific manner

In order to assess the ability of the R9-QQP peptide to inhibit OVA-dependent Th2 cytokine secretion *in vitro*, mice were sensitized with OVA/ alum on day 0 and spleens were harvested and processed into splenocyte suspensions after 7 days as described in Materials and Methods. As illustrated in Figure 8A, separate aliquots of splenocytes were then treated with peptides *in vitro* and 10^6 cells were subsequently added to designated wells of round-bottom 96-well plates. Next, splenocytes were restimulated by the addition of OVA in culture medium and 96-well plates were incubated for 4 days to allow production and secretion of cytokines into the culture medium. As displayed in Figures 8 and 9, cytokine secretion from independent cultures representing different peptide treatments was measured by testing tissue culture supernatant samples for the concentration of IL-4, IL-5, IL-13 and IFN-γ by ELISA which was performed as described in Materials and Methods.

As shown in panels B, D and E of Figure 8, treatment of splenocytes *in vitro* with R9-QQP ranging from 5-100 μM resulted in a dose-dependent reduction of the Th2 cytokines IL-4, IL-5 and IL-13 upon restimulation with OVA. Specifically, IL-4 was reduced from 34.2 pg/mL to 13.0 pg/mL over this range of R9-QQP concentrations representing a 62% decrease in cytokine secretion. Similarly, the level of IL-5 was reduced from 594.3 pg/mL to 318.2

pg/mL and IL-13 was reduced from 2,704 pg/mL to 938.6 pg/mL representing 46.5% and 65.3% decreases in Th2 cytokine secretion, respectively.

In addition to analyzing cytokine secretion, the effect of R9-QQP on cytokine production was assessed using the ELISpot assay which was performed as described in Materials and Methods. Sensitization of mice with OVA/alum, processing of spleens into splenocytes and *in vitro* peptide treatment were performed identically to the experiments described for analyzing cytokine secretion above. For the ELISpot assay, 10^6 splenocytes per well were seeded onto filter plates that were pre-coated with anti-IL-4 capture antibody. After incubation for 4 days, filter plates were developed and spots on the bottom of each well representing individual cytokine producing cells were counted and averaged for replicate wells. As displayed in Figure 8C, treatment of splenocytes *in vitro* with concentrations of R9-QQP ranging from 5-50 μM did not affect the number of cytokine producing cells which varied from 59.5 - 81 spots/well upon restimulation with OVA versus 21.5 spots/well for non-stimulation control as determined by the ELISpot method. In contrast, treatment of splenocytes with R9-QQP across this same range of concentrations resulted in a dose-dependent decrease in cytokine secretion as measured by ELISA (Fig. 8B).

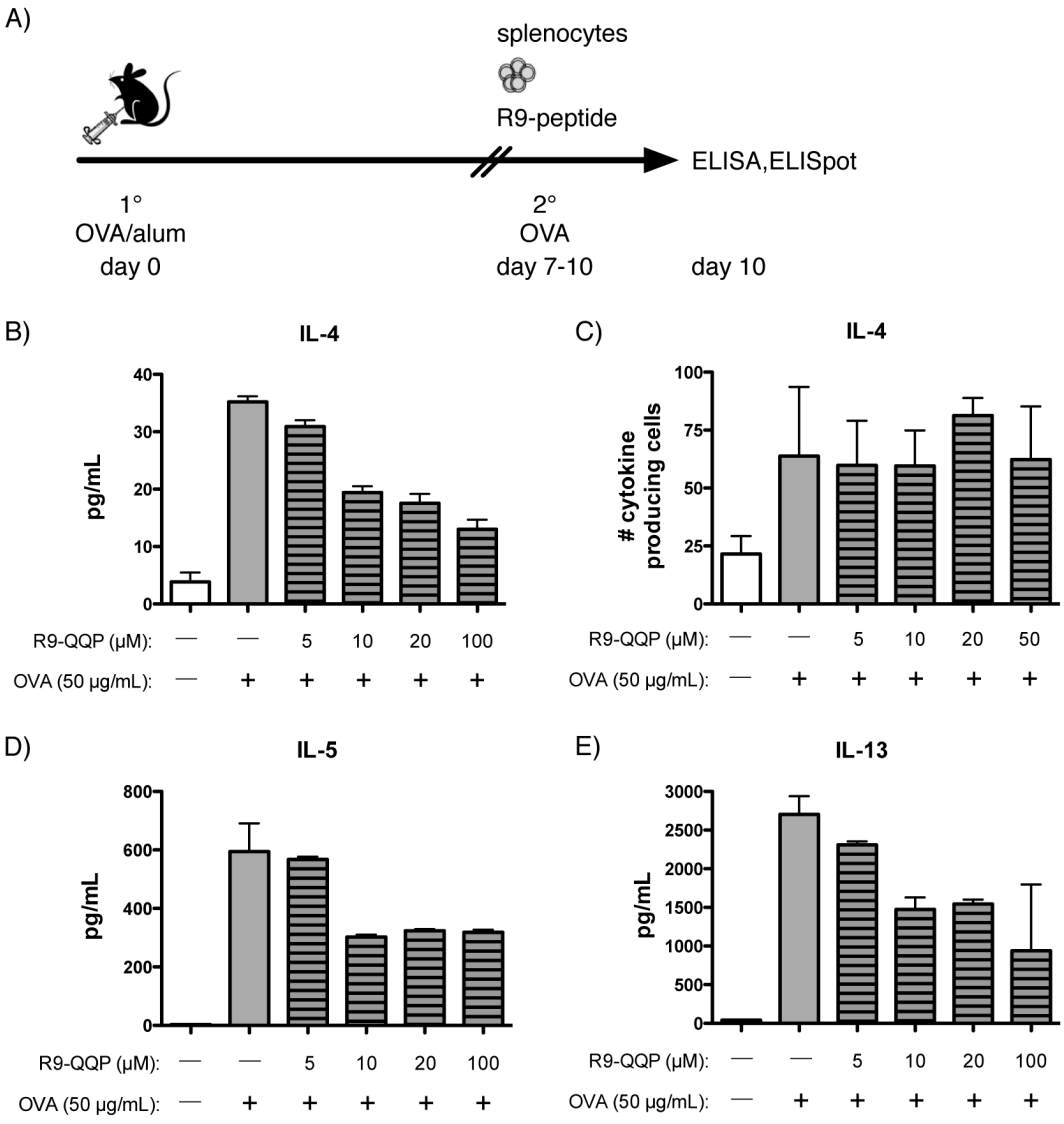
Furthermore, inhibition by R9-QQP was specific for the peptide cargo sequence as determined by treatment of splenocytes at the effective concentration of 20 μM with R9-PQM or R9-QQA control peptides followed by

analysis of cytokine secretion by ELISA. In order to determine statistical significance, a paired comparison was performed between splenocytes treated with 20 μM of R9-QQP or R9-PQM / R9-QQA peptides to splenocytes derived from the same mouse that were treated with vehicle and restimulated with OVA. For these experiments, independent replicates for statistical analysis were generated from splenocytes cultures that were obtained from 5-6 separate mice. Overall, these experiments revealed that treatment with R9-QQP at 20 μM significantly and specifically inhibited Th2 cytokines while the signature Th1 cytokine IFN-γ was elevated independently of restimulation with OVA and was not affected by treatment with either peptide. Specifically, treatment of splenocytes with 20 μM of R9-QQP significantly inhibited IL-4 secretion from an average concentration of 145.9 pg/mL for treatment with vehicle to 95.4 pg/mL representing a decrease of 34.6% ($p < 0.01$) while treatment with 20 μM of R9-PQM control peptide resulted in an average IL-4 concentration of 152.7 pg/mL (Fig. 9A). Similarly, treatment with 20 μM of R9-QQP significantly inhibited IL-5 secretion from 700.5 pg/mL to 486.7 pg/mL representing a 30.5% decrease ($p < 0.05$) while treatment with 20 μM of R9-QQA resulted in an average IL-5 concentration of 742.1 pg/mL (Fig. 9B). In addition, treatment with 20 μM of R9-QQP significantly inhibited IL-13 secretion from 5,670 pg/mL to 3,616 pg/mL representing a 36.2% decrease while treatment with 20 μM of R9-QQA resulted in an average IL-13 concentration of 5,672 pg/mL (Fig. 9C). Lastly, IFN-γ secretion was not

affected by either restimulation with OVA or by treatment with either R9-QQP inhibitor or R9-PQM control peptide and ranged from 3,661 - 4,559 pg/mL (Fig. 9D).

Figure 8: R9-QQP added *in vitro* inhibits OVA-dependent Th2 cytokine production by splenocytes in a dose-dependent manner.

As described in Materials and Methods, BL/6 mice were sensitized i.p. with OVA/alum and spleens were harvested after 7 days. Splenocytes were then treated with 5-100 μM of R9-QQP and dispensed to designated wells of 96-well plates. Next, OVA in culture medium was added to designated wells for restimulation at a final concentration of 50 μg/mL. After 4 days of culture *in vitro*, supernatants were harvested and tested for cytokines by ELISA or filter plates were processed to detect cytokine producing cells by ELISpot as described in Materials and Methods. B, D & E) Bar graphs display pg/mL of IL-4, IL-5 and IL-13 measured from culture supernatants by ELISA. C) Bar graph displays number of IL-4 producing cells as determined by ELISpot. B-E) Bar graphs display average values and standard deviation from replicate wells.


A)
splenocytes
R9-peptide
ELISA,ELISpot
1°
OVA/alum
day 0
2°
OVA
day 7-10
day 10
B)
IL-4
pg/mL
R9-QQP (μM):
OVA (50 μg/mL):
C)
IL-4
cytokine producing cells
R9-QQP (μM):
OVA (50 μg/mL):
D)
IL-5
pg/mL
R9-QQP (μM):
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R9-QQP (μM):
OVA (50 μg/mL):

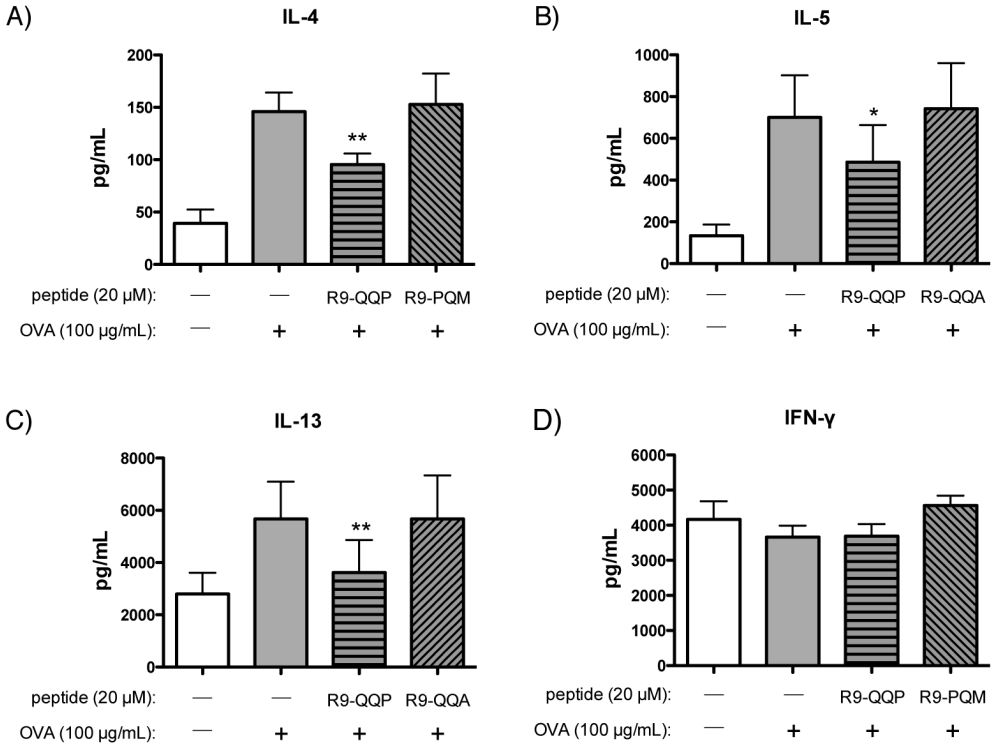


Figure 9: R9-QQP added *in vitro* specifically inhibits OVA-dependent Th2 cytokine production by splenocytes.

Similar to the experiment described in figure 1, BALB/c mice were sensitized i.p. with OVA/alum and spleens were harvested after 7 days. Splenocytes were then treated with 20 μM of R9-QQP or R9-PQM / R9-QQA (control peptides) and dispensed to designated wells of 96-well plates. Next, OVA in culture medium was added to designated wells for restimulation at a final concentration of 100 μg/mL. After 4 days of culture *in vitro*, supernatants were harvested and tested for cytokines by ELISA. A-D) Bar graphs display the average and SEM of 5-6 mice analyzed per group for IL-4, IL-5, IL-13 and IFN-γ ELISA, respectively. Statistical significance was calculated using a paired student's t test comparing cytokine amounts of peptide-treated to untreated (positive control) culture supernatants for each mouse (* = $p < .05$; ** = $p < 0.01$).

3.3. Itk$^{-/-}$ mice have reduced eosinophilic inflammation as compared to WT mice upon challenge with low but not high doses of OVA

Prior to investigating the effects of the R9-QQP inhibitor peptide, the previously reported defective inflammatory responses of Itk$^{-/-}$ mice upon challenge in the OVA-induced asthma model were verified by evaluation of inflammatory cells collected from bronchoalveolar lavage fluid (BALF) (11, 76). First, WT and Itk$^{-/-}$ mice were sensitized with 20 μg OVA / 2 mg alum delivered intraperitoneally (i.p.) on days 0 and 5. During the challenge period from days 12-15, WT and Itk$^{-/-}$ mice were briefly anesthetized using isoflurane and challenged with 10 μg of OVA delivered intranasally (i.n.) for a total exposure of 40 μg of OVA (Figure 10A). On day 16, mice were sacrificed and BALF was collected and processed for each mouse as described in Materials and Methods. Next, BAL cells were attached to separate cytospin slides and stained using a modified Wright-Giemsa dye to qualitatively evaluate the presence of inflammatory cells. In contradiction to previously published reports, a large infiltrate of granulocytes characteristic in appearance to eosinophils was observed on cytospin slides prepared from both WT and Itk$^{-/-}$ mice that were challenged with a total of 40 μg of OVA (Figure 10B).

In order to confirm the genotype of the Itk$^{-/-}$ mice used in these experiments, tail fragments were obtained from both WT and Itk$^{-/-}$ mice and genomic DNA was purified as described in Materials and Methods. Next, genomic DNA was amplified by PCR using primers for Itk Exon 2 and Intron 4

as well as for the neomycin cassette that was used to replace Exon 3 to generate the Itk$^{-/-}$ mice. Lastly, PCR products were resolved by agarose gel electrophoresis prior to the genomic diagnosis of each mouse. Analysis of gel images obtained using this method confirmed the presence of the predicted 7.5 and 1.9 kb band fragments corresponding to the WT and Itk$^{-/-}$ alleles for each mouse strain, respectively (data not shown). Furthermore, the lack of Itk protein expression by Itk$^{-/-}$ mice was confirmed by immunoblotting. For this analysis, spleens were obtained from WT and Itk$^{-/-}$ mice followed by processing to generate splenocyte lysate samples. Next, lysates were resolved by SDS-PAGE and transferred to a PVDF membrane followed by incubation with anti-Itk (clone 2F12) antibody. After secondary incubation with HRP-conjugated goat anti-mouse antibody, protein expression was detected by the addition of enhanced chemiluminescent (ECL) substrate. As displayed in Figure 10C, analysis of gel images revealed a 72 kDa band corresponding to the predicted size of Itk from lysate samples prepared from WT but not Itk$^{-/-}$ mice. Thus, the unexpected Itk knockout phenotype observed in the OVA-induced asthma model could not be attributed to mis-identification of the Itk$^{-/-}$ mice used in these studies.

In pursuit of the previously reported defect, the total amount of OVA provided during the challenge period was reduced by a factor of 10. For these experiments, WT and Itk$^{-/-}$ mice were sensitized on days 0 and 5 as described above and challenged with 1 μg of OVA delivered intranasally on days 12-15

for a total exposure of 4 μg (Figure 10A). While BALF samples obtained from both WT and Itk$^{-/-}$ mice indicated a high degree of inflammation upon challenge with 40 μg of OVA, examination of cytospin slides prepared from mice challenged with a total exposure of 4 μg of OVA revealed a greatly reduced eosinophilic infiltrate obtained from both WT and Itk$^{-/-}$ mice as expected. Furthermore, detailed examination of cytospin slides at 400x magnification revealed an almost complete lack of eosinophils obtained from Itk$^{-/-}$ mice challenged with 4 μg of OVA as compared to WT mice which displayed moderate eosinophilic inflammation (Figure 10B).

In order to quantify the inflammatory phenotype described above, fluorophore-conjugated antibodies were used to stain eosinophils followed by FACS analysis performed according to the method described by Stevens and Braciale (133). Briefly, cells obtained from BALF samples were stained using an antibody cocktail containing PE-conjugated anti-Siglec-F, PerCP-Cy5.5-conjugated anti-CD45 and FITC-conjugated anti-Cd11c as described in Materials and Methods. Fluorescence of stained cells was then acquired by FACS followed by multi-color analysis to identify eosinophils. First, total leukocytes contained in BALF samples were gated as defined by expression of the surface marker CD45. Next, gated CD45+ cells were further analyzed using two-color density plots to display expression of Siglec-F versus Cd11c. As identified by a Siglec-F+ Cd11c- expression profile, eosinophils were gated and quantified as a percentage of the parent CD45+ population. Furthermore,

alveolar macrophages were quantified based on a Siglec-F+ Cd11c+ expression profile that inversely correlates with the percentage of eosinophils with respect to the parent CD45+ population.

As displayed in Figure 11A, the representative density plots generated as described above indicate that eosinophils represent ~70% of total leukocytes obtained from both WT and Itk$^{-/-}$ mice that are challenged with 40 μg of OVA. Consistent with the qualitative evaluation of the cytospin slides, eosinophilia was less robust for WT mice challenge with 4 μg of OVA (i.e., ~30% versus ~70% of total leukocytes) while Itk$^{-/-}$ mice displayed an almost complete reduction in eosinophilia at this dose of OVA. Significantly, the cumulative data displayed in Figure 11B reveal that eosinophilic inflammation as measured by the percentage of total leukocytes is reduced in BALF samples obtained from Itk$^{-/-}$ versus WT mice that are challenged with a total of 4 μg of OVA (average of 31.7% eosinophilia for WT mice versus 2.2% for Itk$^{-/-}$ mice; $p < 0.01$). Conversely, the cumulative data displayed in Figure 11C reveal that the percentage of alveolar macrophages from Itk$^{-/-}$ mice is significantly increased as compared to WT mice (average of 52.4% alveolar macrophages for WT mice versus 78.8% for Itk$^{-/-}$ mice; $p < 0.05$). Thus, Itk$^{-/-}$ mice display defective eosinophilic inflammation from BALF upon challenge with low but not high doses of OVA.

Consistent with the analysis of BALF samples, eosinophilia is also reduced in the lung tissue of Itk$^{-/-}$ mice upon challenge with low but not high

doses of OVA (Figure 12). After collecting BALF samples, individual lung lobes were extracted from mice and lung tissue was dissociated into a cell suspension as described in Materials and Methods. Next, lung cells were stained with fluorophore-conjugated antibodies for quantification of eosinophils and macrophages as described above. Overall, eosinophilia as a percentage of total leukocytes was reduced in lung tissue as compared to BALF samples. For example, eosinophils accounted for ~70% of BALF leukocytes versus ~15% of lung cell leukocytes obtained from both WT and $Itk^{-/-}$ mice that were challenged with 40 μg of OVA (Figures 11B and 12B). Despite the smaller detection range in lung tissue, the cumulative data displayed in Figure 12B indicate that eosinophilia is significantly reduced in $Itk^{-/-}$ versus WT mice that are challenged with 4 μg of OVA (average of 7.6% eosinophilia for WT mice versus 3.7% for $Itk^{-/-}$ mice; $p < 0.05$). However, no differences were detected in terms of macrophage percentages in the lung tissue (Figure 12C).

In order to determine the half maximal effective dose of OVA (EC_{50}), WT and $Itk^{-/-}$ mice were challenged with total doses of OVA ranging from 0.4 μg to 160 μg. Next, eosinophil percentages obtained from BALF and lung tissue were plotted as a function of total OVA exposure and a sigmoidal cure fit was applied to the recorded data points (Figures 11D and 12D). In summary, the EC_{50} response to OVA as determined from BALF eosinophilia was 4.1μg and 10.2 μg for WT and $Itk^{-/-}$ mice, respectively (Figure 11D).

Furthermore, the EC_{50} response to OVA as determined from lung tissue eosinophilia was 4.2 μg and 25.3 μg for WT and Itk$^{-/-}$ mice, respectively (Figure 12D). Taken together, the qualitative and quantitative approaches described above indicate that Itk$^{-/-}$ mice have reduced but not completely defective inflammatory responses to OVA as determined by BALF and lung tissue eosinophilia. Informed by these studies, an OVA dose within the Itk-sensitive range was established for investigation of the inhibitory effects of R9-QQP applied to the asthma model.

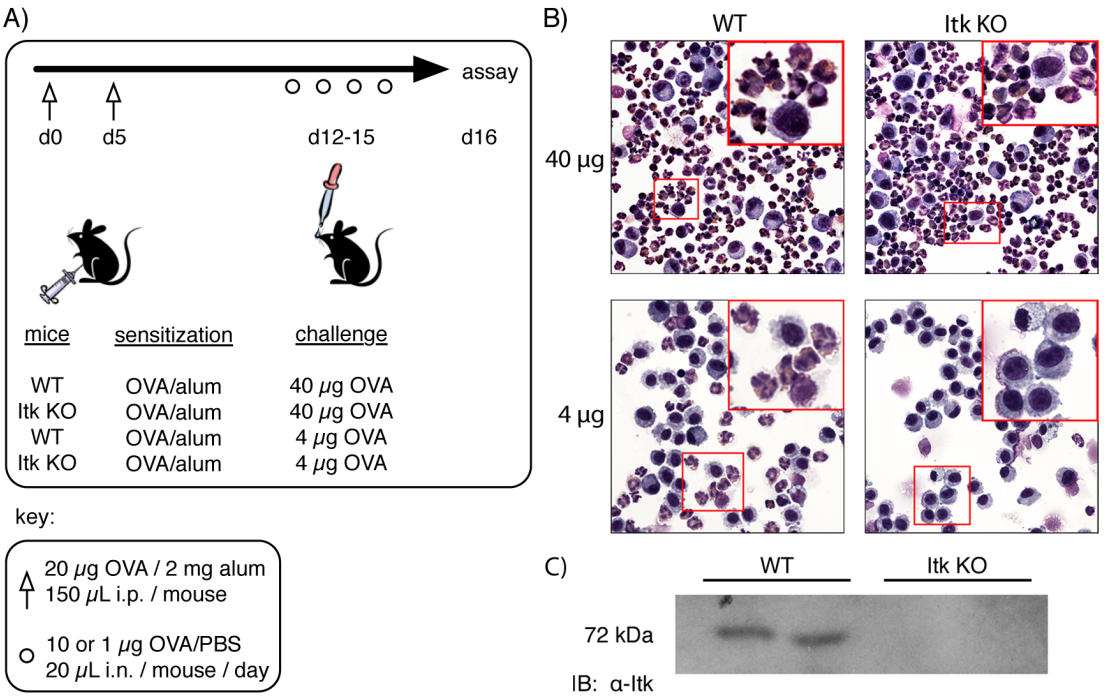


Figure 10: BALF inflammation is reduced in Itk KO mice when challenged with a low amount of OVA.

C57BL/6 (WT) and Itk KO mice were sensitized and challenged intranasally with either a total of 40 μg or 4 μg of OVA. B) Micrographs display representative fields acquired at 400x magnification from BALF cytospin slides that were stained using a modified Wright-Giemsa dye to identify infiltrating leukocytes. Inset box in upper right corner of each micrograph shows additional digital magnification of corresponding region outlined in original image. C) Next, splenocytes from WT an Itk KO mice were lysed and samples were resolved by SDS-PAGE gel electrophoresis followed by immunoblotting for detection of Itk expression as described in Materials and Methods.

Figure 11: BALF eosinophilia is reduced in Itk KO mice challenged with a low amount of OVA.

FACS plots display eosinophils (eos) and macrophages (Mac) of representative wild type (WT) and Itk KO mice challenged with a total of 40 or 4 μg of OVA. Numbers above each gate indicate the percentage of CD45+ cells for each population and tick marks indicate fluorescence intensity on a log scale. B & C) Bar graphs display the average and SEM of eosinophil and macrophage percentages of CD45+ cells for the indicated numbers (N) of WT and Itk KO mice challenged with either a total of 40 or 4 μg of OVA. Statistical significance was determined using an unpaired student's t test comparing WT and Itk KO mice for each amount of OVA (* = $p < 0.05$; ** = $p < 0.01$). D) XY graph displays the eosinophil percentage of CD45+ cells as a function of the total amount of OVA (μg) delivered i.n. during the challenge period on a log scale for WT and Itk KO mice. A sigmoidal dose response cure was applied in order to determine a half maximal effective dose (EC_{50}) of OVA for WT as compared to Itk KO mice.

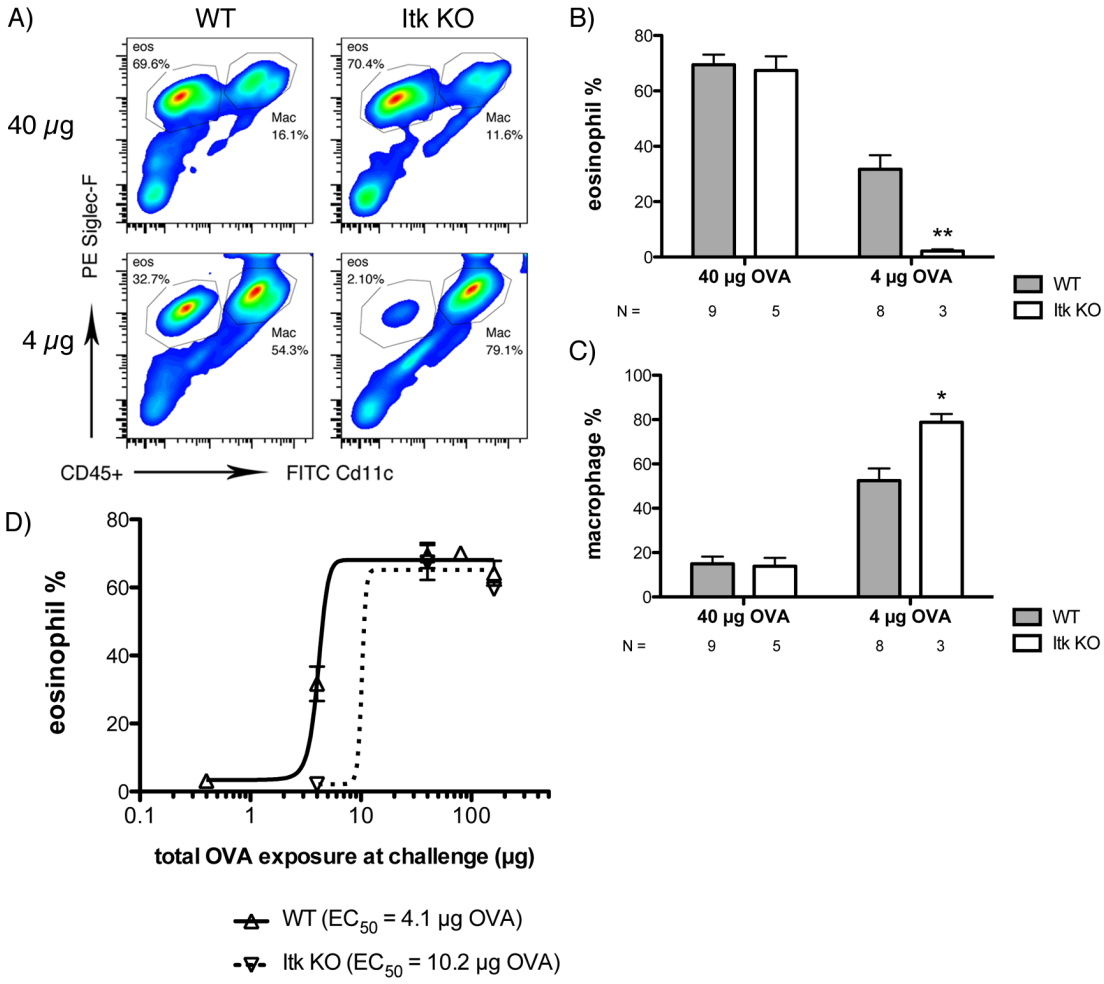
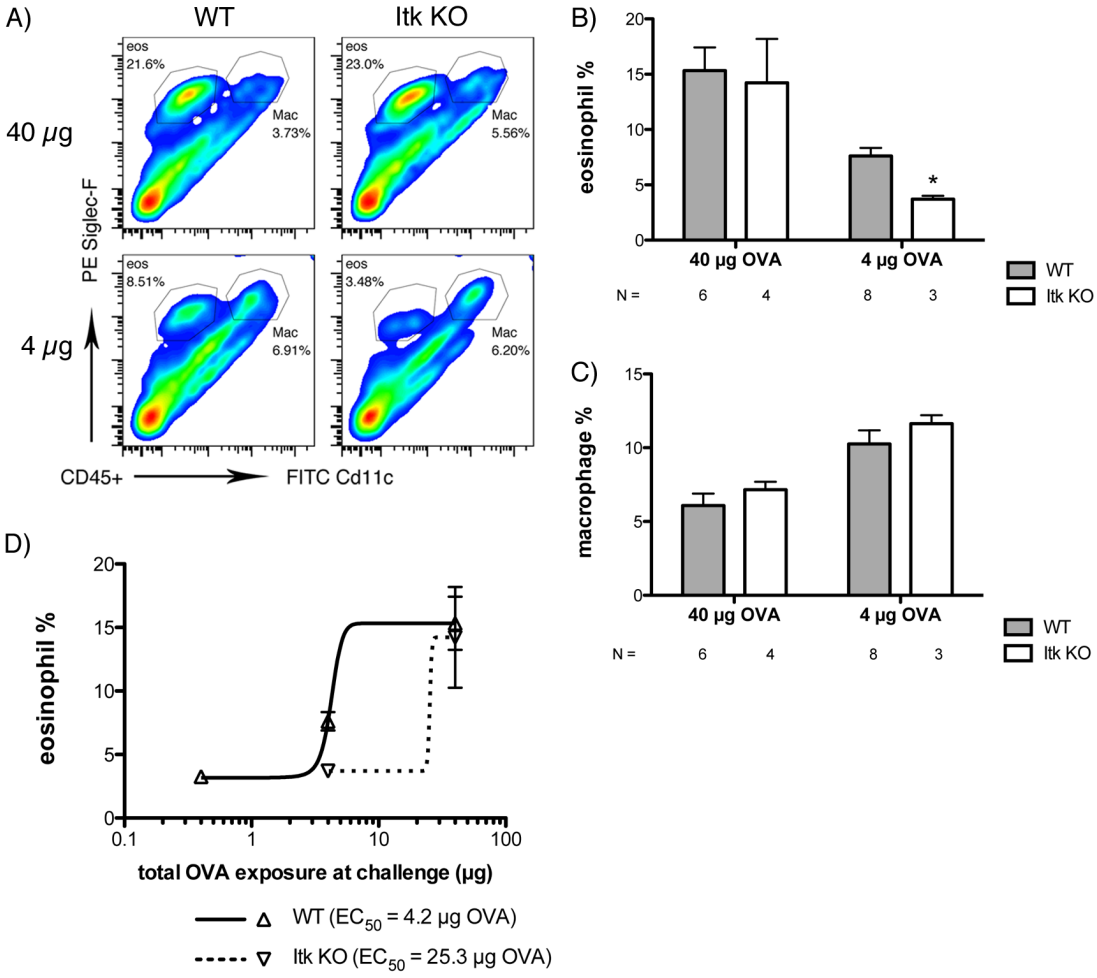

A)
WT
Itk KO
40 μg
4 μg
PE Siglec-F
eos
69.6%
Mac
16.1%
eos
70.4%
Mac
11.6%
eos
32.7%
Mac
54.3%
eos
2.10%
Mac
79.1%
CD45+
FITC Cd11c
B)
eosinophil %
40 μg OVA
4 μg OVA
**
N =
9
5
8
3
WT
Itk KO
C)
macrophage %
*
D)
eosinophil %
total OVA exposure at challenge (μg)
WT (EC50 = 4.1 μg OVA)
Itk KO (EC50 = 10.2 μg OVA)

Figure 12: Lung tissue eosinophilia is reduced in Itk KO mice when challenged with a low amount of OVA.

FACS plots display eosinophils (eos) and macrophages (Mac) of representative wild type (WT) and Itk KO mice challenged with a total of 40 or 4 μg of OVA. Numbers above each gate indicate the percentage of CD45+ cells for each population and tick marks indicate fluorescence intensity on a log scale. B & C) Bar graphs display the average and SEM of eosinophil and macrophage percentages of CD45+ cells for the indicated numbers (N) of WT and Itk KO mice challenged with either a total of 40 or 4 μg of OVA. Statistical significance was determined using an unpaired student's t test comparing WT and Itk KO mice for each amount of OVA (* = $p < 0.05$). D) XY graph displays the eosinophil percentage of CD45+ cells as a function of the total amount of OVA (μg) delivered i.n. during the challenge period on a log scale for WT and Itk KO mice. A sigmoidal dose response curve was applied in order to determine a half maximal effective dose (EC_{50}) of OVA for WT as compared to Itk KO mice.


A)
WT
Itk KO
40 μg
4 μg
PE Siglec-F
eos
21.6%
Mac
3.73%
eos
23.0%
Mac
5.56%
eos
8.51%
Mac
6.91%
eos
3.48%
Mac
6.20%
CD45+
FITC Cd11c
B)
eosinophil %
40 μg OVA
4 μg OVA
N =
6
4
8
3
WT
Itk KO
*
C)
macrophage %
40 μg OVA
4 μg OVA
N =
6
4
8
3
WT
Itk KO
D)
eosinophil %
total OVA exposure at challenge (μg)
WT (EC50 = 4.2 μg OVA)
Itk KO (EC50 = 25.3 μg OVA)

3.4. FITC-R9-QQP delivered i.p. and i.n. is taken up by CD4+ T cells

After optimizing the amount of OVA provided at challenge, translation of R9-QQP to the OVA-induced asthma model was next developed by investigation of the pharmacokinetics of peptide delivered *in vivo* using FITC-conjugated R9-QQP. While previous *in vitro* experiments established that R9-QQP and control peptides were efficiently taken up by Jurkat cells (Figure 7), the ability of R9-QQP delivered intraperitoneally to enter primary CD4+ T cells was determined using a similar method that was modified to include staining for additional surface markers. As displayed in Figure 13A, 1 mg of FITC-R9-QQP was delivered i.p. at 24 hr. and/or 0.5 hr. prior to harvesting spleens. After processing into splenocytes, cells were washed with ice-cold PBS and treated with trypsin to enhance detection of internalized peptide as described for uptake studies performed in Jurkat cells. Next, splenocytes were washed an additional three times in ice-cold PBS and cells were then resuspended in FACS buffer containing PB-conjugated anti-CD4 and APC-conjugated anti-TCR antibodies as described in Materials and Methods. After surface staining, splenocytes were fixed with 2% paraformaldehyde and resuspended in FACS buffer prior to acquisition of cellular fluorescence by FACS.

FACS analysis for quantification of peptide uptake was performed by gating lymphocytes based on their signature forward and side scatter profile followed by gating based on expression of the TCR. Subsequently, TCR+ lymphocytes were analyzed using two-color density plots to display CD4

expression versus FITC fluorescence. For quantification of peptide uptake as a percentage of total T cells, density plots were gated into quadrants based on background fluorescence of mice receiving vehicle control (Figure 13B) and the percentage of CD4+ FITC+ T cells was recorded for mice treated with peptide at various time points (Figure 13C-E). As displayed in the cumulative graph (Figure 13F), 1 mg of FITC-R9-QQP delivered i.p. at both time points was taken up by 37% of T cells in the spleen and this percentage was reduced to 26% and 5% when delivered only at the -24 hr. or -0.5 hr time points, respectively. In addition, the FITC mean fluorescence intensity (MFI) of CD4+ T cells was quantified by gating CD4+ cells as displayed in the accompanying histograms for visual comparison (Figure 13B-F). Consistent with the analysis of peptide uptake as a percentage of total T cells, 1 mg of FITC-R9-QQP delivered at both time points resulted in a FITC MFI of 208 relative fluorescence units that was reduced to 266 units and 127 units when peptide was delivered at only the -24 hr. or -0.5 hr. time points, respectively (Figure 13G).

In addition to demonstrating systemic delivery by i.p. injection, the ability of FITC-R9-QQP to enter CD4+ T cells in the lungs was quantified following intranasal (i.n.) delivery of peptide to OVA-sensitized and challenged mice. For these studies, robust lung inflammation was induced by challenging OVA-sensitized mice with 40 μg of OVA on each day of the challenge period from days 7-9 for a total exposure of 120 μg. Furthermore, mice were

anesthetized 2 hrs. prior to each challenge with OVA and treated with 200 μg of FITC-R9-QQP delivered i.n. for a total exposure of 0.6 mg (Figure 14A). 24 hours after the final challenge, mice were sacrificed and lungs were lavaged as described in Materials and Methods. Next, cells contained in BALF samples were washed, treated with trypsin and stained for TCR and CD4 surface expression as described above for splenocytes. After fixation with 2% paraformaldehyde, acquisition of cellular fluorescence and FACS analysis were performed as described above. As displayed in Figure 14B-E, gated density plots indicate that FITC-R9-QQP was taken up by 7% of total T cells obtained from BALF as compared to a background of 1% for mice treated with vehicle. Furthermore, i.n. treatment with FITC-R9-QQP resulted in a FITC MFI of 112 relative fluorescence units for gated CD4+ T cells as compared to a background of 45 units. Based on the detection of FITC reporter fluorescence, R9-QQP delivered *in vivo* either by i.p. injection or by i.n. administration results in uptake by CD4+ T cells, which are the relevant cellular target for Itk-dependent inhibition of asthma pathogenesis.

Figure 13: FITC-conjugated R9-QQP injected intraperitoneally is efficiently taken up by CD4+ T cells in the spleen.

BL/6 mice were injected with 1 mg of FITC-R9-QQP at -24 and/or -0.5 hrs. prior to harvesting spleens. Next, spleens were processed into splenocytes, treated with trypsin and washed to enhance detection of internalized peptide. Splenocytes were then stained with APC-conjugated anti-TCR and Pacific Blue (PB)-conjugated anti-CD4 antibodies and fixed prior to analysis by FACS. Pseudo-colored density plots display TCR+ splenocytes analyzed for CD4 expression versus FITC fluorescence and are gated to measure the percentage of CD4+ FITC+ T cells. Histograms display the FITC fluorescence intensity of the gated CD4+ T cells. B-E) Density plots display fluorescence data from mice receiving B) vehicle at -24 and -0.5 hr. time points, C) FITC-R9-QQP at both time points, D) vehicle at -24 hrs. followed by FITC-R9-QQP at -0.5 hr. time point or E) FITC-R9-QQP followed by vehicle at -0.5 hr. time point. Numbers above gates in density plots indicate the percentage of T cells and tick marks indicate fluorescence intensity on a log scale for all fluorescence channels. Open histogram in panel B represents vehicle treatment and is overlaid with shaded histograms representing treatment with FITC-R9-QQP in panels C-E for comparison of fluorescence intensity. F-G) Bar graphs display the F) percentage of CD4+ FITC+ T cells or G) the FITC MFI of CD4+ T cells.

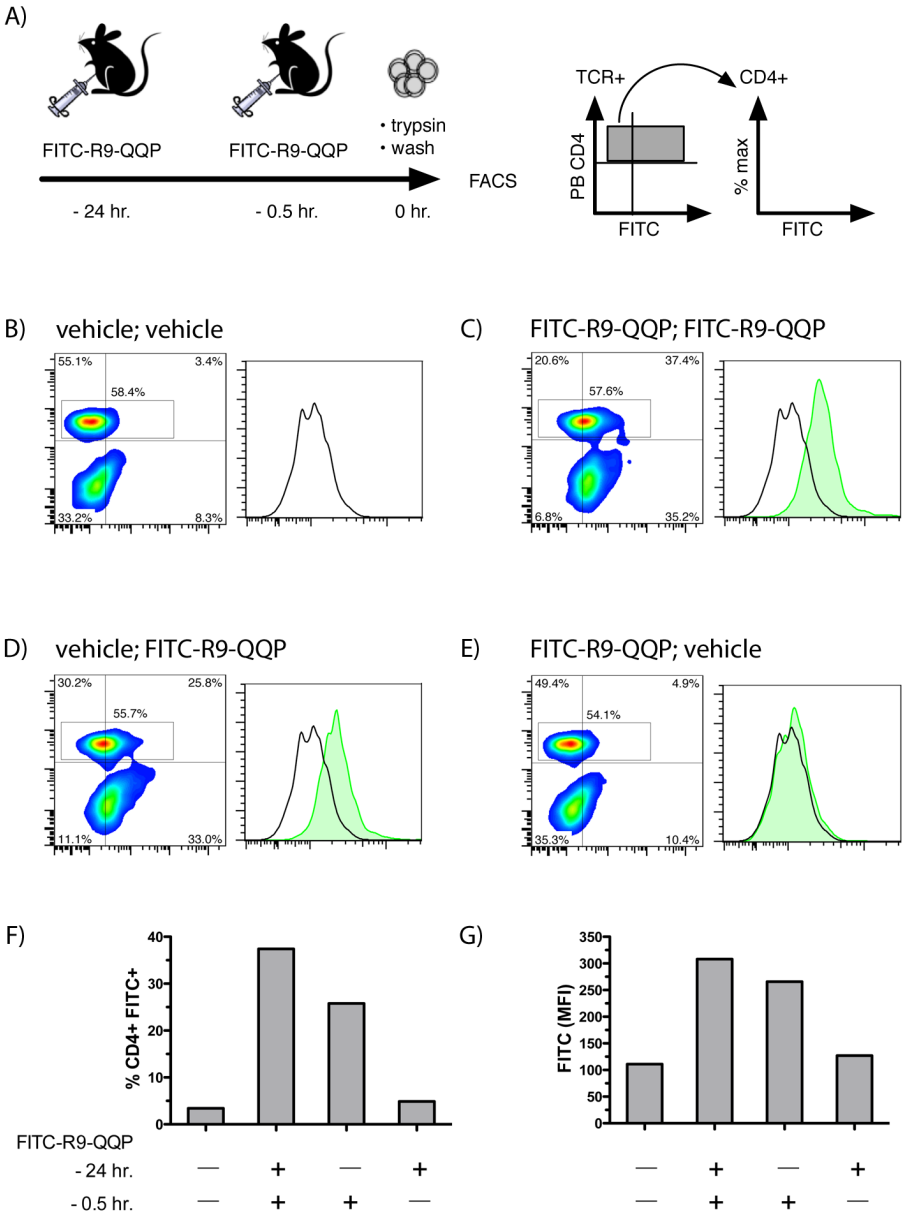
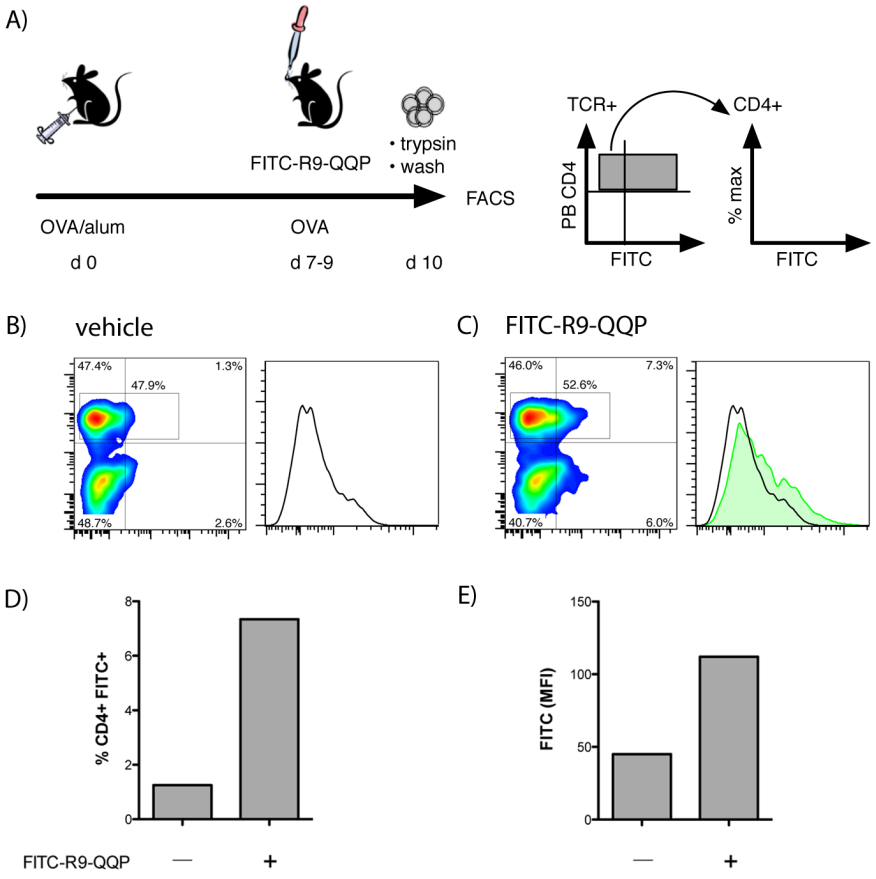

A)
FITC-R9-QQP
FITC-R9-QQP
• trypsin
• wash
- 24 hr.
- 0.5 hr.
0 hr.
FACS
TCR+
CD4+
PB CD4
FITC
% max
FITC
B) vehicle; vehicle
55.1%
3.4%
58.4%
33.2%
8.3%
C) FITC-R9-QQP; FITC-R9-QQP
20.6%
37.4%
57.6%
6.8%
35.2%
D) vehicle; FITC-R9-QQP
30.2%
25.8%
55.7%
11.1%
33.0%
E) FITC-R9-QQP; vehicle
49.4%
4.9%
54.1%
35.3%
10.4%
F)
% CD4+ FITC+
40
30
20
10
0
FITC-R9-QQP
- 24 hr. — + — +
- 0.5 hr. — + + —
G)
FITC (MFI)
350
300
250
200
150
100
50
0
— + — +
— + + —

Figure 14: FITC-conjugated R9-QQP delivered intranasally (i.n.) is efficiently taken up by CD4+ T cells in the lungs.

BL/6 mice were sensitized with OVA/alum on day 0. On days 7-9, mice were first treated i.n. with 200 μg of FITC-R9-QQP (or vehicle control) and challenged ~2 hrs. later with 40 μg of OVA delivered i.n. On day 10, mice were lavaged and BAL cells were treated with trypsin and washed to enhance detection of internalized peptide. BAL cells were then stained with APC-conjugated anti-TCR and Pacific Blue anti-CD4 antibodies and fixed prior to analysis by FACS. Pseudo-colored density plots display TCR+ BAL cells analyzed for CD4 expression and FITC fluorescence and are gated to measure the percentage of CD4+ FITC+ T cells. Histograms display the FITC fluorescence intensity of the gated CD4+ T cells. B-C) Density plots display fluorescence data from mice receiving B) vehicle or C) FITC-R9-QQP i.n. Numbers above gates in density plot indicate the percentage of T cells and tick marks indicate fluorescence intensity on a log scale for all fluorescence channels. Open histogram in panel B represents vehicle treatment and is overlaid with shaded histogram representing treatment with FITC-R9-QQP in panel C for comparison of fluorescence intensity. D-E) Bar graphs display the D) percentage of CD4+ FITC+ T cells or E) the FITC MFI of CD4+ T cells.


A)
FITC-R9-QQP
• trypsin
• wash
FACS
OVA/alum
d 0
OVA
d 7-9
d 10
TCR+
CD4+
PB CD4
FITC
% max
FITC
B)
vehicle
47.4%
1.3%
47.9%
48.7%
2.6%
C)
FITC-R9-QQP
46.0%
7.3%
52.6%
40.7%
6.0%
D)
% CD4+ FITC+
FITC-R9-QQP
—
+
E)
FITC (MFI)
—
+

3.5. Mice treated with R9-QQP display reduced AHR, mucus production and inflammation upon challenge in the OVA-induced asthma model

Informed by the previous studies, R9-QQP was next translated to the OVA-induced asthma model for investigation of inhibitory effects *in vivo*. For each independent asthma experiment, separate groups of 3-4 mice per group were treated with either vehicle (positive control; PC), R9-QQP inhibitor peptide or R9-QQA control peptide and experimental asthma was induced as described in Materials and Methods. In these studies, C57BL/6 mice were sensitized i.p. with OVA/alum on day 0 and challenged i.n. with 10 μg of OVA on days 7-9 for a total exposure of 30 μg of OVA. In order to maximize bioavailability and thus effectiveness, R9-QQP or control peptide was delivered to each mouse throughout the entire sensitization and challenge periods as illustrated in Figure 15 and described in Materials and Methods. First, 0.5 mg (~25 mg/kg) of R9-QQP or R9-QQA peptide was delivered i.p. at 24 hrs. and 0.5 hr. prior to sensitization with OVA/alum on day 0. Next, peptide was delivered i.p. as described previously at 24 hrs. prior to the first challenge with OVA on day 7. In addition, peptide was delivered both i.p. as described before as well as by i.n. administration at 2 hrs. prior to each challenge with OVA on days 7-9. For the i.n. peptide treatment, mice were briefly anesthetized using isoflurane and 200 μg of peptide was delivered by inhalation of a 20 μL bolus provided drop-wise to alternating nares. As diagrammed in Figure 16, parameters of lung inflammation were assayed 24

hours after the final challenge with OVA on day 10 of the experimental protocol.

For assessment of airway hyperresponsiveness (AHR), mice were deeply anesthetized using a cocktail composed of ketamine and xylazine delivered i.p. and the trachea was surgically exposed as described in Materials and Methods. After creating a small opening, an 18 gauge cannula was inserted into the trachea that was secured using surgical suture. Next, mice were connected to a small rodent ventilator and AHR of passively breathing mice was measured in response to increasing doses of aerosolized methacholine. As displayed in Figure 17A, AHR of mice treated with R9-QQP for 4-7 independent experiments representing an average of 2 mice per group was significantly reduced at the 12 mg/mL dose of methacholine as compared to mice in the positive control (PC) group that received vehicle (average airway resistance of 4.2 $cmH_20.s/mL$ for PC group versus 2.8 $cmH_20.s/mL$ for R9-QQP group; $p < 0.05$). Furthermore, the effect was specific as indicated by a representative experiment comparing mice treated with R9-QQA control peptide versus vehicle control (average airway resistance of 4.1$cmH_20.s/mL$ for both PC and R9-QQA groups; Fig. 17B). Taken together, these data indicate that R9-QQP specifically inhibits a physiological measurement of airway obstruction that is indicative of asthma.

In order to examine the histological basis for the observed reduction in AHR, lung tissue was harvested from mice that were treated as described

above followed by processing for evaluation of mucus production and lung inflammation. For these experiments, mice were sacrificed 24 hours after the final challenge with OVA and lungs were lavaged as described in Materials and Methods. Next, a single lung lobe was removed from each mouse that was rinsed in PBS and fixed in formalin. Lung tissue was then sectioned, mounted onto microscopy slides and stained with periodic acid schiff (PAS) reagent for detection of mucus production or with hematoxylin and eosin (H&E) reagent for detection of lung inflammation. For evaluation of histological staining, slides prepared from each lung specimen were labeled with randomly assigned numbers, examined using a light microscope and blindly scored using criteria described in Materials and Methods.

As displayed in Figure 18A, representative images of bronchioles acquired at 200x magnification indicate that mucus production by goblet cells as revealed by cytoplasm staining fuchsia is reduced in mice treated with R9-QQP as compared to bronchioles of mice from both vehicle (PC) and R9-QQA peptide control groups. For quantification of mucus production, individual bronchioles were assigned a mucus severity score ranging from 0-5 according to the percentage of the bronchiole circumference staining positive for mucus. Due to the low overall mucus production induced upon challenge with low doses of OVA, the raw mucus severity score for each mouse was normalized to the average score of the PC group which was defined as 100%. As displayed in Figure 18B, the cumulative scores acquired from 3-5 mice

with an average of 32 bronchioles examined per specimen reveal that treatment with R9-QQP significantly inhibits mucus production as compared to treatment of mice with vehicle (PC) while the R9-QQA control peptide has no significant effect (average normalized score of 100% for PC group versus 12.1% for R9-QQP group and 81.5% for R9-QQA group; $p < 0.01$).

Similarly, representative images of bronchioles acquired at 200x magnification show a reduced accumulation of nuclei staining dark purple in the lung tissue of mice treated with R9-QQP as compared to mice from both vehicle (PC) and R9-QQA control groups (Figure 19A). For quantification of inflammation, the degree of peribronchiole as well as perivascular cuffing was assigned a score ranging from 0-4 according to the depth cells infiltrating the lung tissue directly surrounding each bronchiole or blood vessel. Again, raw scores obtained for each mouse were normalized to the average score of the PC group due to the purposefully low overall level of inflammation induced with OVA at challenge. As displayed in Figure 19B, the cumulative inflammation scores representing 3-4 mice with an average of 45 combined bronchioles and blood vessels examined per mouse indicate that R9-QQP significantly inhibits lung tissue inflammation while the R9-QQA control peptide has no significant effect as compared to the PC group (average normalized score of 100% for PC group versus 28.7% for R9-QQP group and 143.5% for R9-QQA group; $p < 0.05$). Thus, inhibition of AHR by R9-QQP

correlates with a peptide-specific reduction in mucus production and inflammation occurring in the lung tissue.

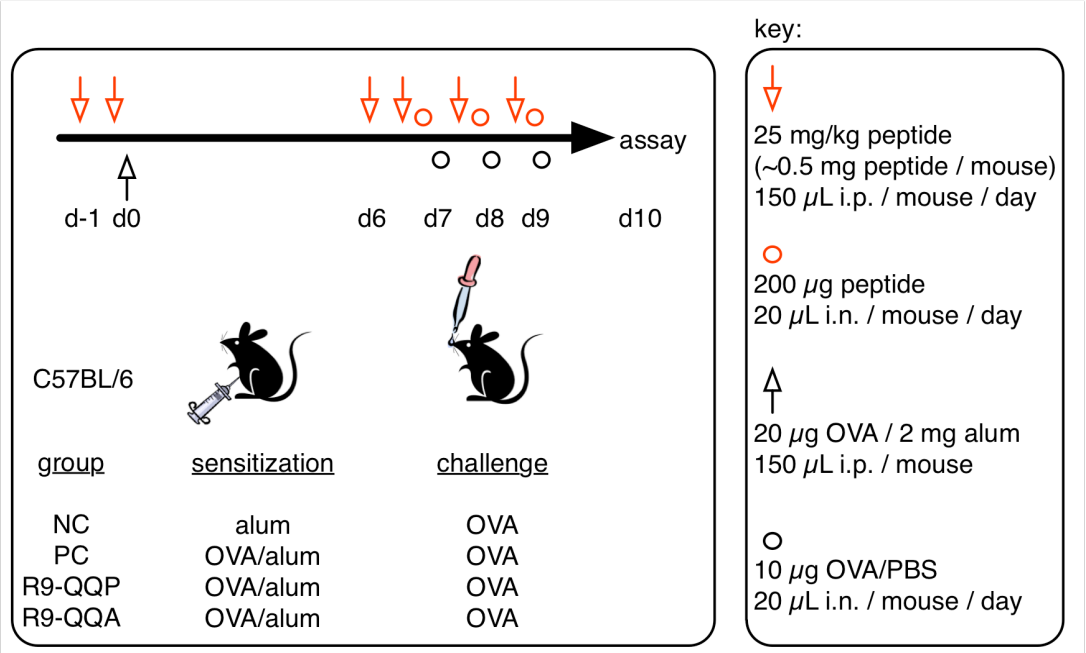


Figure 15: Schematic diagram summarizes the *in vivo* peptide delivery protocol used for treatment of experimental asthma as described in Materials & Methods.

BL/6 mice were treated with 25 mg/kg of peptide by intraperitoneal (i.p.) injection at -24 and -0.5 hr. prior to sensitization with 20 μg OVA/ 2 mg alum on day 0. Next, peptide was again delivered i.p. 24 hours prior to the first challenge with OVA on day 7. In addition, mice were treated with peptide i.p. as before and 200 μg of peptide was delivered intranasally (i.n.) to anesthetized mice 2 hours prior to each challenge with 10 μg of OVA on days 7-9.

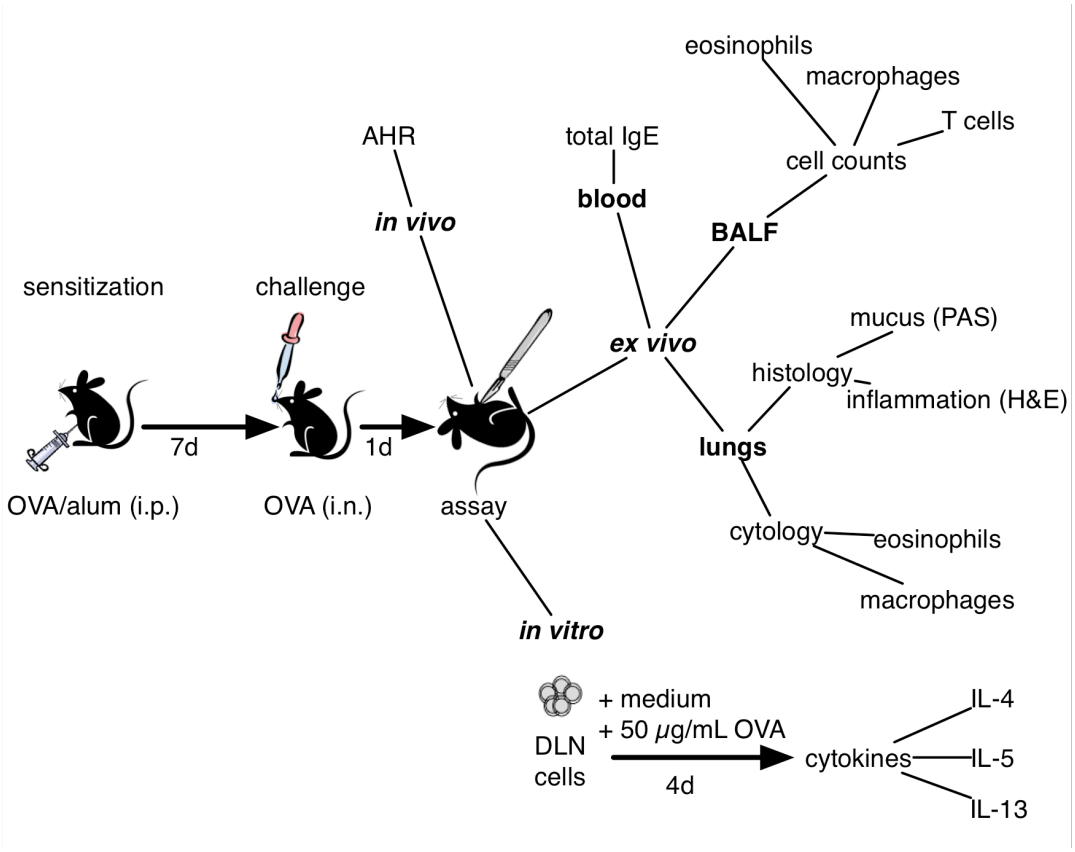


Figure 16: Schematic diagram summarizes parameters analyzed following induction of experimental asthma.

24 hours after the final challenge with OVA, mice were assayed for various parameters of lung inflammation as described in detail Materials and Methods. Briefly, *in vivo* measurement of airway hyperresponsiveness (AHR) was performed using mice anesthetized with a cocktail of ketamine / xylazine administered i.p. In separate experiments, mice were anesthetized with isoflurane and retroorbital blood was collected for measurement of serum IgE. After sacrificing anesthetized mice, lungs were lavaged and bronchoalveolar lavage fluid (BALF) was collected for identification of infiltrating cells. In addition, separate lung lobes were excised and fixed for tissue processing and histological staining or dissociated and analyzed for the identification of infiltrating cells. Lastly, lung draining lymph nodes (DLN) were harvested and processed to prepare a cell suspension. DLN cells were then restimulated for 4 days and *in vitro* culture supernatants were analyzed for Th2 cytokines.

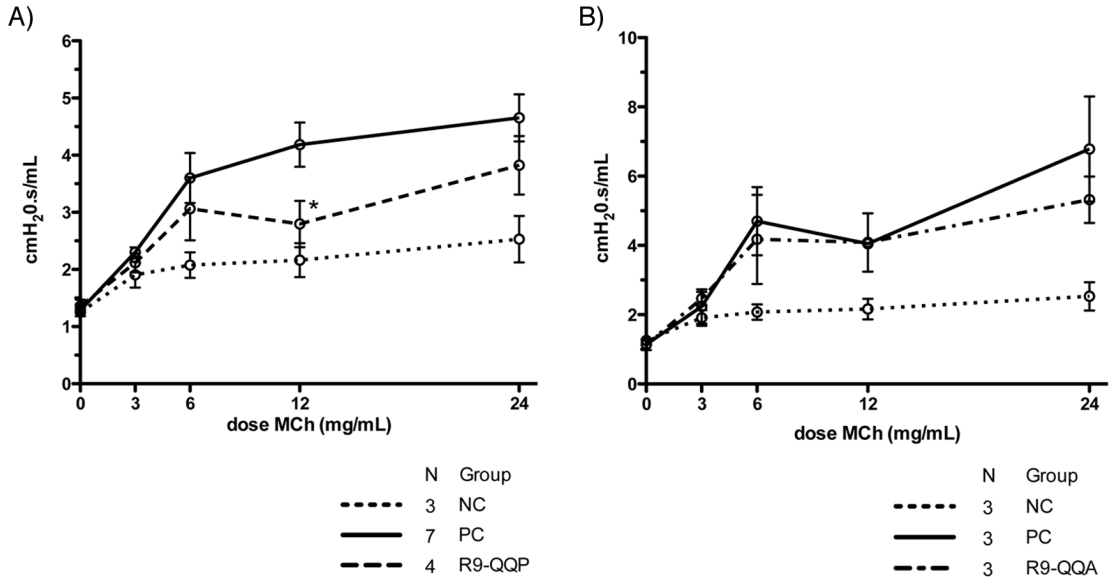


Figure 17: Mice treated with R9-QQP have reduced airway hyperresponsiveness (AHR) as compared to mice treated with control peptide.

BL/6 mice were treated with peptides and experimental asthma was induced as described in figure 7. 24 hours after the final challenge with OVA, mice were anesthetized and AHR was measured in response to increasing doses of aerosolized methacholine as described in Materials and Methods. A & B) XY graphs display average airway resistance (+/- SEM) versus dose of methacholine. A) Graph compares mice treated with R9-QQP to positive and negative control (PC, NC) groups for indicated number (N) of independent experiments representing an average of 2 mice per group. B) Graph displays results of a representative control experiment including R9-QQA group for indicated number (N) of individual mice. Statistical significance was determined using an unpaired student's t test comparing each peptide treated group to the positive control (PC) group at the 12 mg/mL dose of methacholine (* = $p < 0.05$).

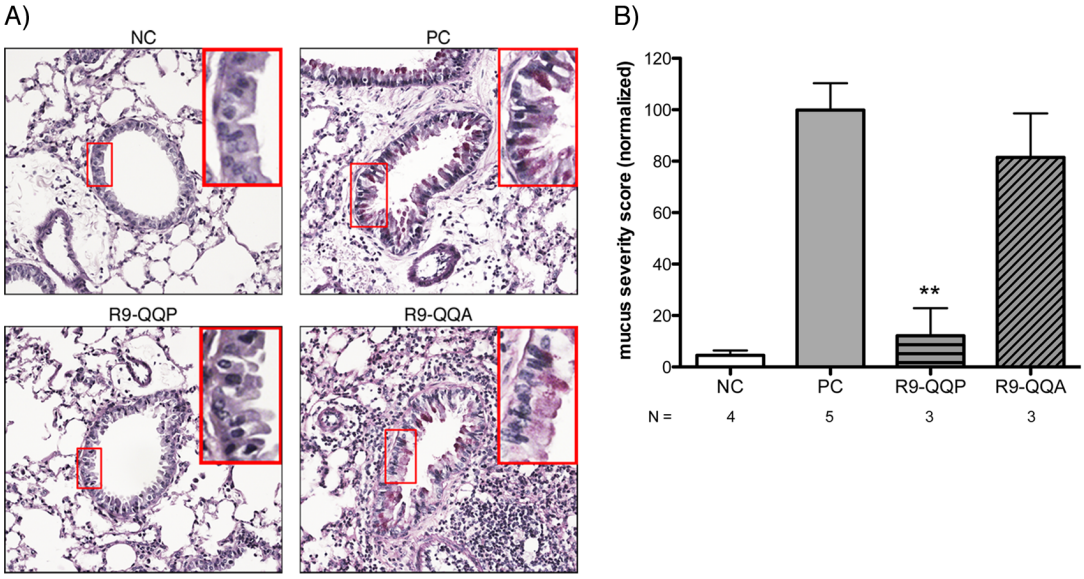


Figure 18: R9-QQP specifically inhibits mucus production.

BL/6 mice were treated with peptides and experimental asthma was induced as described in figure 7. After sacrificing mice and performing bronchoalveolar lavage, lung tissue from each mouse was sectioned, stained with PAS and analyzed as described in Materials and Methods. A) Micrographs display representative bronchiole cross sections acquired at 200x magnification from slides stained with PAS for each group. Inset box in upper right corner of each micrograph shows additional digital magnification of corresponding region outlined in original image. Cytoplasm staining fuchsia indicates mucus producing goblet cells. B) Bars display average and SEM of normalized mucus severity score for cumulative number (N) of mice per group (** $p < 0.01$).

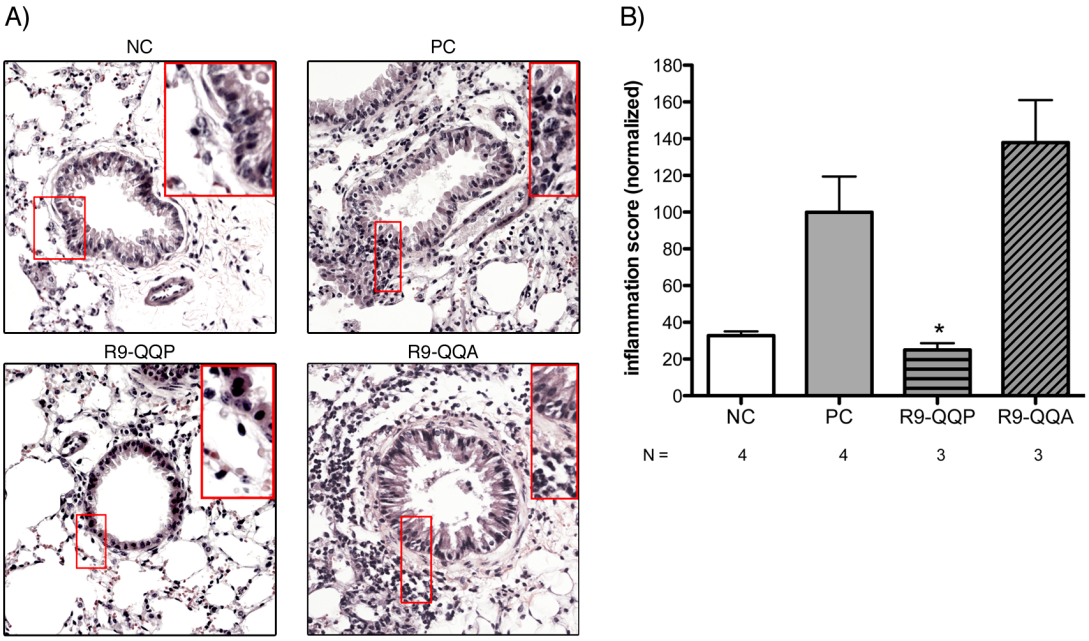


Figure 19: R9-QQP specifically inhibits lung tissue inflammation.

BL/6 mice were treated with peptides and experimental asthma was induced as described in figure 7. After sacrificing mice and performing bronchoalveolar lavage, lung tissue from each mouse was sectioned, stained with H&E and analyzed as described in Materials and Methods. A) Micrographs display representative bronchiole cross sections acquired at 200x magnification from slides stained with H&E for each group. Inset box in upper right corner of each micrograph shows additional digital magnification of corresponding region outlined in original image. Nuclei staining dark purple surrounding bronchioles indicate the presence of inflammatory cells. B) Bars display average and SEM of normalized mucus severity score for cumulative number (N) of mice per group (* = $p < 0.05$).

3.6. R9-QQP specifically inhibits BAL and lung cell eosinophilia in the OVA-induced asthma model

In order to characterize the inflammatory cells affected by treatment with R9-QQP, cells obtained from BALF samples were counted using a hemacytometer, attached to cytospin slides and stained using a modified Wright-Giemsa dye for hematological identification as described previously. As displayed in Figure 20A, the total number of cells obtained from mice treated with R9-QQP in the OVA-induced asthma model was significantly reduced as compared to mice treated with vehicle while treatment with R9-QQA had no effect on total BAL cell counts (i.e., from an average of 374,000 cells for PC group to 173,000 cells for R9-QQP group representing a 53.7% decrease and 344,000 cells for R9-QQA group; $p < 0.0001$). Furthermore, microscopic evaluation of cytospin slides prepared from mice treated with vehicle and R9-QQA control peptide revealed a high frequency of granulocytes consistent in appearance to eosinophils while BAL cells on slides prepared from mice treated with R9-QQP consistently mostly of alveolar macrophages with few granulocytes (Fig. 20B).

For quantification of the inflammatory phenotype, separate aliquots of BAL cells obtained from each mouse were stained with fluorophore-conjugated antibodies according to the previously described method for evaluation of eosinophils and macrophages as a percentage of total leukocytes by FACS. Consistent with the representative cytospin images (Fig.

20A), the representative density plots displayed in Figure 21A revealed that a high relative frequency of eosinophils as defined by a CD45+ Siglec-F+ FITC- staining profile were obtained from mice treated with vehicle (PC) and R9-QQA control peptide while the majority of BAL cells obtained from mice treated with R9-QQP were characterized as alveolar macrophages as defined by a CD45+ Siglec-F+ FITC+ staining profile. As substantiated by the cumulative analysis displayed in Figure 21B, treatment of mice with R9-QQP resulted in a highly significant reduction in the percentage of BAL eosinophils as compared to treatment with vehicle while the R9-QQA control peptide had no significant effect on eosinophilia (i.e., from an average of 53.2% eosinophils for PC group to 28.1% for R9-QQP group representing a 47.2% decrease and 49.9% for R9-QQA group; $p < 0.0001$). Conversely, the percentage of alveolar macrophages was significantly higher for mice treated with R9-QQP as compared to treatment with vehicle as displayed in Figure 21C (i.e., 26.1% macrophages for PC group versus 55.1% for R9-QQP group and 33.9% for R9-QQA group; $p < 0.0001$).

Using a similar method, separate aliquots of BAL cells were stained for quantification of T cells. For this assay, PerCP-Cy5.5-conjugated anti-CD45 and PE-Cy7-conjugated anti-CD3ε antibodies were added to Fc receptor-blocked cells followed by FACS analysis as described in Materials and Methods. After gating CD45+ lymphocytes, T cells as defined by positive staining for CD3ε were expressed as a percentage of total leukocytes.

Although the detection range was narrow (i.e., from 1.6% T cells for NC group to 7.1% for PC group), no difference in the percentage of T cells was observed for mice treated with either R9-QQP or R9-QQA peptides as compared to vehicle control (Figure 21D-E).

To further validate the effects of R9-QQP, lung tissue was dissociated as described in Materials and Methods and lung cells were stained and analyzed by FACS as described for quantification of BAL cell eosinophilia (Fig. 22A-C). Although the detection range was much more narrow for lung tissue (i.e., 12.4 percentage point difference in eosinophils between NC and PC groups as compared to a 50.9 difference for BAL eosinophilia), the percentage of eosinophils obtained from lung tissue was significantly reduced for mice treated with R9-QQP as compared to treatment with vehicle in a manner that was consistent with the reduction in BAL eosinophilia while treatment with the R9-QQA control peptide had no significant effect (i.e., from 15.8% eosinophils for PC group to 8.2% for R9-QQP group representing a 48.1% decrease and 13.0% for R9-QQA group; $p < 0.001$). Furthermore, the contribution of macrophages was significantly increased for mice treated with R9-QQP as compared to treatment with vehicle as observed for BAL cells (i.e., 6.3% macrophages for PC group versus 9.5% for R9-QQP group; $p < 0.01$). Together, the characterization of BAL and lung cells indicates that R9-QQP specifically reduces eosinophilic inflammation upon challenge with OVA in the mouse asthma model.

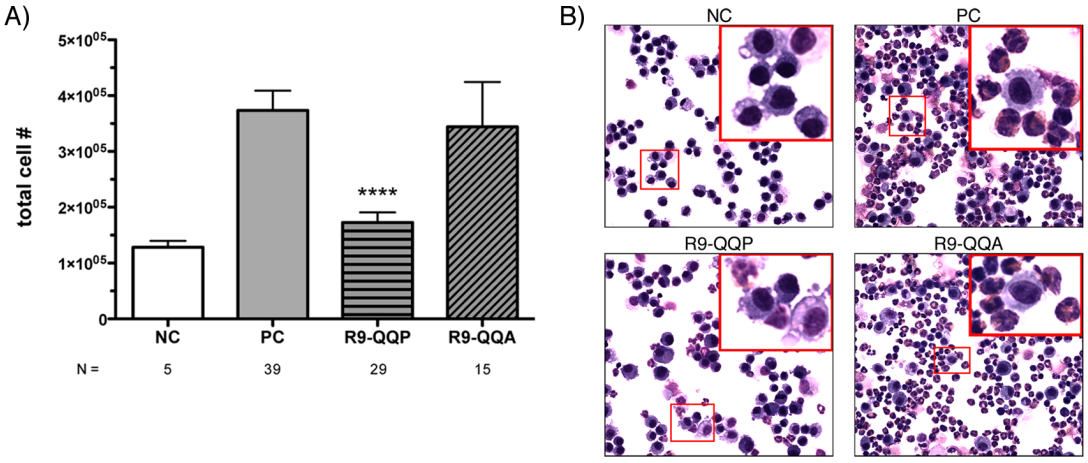
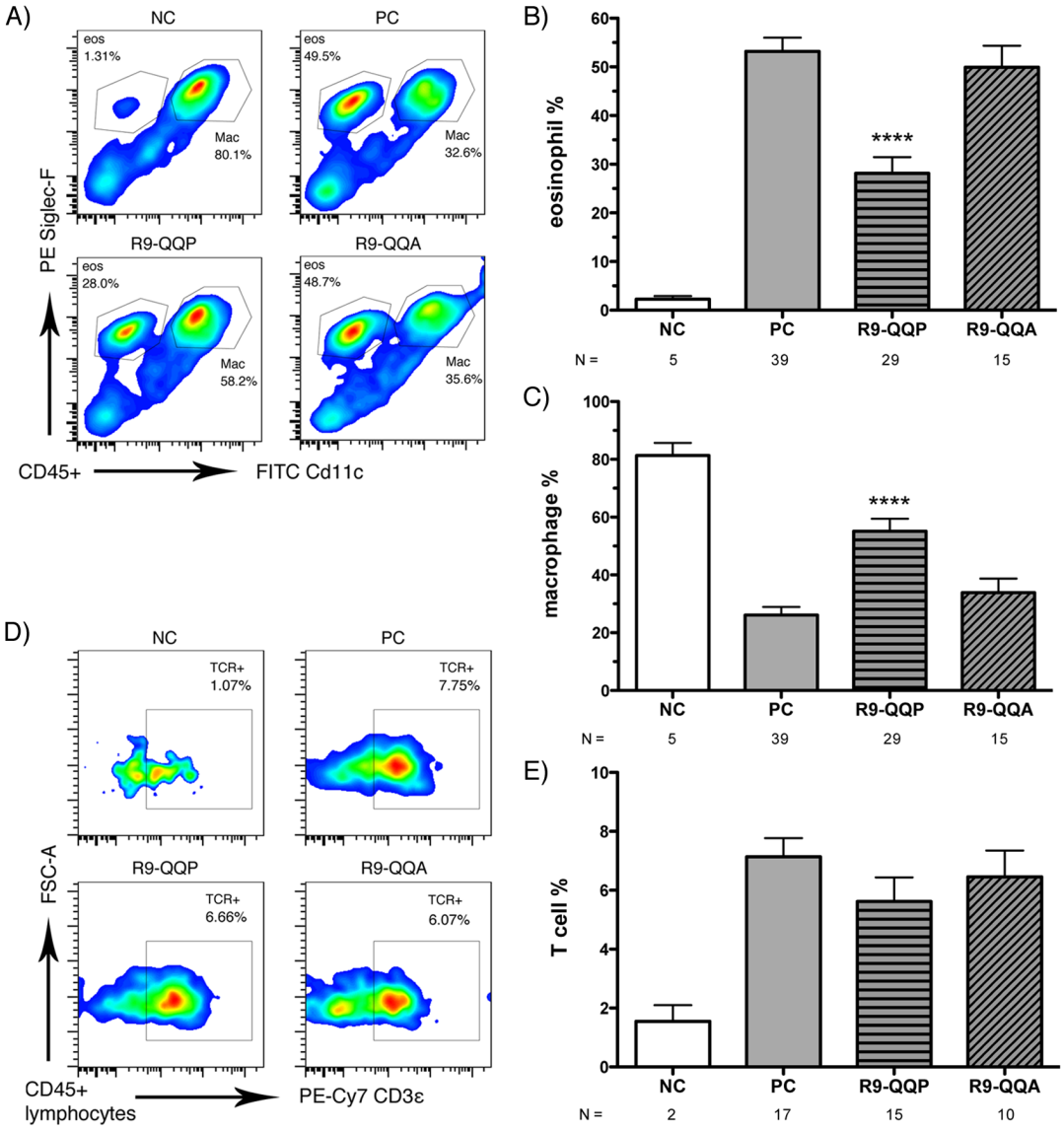


Figure 20: R9-QQP specifically inhibits BALF infiltration.

BL/6 mice were treated with peptides and experimental asthma was induced as described in figure 7. A) After sacrificing mice, bronchoalveolar lavage fluid (BALF) was collected and total infiltrating cells were counted as described in Materials and Methods. Bars display average total cell number and SEM for cumulative number (N) of mice per group. Statistical significance was determined using an unpaired student's t test comparing each peptide treated group to the positive control group (**** = $p < 0.0001$). B) Micrographs display representative fields acquired at 400x magnification from cytospin slides that were stained using a modified Wright-Giemsa dye to identify infiltrating leukocytes. Inset box in upper right corner of each micrograph shows additional digital magnification of corresponding region outlined in original image.

Figure 21: R9-QQP specifically inhibits BALF eosinophilia.

BL/6 mice were treated with peptides and experimental asthma was induced as described in figure 7. A) After performing bronchoalveolar lavage, BALF cells were stained for eosinophils / macrophages and analyzed by FACS as described in Materials and Methods. FACS plots display eosinophils (eos) and macrophages (Mac) of representative mice from each group. B & C) Bar graphs display cumulative eosinophil and macrophage percentages of CD45+ cells for each group. D) In addition, BALF cells were stained for T cells as described in Materials and Methods. FACS plots display T cells (TCR +) of representative mice from each group. E) Bar graph displays cumulative T cell percentages of CD45+ cells for each group. A & D) Numbers above each gate indicate the percentage of CD45+ cells for each population and tick marks indicate fluorescence intensity on a log scale for all fluorescence channels and on a linear scale for FSC-A. B, C & E) Bar graphs display average and SEM of cumulative number (N) of mice per group and statistical significance was determined using an unpaired student's t test comparing each peptide treated group to the positive control for each parameter (**** = $p < 0.0001$).


A)
NC
PC
R9-QQP
R9-QQA
eos
1.31%
Mac
80.1%
eos
49.5%
Mac
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eos
28.0%
Mac
58.2%
eos
48.7%
Mac
35.6%
PE Siglec-F
CD45+
FITC Cd11c
B)
eosinophil %
****
N =
5
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29
15
C)
macrophage %
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N =
5
39
29
15
D)
NC
PC
R9-QQP
R9-QQA
TCR+
1.07%
TCR+
7.75%
TCR+
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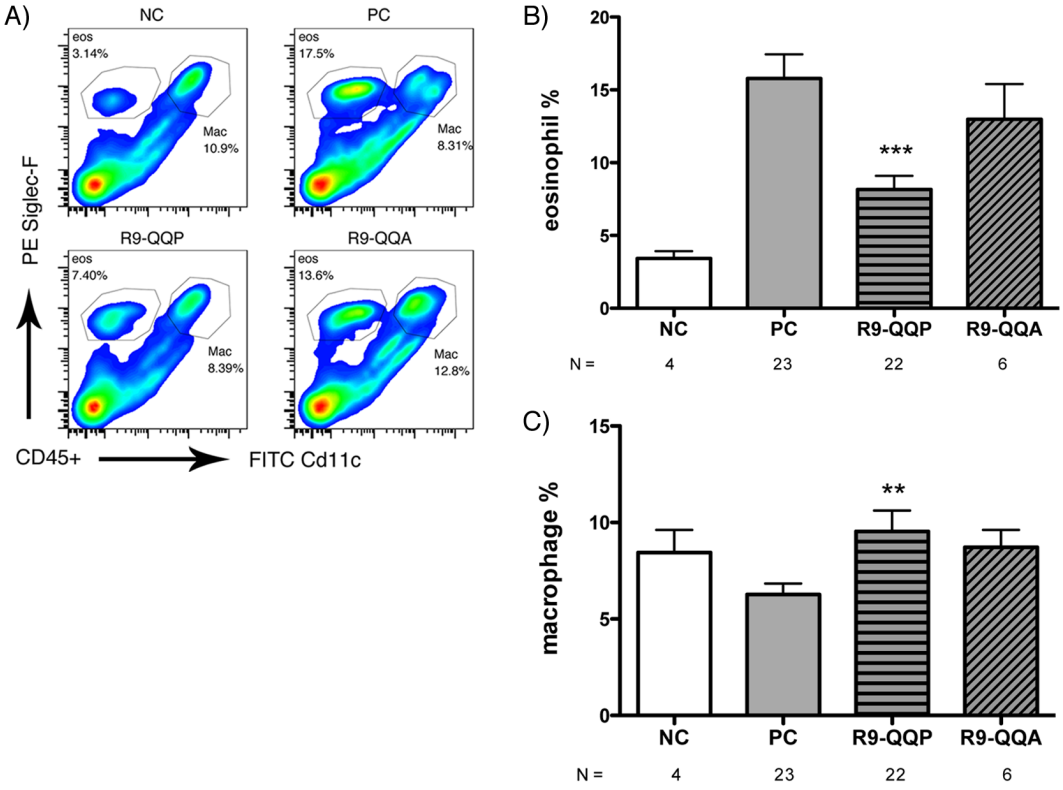


Figure 22: R9-QQP specifically inhibits lung tissue eosinophilia.

BL/6 mice were treated with peptides and experimental asthma was induced as described in figure 7. After sacrificing mice and performing bronchoalveolar lavage, lung tissue from each mouse was harvested, rinsed in PBS and stained for eosinophils / macrophages as described in Materials and Methods. A) FACS plots display eosinophils (eos) and macrophages (Mac) of representative mice from each group. Numbers above each gate indicate the percentage of CD45+ cells for each population and tick marks represent fluorescence intensity on a log scale for fluorescence channels. B & C) Bar graphs display average and SEM of eosinophil and macrophage percentages of CD45+ cells for cumulative number (N) of mice per group. Statistical significance was determined using an unpaired student's t test comparing each peptide treated group to the positive control for each parameter (** = $p < 0.01$; *** = $p < 0.001$).

3.7. OVA-dependent serum IgE is not affected by treatment with R9-QQP

As a known mediator of asthma pathogenesis, serum IgE levels were investigated for mice treated with R9-QQP in the OVA-inducible asthma model. For this assay, mice were anesthetized and retroorbital blood was collected 24 hours after the final challenge with OVA as illustrated in the protocol and assay diagrams (Fig. 15-16). After clot formation, serum was separated by centrifugation and aliquots obtained from each mouse were tested in duplicate for total IgE by ELISA as described in Materials and Methods. As displayed in Figure 23, total serum IgE levels were highly induced for OVA-sensitized and challenged mice as compared to non-immunized negative control mice (i.e., from an average of 61 pg/mL for NC group to an average of 671 pg/mL for PC group). However, OVA-inducible IgE was not affected for mice treated with R9-QQP as compared to treatment with vehicle (i.e., average total IgE of 659 pg/mL for R9-QQP group versus 671 for PC group). Thus, the inhibitory effects of R9-QQP on parameters of lung inflammation as described in this report were apparently independent of IgE.

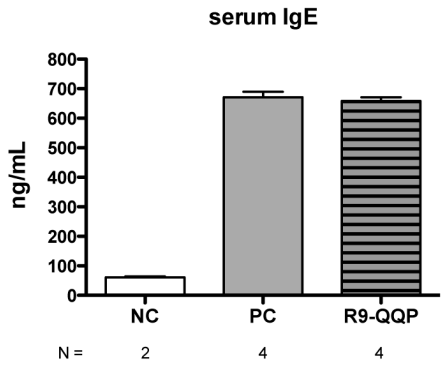


Figure 23: R9-QQP does not affect total serum IgE.

BL/6 mice were treated with R9-QQP and experimental asthma was induced as described in figure 7. 24 hours after the final challenge with OVA, serum was obtained from indicated number (N) of mice for each group. Bars display average and SEM of total serum IgE as determined by ELISA.

3.8. R9-QQP specifically inhibits Th2 cytokine production by lung DLN cells upon restimulation with OVA *in vitro*

In order to identify the inflammatory mediators affected by treatment of mice with R9-QQP, Th2 cytokine production by lung draining lymph node cells was investigated following restimulation with OVA *in vitro*. For these experiments, mice were treated with peptides and asthma was induced in response to OVA as described previously. Twenty-four hours after the final challenge with OVA, mice were sacrificed and lung draining lymph nodes (DLN) were harvested following dissection of the chest cavity. Next, DLN from 2-3 mice for each treatment group were combined and lymphoid tissue was dissociated into separate cell suspensions. For restimulation *in vitro*, 10^5 or 10^6 DLN cells were seeded to replicate wells of round-bottom 96-well plates followed by addition of OVA at a final concentration of 50 μg/mL as described in Materials and Methods. After incubation of DLN cells for 4 days, tissue culture supernatants corresponding to separate treatment groups were harvested and tested in duplicate for quantification of IL-4, IL-5 and IL-13 by ELISA., respectively. Importantly, no additional peptides were added during the *in vitro* incubation as this assay was designed to reflect the effects of R9-QQP *in vivo*.

As represented for 6-7 independent experiments (Fig. 24A-B), OVA-dependent restimulation resulted in robust secretion of IL-13 by DLN cells isolated from mice treated with vehicle (PC) that were seeded at 10^5 cells per

well while IL-13 was significantly reduced for independent cultures derived from mice treated with R9-QQP (i.e., average IL-13 concentration of 428.7 pg/mL for PC group as compared to 64.5 pg/mL for R9-QQP group; $p < 0.01$). Importantly, the inhibitory effect of R9-QQP was specific as restimulation of DLN cells from mice treated with R9-QQA was not significantly different as compared to treatment with vehicle (average IL-13 concentration of 378.6 pg/mL for R9-QQA group). Similarly, analysis of the same supernatant samples for quantification of IL-5 revealed a more narrow detection range that nevertheless revealed a significant reduction in IL-5 secretion by DLN cells of mice treated with R9-QQP as compared to vehicle while treatment with R9-QQA had no effect (i.e., average IL-5 concentration of 17.1 pg/mL for PC group as compared to 4.7 pg/mL for R9-QQP group and 18.5 pg/mL for R9-QQA group; $p < 0.05$). However, levels of the Th2 signature cytokine IL-4 were beyond the detection limit for the IL-4 ELISA assay using the culture conditions described above (data not shown).

In order to maximize the ability to detect low levels of IL-4, parallel cultures were established that were seeded at 10^6 DLN cells per well and restimulated with OVA as described above. As represented for 4-7 independent experiments (Fig. 24C-D), restimulation with OVA resulted in significantly reduced secretion of IL-4 by DLN cells from mice that were treated with R9-QQP as compared to treatment with vehicle while treated with the R9-QQA control peptide had no significant effect (i.e., average IL-4

concentration of 73.4 pg/mL for PC group as compared to 20.0 pg/mL for R9-QQP group and 54.1 pg/mL for R9-QQA group; $p < 0.05$). Likewise, analysis of the same culture supernatants for IL-5 revealed a significant and peptide specific effect while increasing the overall levels of IL-5 as compared to cultures seeded at 10^5 cells per well (i.e., average IL-5 concentration of 1,186.5 pg/mL for PC group as compared to 657.7 pg/mL for R9-QQP group and 1,217.0 pg/mL for R9-QQA group; $p < 0.05$). In summary, these results indicate that R9-QQP specifically inhibits secretion of the Th2 cytokines IL-4, IL-5 and IL-13 by lung DLN cells, which are critical mediators involved in asthma pathogenesis.

Chapter 3, in part, is currently being prepared for submission for publication of the material. Guimond, D.M., Cam, N.R., Hirve, N., Duan, W., Lambris, J.D., Croft, M., & Tsoukas, C.D (2012). Treatment of Experimental Asthma Using a Novel Peptide Inhibitor of the Inducible T Cell Kinase. The dissertation author was the primary author of this material.

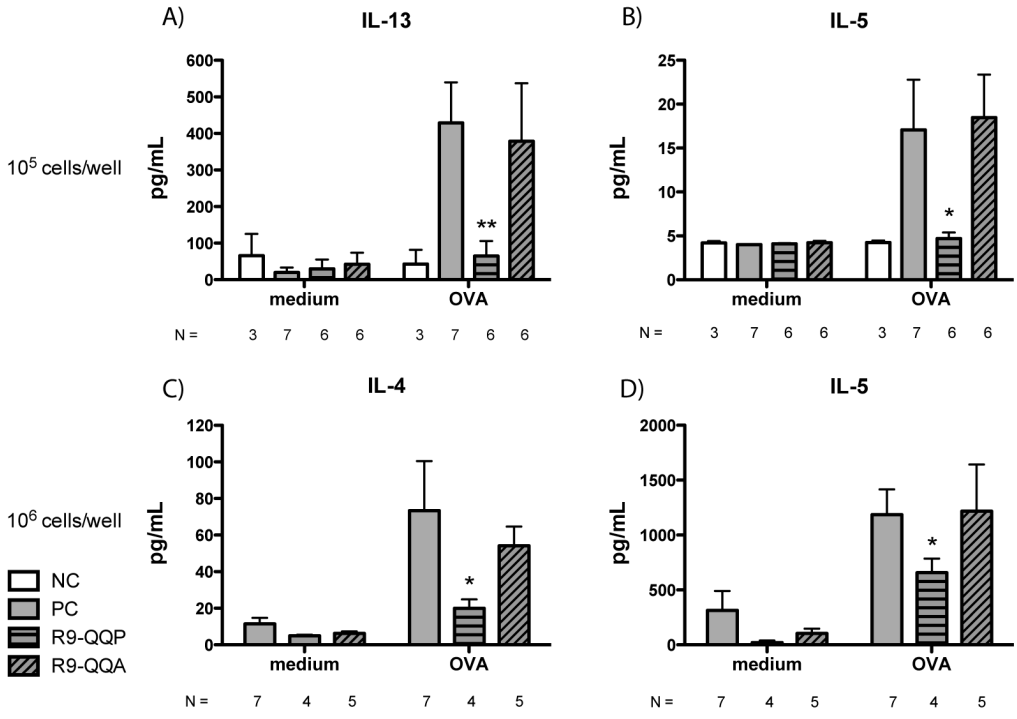


Figure 24: R9-QQP specifically inhibits Th2 cytokine production by lung draining lymph node (DLN) cells upon *in vitro* restimulation with OVA.

BL/6 mice were treated with peptides and experimental asthma was induced as described in figure 7. 24 hours after the final challenge with OVA, mice were sacrificed and lung DLN were harvested and pooled from 2-4 mice per group as described in Materials & Methods. After preparing a cell suspension, pooled DLN cells for each group were seeded at 10^5 or 10^6 DLN cells per well to designated wells of a round-bottom 96-well plate and restimulated with OVA. After culture for 4 days *in vitro*, supernatants were harvested and tested for Th2 cytokines by ELISA as described in Materials and Methods. A-D) Bar graphs display the average and SEM of Th2 cytokine amounts from supernatants of cell cultures seeded at (A-B) 10^5 or (C-D) 10^6 DLN cells per well. Statistical significance was calculated using an unpaired student's t test comparing cytokine amounts of peptide-treated to untreated (positive control) DLN cell culture supernatants for each group (* = $p < .05$; ** = $p < 0.01$).

4. DISCUSSION

As required for its enzymatic and adaptor functions, the inducible T cell kinase (Itk) interacts with the adaptor protein SLP-76 downstream of signals received via the T cell receptor (TCR). Upon stimulation, the Itk–SLP-76 association is mediated by binding of the SH2 and SH3 domains of Itk to specific phosphotyrosine (pY 145) and polyproline ligands present on SLP-76, respectively (81, 117). Furthermore, previous *in vitro* binding studies using purified Itk domain fragments identified the QQPPVPPQRPMA peptide sequence derived from the SLP-76 proline rich domain as the minimal polyproline ligand for the Itk SH3 domain with a reported affinity of 0.77 mM (1, 81).

In order to study the biological effects of the Itk–SLP-76 interaction, Grasis et al. modified the SLP-76 polyproline ligand peptide sequence by the addition of a cell penetration motif at the N-terminus consisting of nine arginines to generate the synthetic peptide named R9-QQP in addition to a control peptide with a scrambled cargo sequence named R9-PQM (2). Previously, studies had demonstrated that peptides as well as protein domains could be rendered capable of penetrating the plasma membrane by the addition of cationic residues including polyarginine motifs (125-130). As a reporter of cellular uptake, FITC-tagged R9-QQP was added to Jurkat T cells and primary splenocytes followed by analysis of fluorescence by confocal

microscopy and FACS. As revealed in the previous and current studies, FITC-tagged versions of R9-QQP, R9-PQM and R9-QQA (proline to alanine substitution control peptide) were all efficiently taken up by cells (2). Furthermore, R9-QQP was non-toxic for the duration of the *in vitro* experiments performed using splenocytes as demonstrated by analysis of cell viability using 7-AAD (2).

Beyond the combined requirement for the Itk SH2 and SH3 domains, the individual contribution of each adaptor domain for binding to SLP-76 had not been previously addressed. As revealed by treatment of T cells with R9-QQP, disruption of the inducible Itk–SLP-76 association indicated that binding of the Itk SH3 domain to its polyproline ligand on SLP-76 is necessary for the biochemical association of the full length molecules occurring in live cells. Thus, these results demonstrated that internalized R9-QQP is capable of competing with full length SLP-76 for binding to the Itk SH3 domain.

After establishing the biochemical importance of the Itk SH3 domain for the association with SLP-76, a series of downstream signaling events resulting in Itk activation and effector function were investigated that were predicted to be disrupted upon treatment of cells with the R9-QQP inhibitor peptide. Upon TCR-mediated stimulation, Itk and SLP-76 are inducibly recruited to the immunological synapse nucleated by the signaling molecule LAT which is constitutively associated within lipid rafts within the plasma membrane (83, 134). Furthermore, recruitment of Itk to the immunological

synapse is defective in Jurkat cells with a disrupted SLP-76 gene (2, 113, 115). Taken together, the requirement of the Itk SH3 domain for association with SLP-76 implied that the recruitment of Itk to the immunological synapse would be impaired upon treatment of cells with R9-QQP. In order to investigate the TCR-mediated recruitment of Itk to the immunological synapse, Jurkat cells were transfected with GFP-tagged Itk and transfectants were conjugated to polystyrene beads coated with anti-TCR antibody which served as surrogate antigen presenting cells (APCs). Consistent with the prediction based on the disrupted biochemical association, analysis of images acquired by confocal microscopy revealed that recruitment of GFP-Itk to the surrogate immunological synapse was defective for Jurkat cells treated with R9-QQP (2).

Once recruited to the immunological synapse, Itk promotes TCR-dependent activation of WASp mediated by its constitutive association with Vav (90-96, 135). In its adaptor role, Itk serves to recruit Vav to the immunological synapse, which activates Cdc42. In this model, Cdc42 then activates WASp, which is inducibly recruited to SLP-76 independently of Itk. As a consequence of its impaired recruitment, the adaptor function of Itk in supporting actin polymerization at the surrogate immunological synapse was investigated for cells treated with R9-QQP. Consistent with the Itk recruitment defect, TCR-dependent actin polymerization was impaired for Jurkat cells treated with R9-QQP as determined by staining of Jurkat-bead conjugates for

filamentous actin followed by analysis of images acquired by confocal microscopy (2).

In its canonical enzymatic role, Itk is activated by phosphorylation in trans on tyrosine 511 of its kinase domain by Lck followed by autophosphorylation in cis on tyrosine 180 of its SH3 domain. While the SH3 domain is necessary for the initial recruitment of Itk to SLP-76 as demonstrated using the R9-QQP inhibitor peptide, additional domains are required that are hypothesized to cooperatively stabilize Itk in a conformation that allows access of the kinase domain for release of catalytic activity. In particular, the PH domain binds to the membrane phospholipid PIP_3 as well as serving as a possible adaptor to enhance recruitment and activation. In addition, the SH2 domain binds to pY 145 in the acidic domain of SLP-76 and is necessary for Itk activation (114, 115). After autophosphorylation in cis, pY 180 in the Itk SH3 domain is hypothesized to allosterically modify the SH3 domain to destabilize a previously described autoinhibitory intramolecular conformation of Itk mediated by interaction with the adjacent proline rich region (107). Taken together, the net effect of the TCR-dependent Itk domain interactions mentioned above leads to sustained Itk kinase activity resulting in phosphorylation the the phospholipase PLC-γ1 on tyrosine residue 783. As demonstrated for both Jurkat T cells and splenocytes, incubation with R9-QQP inhibits Itk activation as measured by phosphorylation of tyrosine 511

located in the kinase domain, which is a substrate targeted by Lck that is necessary for the enzymatic activation of Itk (2).

Once activated, PLC-γ1 hydrolyzes PIP_3 resulting in generation of the second messengers IP_3 and DAG which activate pathways leading to transcriptional activation of inflammatory genes including cytokine genes. As reported for $Itk^{-/-}$ T cells, TCR-mediated secretion of the Th2 cytokines IL-4, IL-5 and IL-13 is defective in the absence of Itk (100-102). In order to characterize the effect of R9-QQP on cytokine secretion, splenocytes from WT C57BL/6 mice were treated *in vitro* with R9-QQP followed by primary stimulation and secondary stimulation using ant-TCR and anti-CD28 antibodies. Consistent with the previously described defect for $Itk^{-/-}$ T cells, analysis of tissue culture supernatants by ELISA as well as analysis of cytokine expression by ICCS revealed that R9-QQP significantly inhibited secretion of the Th2 cytokines IL-4, IL-5 and IL-13 while the signature Th1 cytokine IFN-γ was not affected (2).

In addition to the previously described effects of R9-QQP added to cells *in vitro*, Grasis et al. extended these findings by delivery of R9-QQP to mice *in vivo* followed by functional characterization in the context of Itk activation as described above. For these experiments, 20 mg/kg of R9-QQP was delivered by intraperitoneal (i.p.) injection at 24 and 0.5 hours prior to harvesting spleens. After processing into splenocytes, the TCR-mediated association of Itk with SLP-76, activation of Itk by phosphorylation at tyrosine 511 and

production of Th2 cytokines were analyzed as described previously. Despite concerns about peptide stability *in vivo*, delivery of R9-QQP by i.p. injection resulted in significant inhibitory and peptide specific effects that were consistent with the results of adding R9-QQP to cells *in vitro*. Thus, this study demonstrated the feasibility of using R9-QQP as a competitive inhibitor of the inducible T cell kinase upon delivery to mice *in vivo*.

In the current study, the *in vivo* significance of the Itk–SLP-76 interaction was investigated by application of the R9-QQP inhibitor peptide to the OVA-inducible asthma model. In this model, naive mice are first sensitized with the model allergen OVA which is purified from chicken egg white and adsorbed to the adjuvant aluminum hydroxide followed by delivery by i.p. injection. After 7 days, OVA-specific T cell clones are generated *in vivo* mediated by cells of the innate immune system such as dendritic cells and macrophages which present OVA-derived peptides to CD4+ T cells in the context of MHC class II molecules. Unlike the use of anti-TCR antibodies employed in the initial characterization of R9-QQP, the OVA-inducible system results in a more physiological mode of T cell stimulation.

Prior to the investigation of its effects on asthma pathogenesis, R9-QQP was first characterized in an *in vitro* model of OVA-dependent T cell function as assessed by Th2 cytokine secretion. For these experiments, mice were sensitized i.p. with OVA/alum and spleens were harvested 7 days later. After processing into splenocyte suspensions, separate aliquots of cells were

treated *in vitro* with vehicle, R9-QQP inhibitor or R9-PQM / R9-QQA control peptides according to the *in vitro* peptide treatment protocol established in the initial characterization of R9-QQP. Next, soluble OVA was added to the culture medium for restimulation followed by incubation for 4 days to allow cytokine secretion by OVA-specific T cells.

In agreement with the previously published results, analysis of tissue culture supernatant samples by ELISA revealed that R9-QQP inhibited Th2 cytokine secretion in a dose-dependent and specific manner while the signature Th1 cytokine was not affected. The differential effect of R9-QQP on Th2 but not Th1 cytokine production correlates with the Itk-specific requirement for Th2 effector function while Th1 effector function has been demonstrated to occur independently of Itk expression. Presumably, the Itk-specific requirement for Th2 effector function is due to its selective expression by Th2 effector cells while the related Tec family kinase Rlk is upregulated in Th1 effector cells where it is presumably able to compensate for the lack of Itk expression in $Itk^{-/-}$ T cells.

While analogous to the previous characterization of cytokine production, differences in terms of the timing of inhibition provided by R9-QQP in the two protocols allows additional distinctions to be made with respect to the specific requirement for Itk in the OVA-dependent system. In the previous characterization, splenocytes from naive mice were treated with R9-QQP *in vitro* followed by primary and secondary stimulation separated by

24 hours mediated by anti-TCR and anti-CD28 antibodies. In this protocol, R9-QQP was presumably internalized and effective as a dominant negative inhibitor of Itk activation prior to the primary stimulation as well as being possibly effective 24 hours later at the secondary stimulation time point. In the current work, Itk activation was allowed to occur unperturbed throughout the duration of the primary OVA-dependent stimulation occurring *in vivo* with inhibition mediated by R9-QQP only applied during the secondary stimulation occurring *in vitro*.

As introduced earlier, Itk is required for TCR-mediated Th2 commitment in the absence of additional cytokine-mediated signaling (100). In addition, Itk is required for the TCR-mediated release of effector function by Th2 competent cells upon secondary stimulation (101, 102). In the previous characterization, the inhibitory effect of R9-QQP could be interpreted as affecting either Th2 commitment, Th2 effector function or both due to its likely effectiveness throughout the entire protocol including at the time points of primary and secondary TCR-mediated stimulation. In the current study, R9-QQP is demonstrated to be required at the effector stage of Th2 cytokine release due to the timing of its addition 7 days after the initial sensitization occurring *in vivo*. In reference to the previous characterization of the role of Itk in T cell development, the results reported in the current study are consistent with a model requiring Itk at the effector stage of cytokine secretion upon OVA-mediated restimulation of allergen-specific T cell clones.

In order to further characterize the mode of inhibition provided by treatment of cells with R9-QQP, cytokine expression was compared using the ELISA and ELISpot assays. As determined by the ELISA assay, Th2 cytokine secretion into the culture medium is inhibited by R9-QQP in a dose-dependent manner. However, analysis of the number of cytokine producing cells as determined by the ELISpot assay reveals no effect upon treatment of parallel cultures with R9-QQP. Together, these apparently contradictory results can be resolved by interpretation of the inhibitory effect as suppressing cytokine secretion by previously differentiated Th2 effector cells rather than by affecting the frequency of OVA-specific CD4+ T cells. According to this model, treatment with R9-QQP would be expected to inhibit the amount of Th2 cytokines secreted by a constant population of Th2 effector cells that cumulatively results in a reduction of the total amount of cytokine present in the tissue culture supernatant as measured by the ELISA assay. Thus, these results are consistent with the differentiation of OVA-specific CD4+ T cells that occurs prior to treatment with R9-QQP in this assay and provides additional evidence that R9-QQP is specifically capable of inhibiting Th2 effector function.

In transition to the OVA-inducible asthma model, the previously characterized defective lung inflammation reported for $Itk^{-/-}$ mice was validated prior to application of the R9-QQP inhibitor peptide to the asthma protocol. According to this model, mice are first sensitized i.p. with OVA/alum followed

by a challenge period in which anesthetized mice are treated with OVA provided intranasally. After the final challenge with OVA, parameters of lung inflammation are assessed including airway hyperresponsiveness, mucus production, cellular infiltration into lung tissue and cytokine production by lung draining lymph node cells. As previously described for Itk$^{-/-}$ mice, these parameters of asthma are significantly reduced as compared to WT mice in the OVA-inducible asthma model (11, 76). Indeed, the asthma phenotype for Itk$^{-/-}$ mice described in these reports is nearly identical to non-immunized control mice. Surprisingly, the initial validation of the Itk$^{-/-}$ phenotype in the current study performed according to the previously described protocol resulted in robust lung inflammation that was equivalent to WT mice as indicated by quantification of eosinophils present in bronchoalveolar lavage fluid (BALF) and in lung tissue. Cumulatively, analysis of WT and Itk$^{-/-}$ mice treated according to this protocol revealed a nearly identical ~70% eosinophilia as a percentage of total leukocytes in BALF and ~15% eosinophilia in lung tissue for both groups of mice. Due to the unexpected inflammatory phenotype, the Itk$^{-/-}$ mice used in these studies were evaluated for possible mis-expression of Itk. However, genotyping using Itk-specific PCR primers and immunoblotting for evaluation of protein expression using an anti-Itk monoclonal antibody confirmed the knockout status of the Itk$^{-/-}$ mice. Thus, the lung inflammation observed for the Itk$^{-/-}$ mice treated according to the OVA-inducible asthma protocol was apparently independent of Itk.

Based on the previous characterization of the role of Itk in Th2 development and effector function, Itk is known to be an important regulator of low affinity TCR interactions with high affinity TCR interactions occurring independently of Itk (100). In the context of the paradoxical results described above for Itk$^{-/-}$ mice treated according to the OVA-inducible asthma protocol, the amount of OVA provided intranasally during the challenge period was suspected to be beyond the sensitive range for regulation by Itk. That is, high amounts of OVA provided i.n. during the challenge period were suspected to result in high affinity / avidity TCR interactions that would bypass the role of Itk resulting in Itk-independent inflammatory responses. In order to test this hypothesis, the total amount of OVA provided at challenge was reduced 10-fold from a total of 40 μg to 4 μg of OVA. While differences were not observed after exposure with 40 μg of OVA delivered i.n. according to previous reports, comparison of WT and Itk$^{-/-}$ challenged with a total exposure of 4 μg of OVA resulted in a significant reduction in eosinophilia in BALF and lung tissue for Itk$^{-/-}$ mice as compared to WT mice. Cumulatively, BALF eosinophilia of WT mice challenged with a total exposure of 4 μg of OVA was 32% as compared to 2% for Itk$^{-/-}$ mice while lung eosinophilia was 8% for WT mice versus 4% for Itk$^{-/-}$ mice. Thus, the Itk-dependent defect was reproduced in the OVA-inducible asthma model but only upon intranasal exposure of mice with relatively low amounts of OVA.

While satisfying, these results did not explain the discrepancy with the previous report that described defective lung inflammation upon a total exposure of 40 μg of OVA. In this regard, technical differences in the intranasal delivery of nominally equivalent amounts of OVA to anesthetized mice may explain the disparate results observed in the previous and current reports. For example, previous studies have tracked tissue distribution of equivalent amounts of a dye provided intranasally that was provided in different volumes. Overall, delivery of equivalent amounts of dye in larger volumes resulted in a greater proportion of dye that was detected in the lower respiratory tract (136, 137). In addition, the degree and type of anesthesia impacts the delivery of material to the lower respiratory tract that is provided intranasally. Taken together, these technical differences may individually and in combination result in the delivery of different amounts of OVA to the lower respiratory tract while nominally providing equivalent amounts delivered intranasally according to the same protocol. In summary, validation of the previously described Itk$^{-/-}$ phenotype was invaluable for the development of an OVA-inducible asthma protocol to test the effectiveness of the Itk inhibitor peptide R9-QQP on parameters of asthma.

In the final stage of translation to the OVA-inducible asthma model, FITC-tagged R9-QQP was demonstrated to enter CD4+ T cells upon *in vivo* delivery both by i.p. injection as well as by i.n. treatment. For investigation of uptake by systemic delivery, naive C57BL/6 mice were injected i.p. with FITC-

R9-QQP at 24 hrs. and/or 0.5 hr. prior to harvesting spleens. After extensive washes and treatment with trypsin to reduce detection of membrane-associated peptide, splenocytes were stained using anti-CD4 and anti-TCR fluorophore-conjugated antibodies followed by fixation and analysis by FACS.

As compared to mice treated with vehicle, CD4+ T cells obtained from the spleens of mice treated with FITC-R9-QQP at both time points were positive for FITC fluorescence as determined by the mean fluorescence intensity (MFI) of CD4+ T cells as well as by the percentage of FITC+ CD4+ double positive T cells. According to the MFI analysis of CD4+ T cells, injection of mice with FITC-R9-QQP resulted in a 2.8-fold increase in FITC fluorescence as compared to mice treated with vehicle. Furthermore, analysis of the percentage of FITC+ CD4+ double positive T cells revealed that the 2.8-fold increase in MFI was attributed to 37% of the CD4+ T cell population. Together, these separate analyses suggest that FITC-R9-QQP is not uniformly taken up by all CD4+ T cells obtained from the spleens of mice treated with peptide by i.p. injection. Rather, these results imply that FITC-R9-QQP is disproportionally taken up by a subset of these cells that are perhaps more accessible to peptide at the time of injection.

In further analysis of the pharmacokinetics of peptide uptake, injection of FITC-R9-QQP at either 24 hours or 0.5 hour prior to harvesting spleens resulted in reduced detection of FITC fluorescence as compared to mice treated with peptide at both time points. As likely accounted for by proteolytic

degradation within the peritoneal cavity, the single i.p. injection of FITC-R9-QQP at the 24 hours time point resulted in the least detection of FITC fluorescence as measured by both the MFI of CD4+ T cells as well as by the percentage of FITC+ CD4+ T cells followed in magnitude by the single injection at the 0.5 hour time point with the delivery at both time points resulting the greatest FITC fluorescence.

For investigation of uptake at the lung mucosa, separate OVA-sensitized mice were briefly anesthetized and treated intranasally (i.n.) with FITC-R9-QQP prior to each challenge with OVA. 24 hours after the final challenge, mice were sacrificed and lungs were lavaged. Next, BALF cells were processed as described above for detection of internalized FITC-R9-QQP by CD4+ T cells obtained from spleens. Similar to the analysis of uptake by systemic delivery, FACS analysis of BAL cells revealed a 2.5-fold increase in the FITC MFI of CD4+ T cells as compared to mice treated with vehicle. Furthermore, analysis of the percentage of FITC+ CD4+ T cells revealed that this 2.5-fold increase in FITC MFI was attributed to 7.3% of the CD4+ T cell population. Consistent with the investigation of uptake by systemic delivery, these results suggest that FITC-R9-QQP is disproportionally taken up by a subset of CD4+ T cells at the lung mucosa. According to the same interpretation as before, this phenomenon is likely due to the accessibility of peptide to CD4+ T cells present at the lung mucosa at the time of i.n. delivery. Importantly for inhibition of Itk in the asthma model, investigation of peptide

uptake by i.p. injection as well as by i.n. treatment provides proof of principle that R9-QQP is taken up by CD4+ T cells both systemically and at the lung mucosa, respectively. However, the minor percentage of FITC+ CD4+ T detected upon separate treatment of mice with FITC-R9-QQP by i.p. injection or i.n. administration illustrates the potential issues of peptide stability and availability that may impact effectiveness in the asthma disease model.

Informed by the OVA titration analysis as well as by the peptide uptake studies described above, an experimental protocol was designed for investigation of the role of the Itk–SLP-76 interaction in the OVA-inducible asthma model. In order to maximize the effectiveness of the peptide treatment, R9-QQP inhibitor or R9-QQA control peptides were delivered to designated groups of mice throughout the entire sensitization and challenge periods. To this end, mice were injected i.p. with 25 mg/kg of R9-QQP or R9-QQA control peptides at 24 hrs. and 0.5 hr. prior to sensitization with OVA/alum. In this context, the peptide treatment protocol prior to sensitization with OVA/alum was consistent with the previously described characterization in which 20 mg/kg of R9-QQP was delivered at the these time points resulting in specific inhibition of Itk activation and function upon restimulation of splenocytes ex vivo (2). Furthermore, i.p. delivery of FITC-R9-QQP at both 24 hrs. and 0.5 hr. time points prior to harvesting spleens resulted in the greatest FITC fluorescence observed for CD4+ T cells thus indicating its bio-availability upon delivery using this protocol. Thus, this peptide treatment strategy was

adopted for inhibition of asthma pathogenesis upon sensitization with OVA/ alum.

Seven days after sensitization with OVA/alum, R9-QQP or R9-QQA peptides were delivered both systemically as well as locally to the lung mucosa during the challenge period. Similar to the delivery of peptide prior to sensitization, mice were treated with R9-QQP or R9-QQA by i.p. injection at 24 hours prior to the first i.n. challenge with OVA. In addition, mice were treated with peptides by i.p. injection as well as by i.n. delivery of 0.2 mg of peptides at 2 hrs. prior to each of 3 daily i.n. challenges with OVA. As demonstrated previously, i.n. delivery of 0.2 mg of FITC-R9-QQP according to this protocol resulted in detection of FITC fluorescence as a reporter of uptake by CD4+ T cells obtained from BALF samples. Cumulatively, each mouse treated according to the protocol described above received a total of ~3.6 mg of peptide with 3 mg delivered systemically by 6 i.p. injections of 0.5 mg each and 0.6 mg delivered locally by i.n. administration of 0.2 mg each. Although expensive in terms of peptide consumption (and not to mention intensive in terms of labor), this treatment protocol was designed to maximize effectiveness of R9-QQP by inhibiting Itk-dependent development of Th2 competent cells following sensitization with OVA/alum as well as by inhibiting Th2 effector function upon challenge in the OVA-inducible asthma model.

Separately, the OVA treatment protocol used for the following asthma studies was adopted from the previous characterization of WT versus Itk$^{-/-}$

mice. As discussed above, Itk was demonstrated to be sensitive to the amount of OVA provided i.n. upon challenge in the asthma model. That is, Itk was shown to regulate lung inflammation as measured by BAL and lung eosinophilia at low but not high doses of OVA in this model. In order to measure Itk-dependent inhibition upon treatment with R9-QQP, mice used for the characterization of R9-QQP in the asthma model were challenged with a sub-optimal amount of OVA as determined from the OVA dose response that was performed in these previous studies. As predicted from a sigmoidal curve that was applied to these data, mice treated with peptides were challenged with 10 μg of OVA that was delivered i.n. for 3 daily challenges resulting in a total exposure of 30 μg of OVA. 24 hours after the final challenge with OVA, mice were evaluated for various parameters of asthma as described below.

For evaluation of a composite indicator of asthma, separate groups of mice were treated with peptides or vehicle control as described above followed by assessment of airway hyperresponsiveness (AHR). For measurement of AHR, an 18 gauge cannula was inserted into the trachea of anesthetized mice and external ventilation was initiated upon connection to the Scireq FlexiVent system. Next, airway resistance was recorded in response to increasing doses of aerosolized methacholine ranging from 3 - 24 mg/mL which was aerosolized and delivered directly to the lungs of mice via the intratracheal cannula. As compared to the non-immunized negative control group, airway resistance was significantly increased for OVA-

sensitized and challenged mice at the 12 mg/mL dose of methacholine (from an average of 2.2 to 4.2 $cmH_2O.s/mL$, respectively). Significantly, AHR was reduced at the 12 mg/mL dose of methacholine for mice treated with R9-QQP as compared to vehicle control (average of 2.8 $cmH_2O.s/mL$; $p < 0.05$ as compared to vehicle control). Furthermore, the inhibitory effect of R9-QQP was specific as determined by treatment of mice with the R9-QQA control peptide versus vehicle control. Thus, the *in vivo* assessment of AHR indicated that the R9-QQP peptide was effective as an inhibitor of a physiological parameter of asthma.

In order to examine the histological basis of the inhibitory effect of R9-QQP, intact lungs were extracted and fixed in formalin after treatment of mice with peptides and induction of asthma as described above. Next, 5 μm thick sections were prepared and mounted onto microscopy slides followed by staining for mucus production and for the presence of inflammation, respectively. For analysis of mucus production, lung sections were stained with PAS followed by evaluation of individual bronchioles by light microscopy. For each bronchiole examined, a semi-quantitative score was recorded corresponding to the percentage of the total circumference that was positive for mucus as indicated by fuchsia staining. As revealed by this analysis which was performed blindly, mucus severity was significantly inhibited from the lungs of mice treated with R9-QQP versus vehicle while treatment with the R9-QQA control peptide had no effect (average mucus severity score of

12.1% for mice treated with R9-QQP normalized to vehicle control versus 81.5% for R9-QQA control peptide; $p < 0.01$)

For evaluation of inflammation, lung histology slides were stained with H&E for detection of nuclei followed by semi-quantitative analysis according to the depth of inflammatory cells surrounding bronchioles as well as blood vessels. Similar to the pattern observed for mucus production, inflammation was significantly reduced from the lungs of mice treated with R9-QQP versus vehicle while treatment with the R9-QQA control peptide had no effect (average inflammation score of 28.7% for mice treated with R9-QQP normalized to vehicle control versus 143.5% for R9-QQA; $p < 0.05$). Taken together, the reduced asthma phenotype indicated by the analysis of AHR correlates with the inhibition of mucus production and inflammation detected from the lung histological analyses described above.

In order to characterize the inflammatory cells inhibited by R9-QQP, mice were treated with peptides as described above followed by collection and analysis of bronchoalveolar lavage (BAL) cells. As determined by performing total cell counts, treatment of mice with R9-QQP resulted in a significant 53.7% decrease in the number of BAL cells collected as compared to treatment with vehicle ($p < 0.0001$) while treatment with R9-QQA had no significant effect on total BAL cell number. For morphological analysis, BAL cells obtained from separate mice were attached to cytospin slides followed by staining using a modified Wright-Giemsa dye. Qualitative evaluation of

cytospin slides revealed a robust infiltrate of inflammatory cells containing pink cytoplasmic granules consistent in appearance to eosinophils for slides prepared from mice treated with vehicle while slides from mice treated with R9-QQP had a reduced frequency of granulocytes relative to lung resident alveolar macrophages.

For objective evaluation of inflammatory cells by flow cytometry, aliquots of BAL cells obtained from separate mice were stained with a cocktail of fluorophore-conjugated antibodies followed by FACS analysis for identification and quantification of eosinophils as a percentage of total leukocytes. As revealed by this method, robust eosinophilia was induced upon treatment of mice with vehicle that were sensitized and challenged according to the asthma induction protocol in response to OVA as described above. Significantly, the percentage of eosinophils obtained from BALF samples of mice treated with R9-QQP was reduced by 47.2% ($p < 0.0001$) as compared to treatment with vehicle while the R9-QQA control peptide had no effect. For further investigation of the inhibitory effect of R9-QQP on eosinophilia, lung tissue was harvested from mice after performing bronchoalveolar lavage followed by dissociation into a lung cell suspension and staining for quantification of eosinophilia by flow cytometry. Although less robust overall as compared to BALF samples, eosinophilia in lung tissue was significantly reduced by 48.1% ($p < 0.001$) for mice treated with R9-QQP as compared to treatment with vehicle while R9-QQA had no effect. Thus,

quantitative analysis of BALF samples and lung tissue reveals that treatment of mice with R9-QQP significantly and specifically inhibits eosinophilia. Although their exact role in asthma pathogenesis is not completely understood, eosinophils are inducibly recruited to the lung mucosa in response to inhaled allergens and release inflammatory intermediates which are associated with disease severity (15-17).

As central regulators of the inflammatory response, T helper type 2 (Th2) lymphocytes are recruited to the lung mucosa by chemokines which are secreted by epithelial cells in response to inhaled allergen. Once recruited, activated Th2 effector cells secrete sustained levels of the Th2 cytokines IL-4, IL-5 and IL-13 that together mediate the allergic asthmatic response (63-67). Specifically, IL-4 is involved in the maintenance and differentiation of the Th2 effector population. In addition, IL-4 secreted by Th2 effectors induces cognate B lymphocytes to undergo immunoglobulin class switching to generate and secrete IgE which is involved in the pathogenesis of clinical asthma. Furthermore, IL-5 secreted by Th2 effectors at the lung mucosa recruits and activates eosinophils while IL-13 mediates mucus production and airway hyperresponsiveness of smooth muscle cells surrounding bronchioles during an active inflammatory response.

In order to evaluate the effect of R9-QQP on T lymphocytes, separate aliquots of BAL cells obtained from individual mice that were treated according to the asthma protocol as described above were stained with anti-TCR

antibodies for quantification of T cells as a percentage of total leukocytes by flow cytometry. In contrast to the effect on eosinophilia, analysis of BAL cells revealed that the percentage of T cells was not significantly affected upon treatment of mice with either R9-QQP inhibitor or R9-QQA peptide as compared to treatment with vehicle. In terms of the model of asthma pathogenesis, these results apparently preclude the possibility that the inhibitory effects of R9-QQP on the parameters of asthma discussed above are due to the impaired recruitment of T cells to the lung mucosa as a result of defective chemokine signaling. Instead, these results are consistent with a model of inhibition of Th2 effector function in terms of OVA-inducible cytokine secretion upon treatment with R9-QQP that results in impaired pathogenesis of asthma.

Interestingly, these results seemingly disagree with the previously described defective recruitment of T cells to the lungs of Itk$^{-/-}$ as compared to WT mice that were sensitized and challenged with OVA (11). However, differences in terms of the strategies employed for the inhibition of Itk activity in the two studies may account for the contrasting outcomes in terms of T cell recruitment. For example, targeted inhibition of the Itk–SLP-76 interaction employed in the current study may allow Itk to function in a pathway downstream of chemokine receptor-mediated signaling as opposed to the completely impaired signaling in Itk$^{-/-}$ T cells. In addition, analysis of T cells obtained from BALF samples in the current study versus from the lungs of

mice in the characterization of the Itk$^{-/-}$ mice may account for the different results reported in the two studies (11). That is, the lung tissue may represent an intermediate step in the eventual migration of T cells to the lung mucosa as represented by cells collected following bronchoalveolar lavage. According to this model, the small differences observed in the OVA-inducible recruitment of T cells in the lungs of WT versus Itk$^{-/-}$ mice may not be reflected in BALF samples which represent the final step in the migration of T cells following several challenges with OVA.

In further pursuit of the inhibitory effect of R9-QQP, B cell function was examined as reflected by the levels of OVA-inducible IgE measured from the serum of mice treated according to the protocol described above. As mentioned in the context of asthma pathogenesis, IL-4 secreted by Th2 effector cells induces cognate B cells to secrete IgE which enters systemic blood circulation. At the lung mucosa, IgE binds to high affinity Fc receptors expressed by resident mast cells and, upon IgE-mediated binding to inhaled allergen, results in the immediate release of pre-formed inflammatory intermediates which precipitate an asthma attack. As revealed by ELISA, total serum IgE was highly induced in response to OVA for mice that were sensitized and challenged with OVA as compared to non-immunized control mice. However, levels of serum IgE for mice treated with vehicle versus R9-QQP were similarly induced in response to OVA. One possibility implied by these results is that the Th2-dependent activation of B cells was not affected

by the *in vivo* treatment with R9-QQP. According to this possibility, partial inhibition of Th2 effector function by R9-QQP may allow for the secretion of sufficient levels of IL-4 as required for B cell activation in the B cell germinal center of the lymph node while at the same time inhibiting the pathogenesis of asthma occurring in the lungs. Alternatively, B cell activation may occur independently of Th2 effector function which may explain the lack of an effect of treatment with R9-QQP on the levels of serum IgE. Independently of either interpretation pertaining to the effect of R9-QQP, the previous characterization of $Itk^{-/-}$ mice revealed that OVA-specific IgE was unexpectedly increased as compared to WT mice (11). Later, an aberrant developmental pathway in $Itk^{-/-}$ mice leading to the increased generation of γδ T cells was demonstrated to underlie the dysregulated IgE secretion previously observed for these mice (77).

In order to assess the effect of R9-QQP on Th2 cytokine production in the asthma model, lung draining lymph nodes were harvested and pooled for separate groups of mice that were treated with peptides according to the protocol described above. For each experiment, lymphoid tissue was processed into a single cell suspension and DLN cells were seeded to replicate wells of round-bottom 96-well plates. After incubation for 4 days, tissue culture supernatants were harvested and separate aliquots were tested for IL-4, IL-5 and IL-13 by ELISA. In summary, OVA-inducible secretion of IL-4, IL-5 and IL-13 by DLN cells obtained from mice that were treated with

R9-QQP was significantly reduced as compared to similar cultures established from mice that were treated with vehicle while cytokine secretion was unaffected by DLN cells from mice teated with the R9-QQA control peptide. Consistent with the defect reported for Itk$^{-/-}$ mice, the results obtained for treatment of mice with R9-QQP delivered *in vivo* support a model of inhibition of asthma pathogenesis resulting from impaired Th2 cytokine secretion. Furthermore, the specific effect achieved by the dominant negative R9-QQP peptide in this model implicates the inducible Itk–SLP-76 association as an essential interaction which is intrinsic to T lymphocytes.

As noted in the review of CD4+ Th2 effector development, Itk is differentially required for the generation of Th2 competent and effector cells upon primary and secondary TCR-mediated stimulation, respectively (100-102). As the peptide treatment protocol employed in the current study encompasses both aspects of Th2 effector development, the question of when Itk activation is required in the context of the asthma model cannot be inferred from these data. By analogy, the results of the *in vitro* addition of R9-QQP to splenocytes obtained from OVA-sensitized mice followed by restimulation with OVA suggest that R9-QQP may inhibit Th2 effector function when administered *in vivo* only during the challenge phase. In order directly to address this issue, ongoing studies are aimed at modifying the comprehensive peptide treatment protocol to provide R9-QQP only at the

initial sensitization with OVA or during the challenge period followed by analysis of the asthma phenotype.

In contrast to the previous studies using Itk knockout mice, the use of the cell permeable peptide R9-QQP as a tractable research tool allows for investigation into the Itk-dependent requirements during Th2 development in otherwise intact mice. In terms of a therapeutic strategy, either use of R9-QQP as a prophylactic provided prior to sensitization or as a treatment for ongoing asthma would be possible means of intervention aimed at disrupting asthma pathogenesis by blocking Itk activation. More broadly, allergic and autoimmune disease progression for many other pathological conditions involving Th2 cells may be similarly affected by inhibition of Itk.

Chapter 4, in part, is currently being prepared for submission for publication of the material. Guimond, D.M., Cam, N.R., Hirve, N., Duan, W., Lambris, J.D., Croft, M., & Tsoukas, C.D (2012). Treatment of Experimental Asthma Using a Novel Peptide Inhibitor of the Inducible T Cell Kinase. The dissertation author was the primary author of this material.

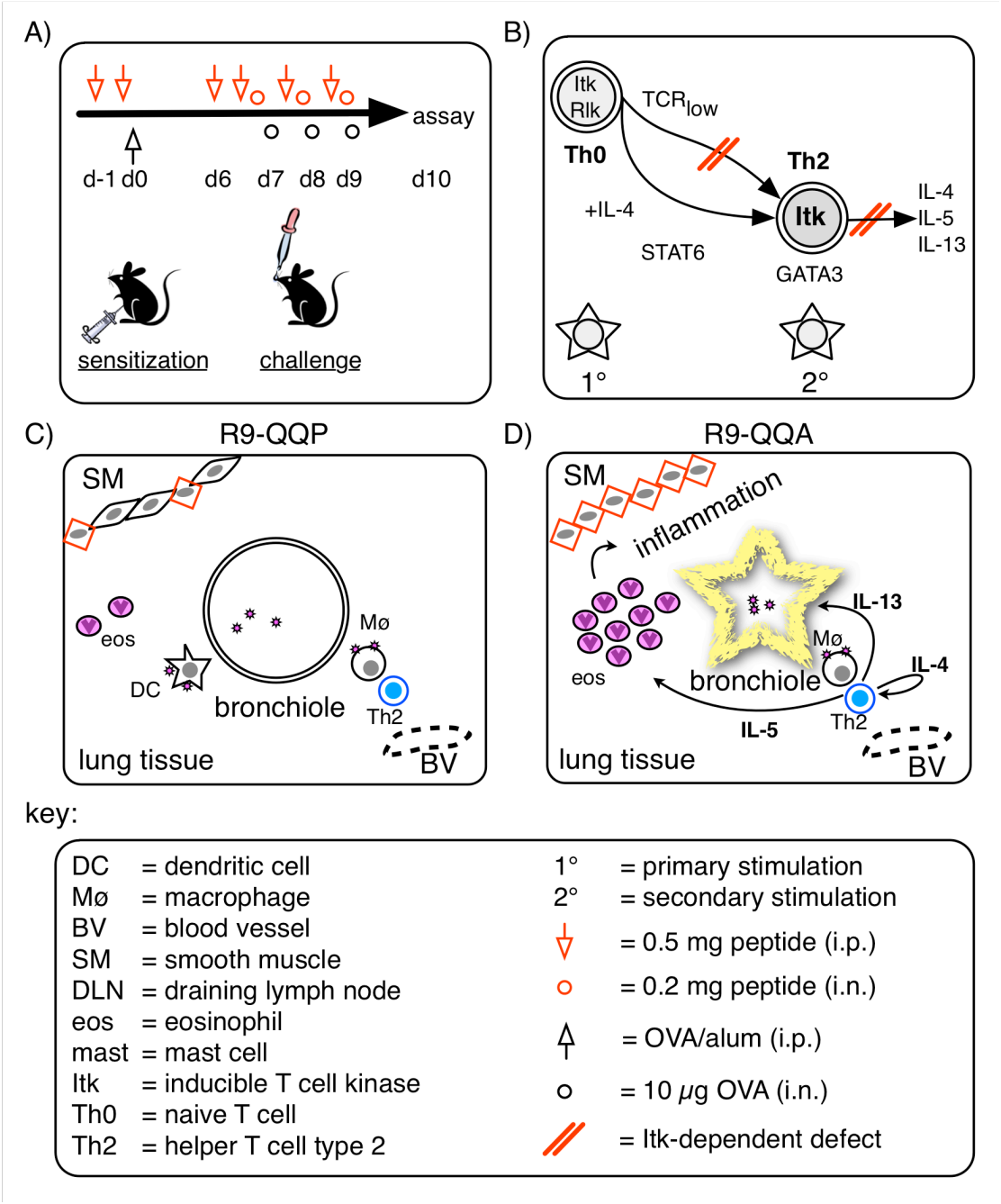


Figure 25: Model of inhibitory mechanism of R9-QQP in the OVA-inducible asthma model.

A) Diagram of in vivo peptide treatment protocol. B) CD4+ Th2 development affected by R9-QQP competitive inhibitory peptide. C-D) Diagram of lung tissue inflammation affected by treatment with R9-QQP or R9-QQA control peptides, respectively.

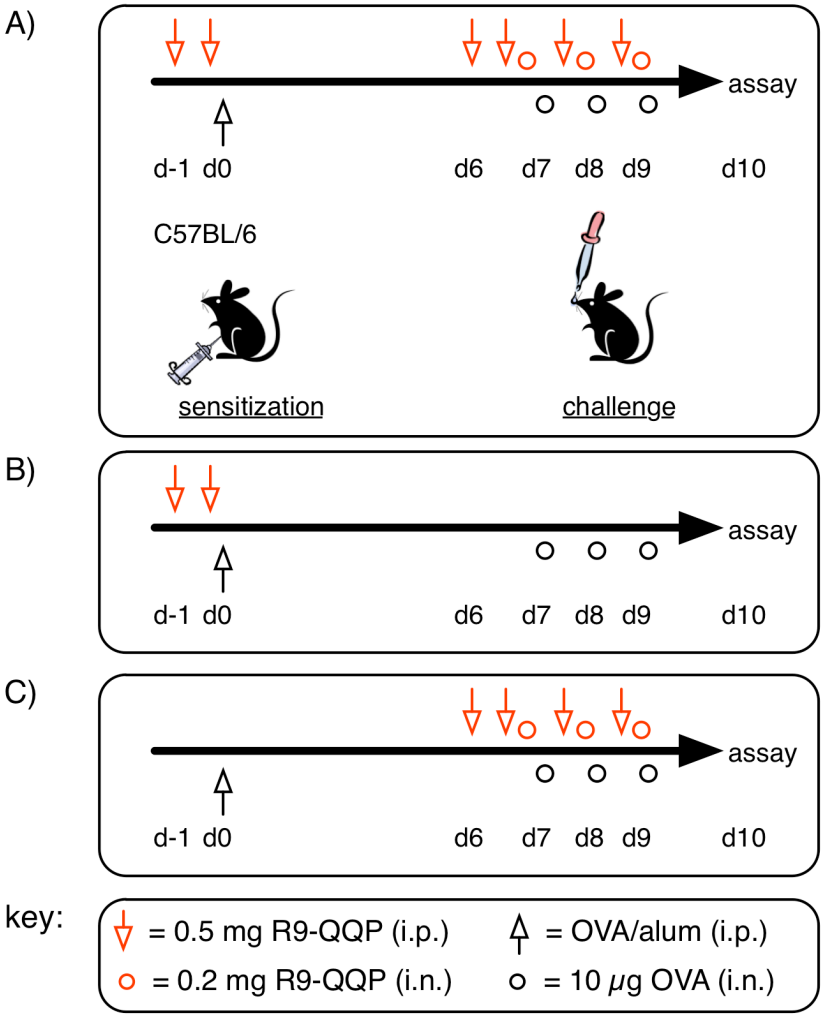


Figure 26: Schematic diagram compares and contrasts peptide treatment protocols.

A) Peptide treatment throughout sensitization and challenge as performed in current study. B-C) Future directions of treating mice with R9-QQP at sensitization or challenge, respectively.

5. APPENDIX

5.1. Buffers and Solutions

5.1.1. General

70% (v/v) Ethanol / dI H_2O

for sterilizing surfaces

- for 2 L, combine:
    - 1.4 L 95% ethanol
    - 0.6 L dI H_2O
- transfer to carboy

10 N NaOH

- work inside chemical fume hood
- for 50 mL, combine:
    - 30 mL ultrapure water
    - 20 g NaOH
        add to water to reduce risk from exothermic reaction
- stir to dissolve
- adjust to final volume with ultrapure water
- transfer to 50 mL tube
- store at room temp.

1 N NaOH

- work inside chemical fume hood
- for 50 mL, combine in 50 mL tube:
    - 45 mL ultrapure water
    - 5 mL 10 N NaOH
- invert to mix
- store at room temp.

0.5 M Tris-HCl, pH 6.8 - 8.0

- for 100 mL, combine:
    - 80 mL ultrapure water
    - 6.1 g Tris base
- for 500 mL, combine:
    - 400 mL ultrapure water
    - 30.5 g Tris base
- stir to dissolve
- adjust pH to 6.8-8.0
    add 10 N HCl drop-wise
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

0.5 M EDTA, pH 8.0

372.24 g/mol

- for 100 mL, combine:
    - 80 mL ultrapure water
    - 18.6 g EDTA
- stir to dissolve overnight
- adjust pH to 8.0
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

0.5 M EGTA, pH 8.0

380.4 g/mol

- for 100 mL, combine:
    - 80 mL ultrapure water
    - 19 g EGTA
- stir to dissolve overnight
- adjust pH to 8.0
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

0.5 M Calcium Chloride

110.99 g/mol

- for 100 mL, combine:
    - 80 mL ultrapure water
    - 5.6 g $CaCl_2$
- stir to dissolve
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

1 mM $CaCl_2$ / PBS

- for 100 mL, combine:
    - 80 mL PBS
    - 0.2 mL 0.5 M $CaCl_2$
- adjust to final volume with PBS
- filter sterilize (0.22 μm PES)
- store at 4°C

Indo-1 Stock, 1,000x

Molecular Probes, Cat. # I-1223

- reconstitute at 2 mg/mL in DMSO
  - *e.g., dissolve 50 µg aliquot in 25 µL of DMSO*
- store at -20°C for ≤ 1 month

30 mM EGTA / 300 mM Tris-HCl, pH 7.4

- for 100 mL, combine:
  - 60 mL Tris-HCl, pH 7.4
  - 6 mL 0.5 M EGTA, pH 8.0
- stir to mix
- adjust final pH to 7.4
- filter sterilize (0.22 µm PES)
- store at 4°C

10x PBS, pH 7.4

- for 1 L, combine:
  - 80 g NaCl
  - 2 g KCl
  - 14.4 g Na_2HPO_4
  - 2.4 g KH_2PO_4
  - 800 mL ultrapure water
- stir to dissolve while heating at 30°C
- adjust to final volume with ultrapure water
- store at 4°C
- pre-heat to 37°C prior to use
  - *to dissolve precipitated salts*

1x PBS, pH 7.4

137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4

- for 1 L, combine:
  - 100 mL 10x PBS
  - 800 mL ultrapure water
- stir to mix
- adjust pH to 7.4
- adjust to final volume with ultrapure water
- filter sterilize (0.22 µm PES)
- store at 4°C

Texas Red Phalloidin Staining Buffer

- *5 U/mL Texas Red Phalloidin in Blocking Buffer*
- *1 unit = amount required to stain 1 microscope slide*
- for each slide, combine:
  - *adjust volumes proportionally according to # of slides*
  - 5 µL 200 U/mL Texas Red Phalloidin stock
    - *Invitrogen / Molecular Probes, Cat. # T7471*
    - *200 U/mL stock in methanol*
  - 200 µL Blocking Buffer (1% BSA/PBS)

0.01 M Tris Base, pH 10.0

- for 1 L, combine:
  - 1.21 g Tris Base
  - 800 mL ultrapure water
- stir to dissolve
- adjust pH to 10.0
- adjust to final volume with ultrapure water
- transfer to glass bottle
- store at 4°C

1 mM Fluorescein Stock Solution

- for 100 mL, combine:
  - 33.2 mg fluorescein
    - *MW: 332 g/mol*
  - 80 mL 0.01 M Tris Base, pH 10.0
- stir to dissolve
- adjust to final volume with 0.01 M Tris Base, pH 10.0
- transfer to glass bottle
- cover bottle with foil to protect from light
- store at 4°C

Kinase Reaction Buffer

- prepare fresh
- for 10 mL, combine:
    - 6 mL ultrapure water
    - 500 μL 1 M HEPES, pH 7.4
        50 mM final
    - 100 μL 200 mM $MnCl_2$
        2 mM final
    - 20 μL 100 mM dATP
        0.2 mM final
    - 1 mL 100 mM $MgCl_2$
        10 mM final
    - 500 μL 200 mM DTT
        10 mM final
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)

5.1.2. DNA

1x LB

- for 1 L, combine in 2 L flask:
  - 10 g bacto-tryptone
  - 5 g bacto-yeast
  - 10 g NaCl
  - 1 L ultrapure water
- cover opening with foil
  adhere using autoclave tape
- place flask inside autoclave-safe container
  add tap water to base
- autoclave for 30 min. on wet cycle; slow exhaust
  takes 1 hr. total time
- cool to room temp.
- store at 4°C

2x YT

- for 1 L, combine in 2 L flask:
  - 16 g bacto-tryptone
  - 10 g bacto-yeast
  - 5 g NaCl
  - 1 L ultrapure water
- cover opening with foil
  adhere using autoclave tape
- place flask inside autoclave-safe container
  add tap water to base
- autoclave for 30 min. on wet cycle; slow exhaust
  takes 1 hr. total time
- cool to room temp.
- store at 4°C

1,000x Ampicillin

50 mg/mL ampicillin in ultrapure water

- solid stored at -20°C
- prepare aliquots and store at -20°C
- use at 50 μg/mL final

500x Kanamycin

10 mg/mL kanamycin in ultrapure water

- solid stored at -20°C
- prepare aliquots and store at -20°C
- use at 20 μg/mL final

Resuspension Buffer (#1), pH 7.5

- for 500 mL, combine:
    - 3.9 g Tris base
    - 1.9 g EDTA
    - 50 mg RNase A
    - 400 mL ultrapure water
- stir to dissolve
- adjust pH to 7.5
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

Lysis Buffer (#2)

- for 500 mL, combine:
    - 25 g SDS
    - 4 g NaOH
    - 400 mL ultrapure water
- stir to dissolve
- adjust to final volume with ultrapure water
- store at room temp.

Neutralization Buffer (#3), pH 4.8

- for 500 mL, combine:
    - 64.8 g KAc
    - 400 mL ultrapure water
- stir to dissolve
- adjust pH to 4.8
    add 10 N HCl drop-wise
- adjust to final volume with ultrapure water
- store at 4°C

Column Wash

- for 1 L, combine:
    - 7.9 g KAc
    - 400 mL ultrapure water
- stir to dissolve
- continue to add:
    - 16.6 mL 0.5M Tris-HCl, pH 7.4
    - 80 μL 0.5M EDTA, pH 8.0
    - 579 mL ethanol (molecular biology grade)
- adjust pH to 7.5
- adjust to final volume with ultrapure water
- store at 4°C

3.0 M Sodium Acetate, pH 5.2

- for 100 mL, combine:
    - 40.8 g sodium acetate
        136.1 g/mol
    - 80 mL ultrapure water
- adjust pH to 5.2
- adjust to final volume with ultrapure water
- store at 4°C

50x TAE Buffer

- for 500 mL, combine:
    - 121 g Tris base
    - 250 mL ultrapure water
- stir to dissolve
- continue to add:
    - 28.6 mL glacial acetic acid
    - 50 mL 0.5 M EDTA, pH 8.0
- adjust to final volume with ultrapure water
- store at room temp.

1x TAE Buffer

- for 500 mL, combine:
    - 10 mL 50x TAE Buffer
    - 490 mL ultrapure water
- store at room temp.

1x TE Buffer, pH 7.5 / 8.0

- for 100 mL, combine:
    - 80 mL ultrapure water
    - 2 mL 0.5 M Tris-HCl, pH 7.5 / 8.0
        10 mM final
    - 200 μL 0.5 M EDTA, pH 8.0
        1 mM final
- stir to mix
- adjust pH to 7.5 / 8.0
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

0.1x TE Buffer, pH 7.5 / 8.0

- for 100 mL, combine:
    - 80 mL ultrapure water
    - 10 mL 1x TE Buffer, pH 7.5 / 8.0
        1 mM Tris-HCl, 0.1 mM EDTA final
- stir to mix
- adjust pH to 7.5 / 8.0
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

5.1.3. Mouse Tail Digestion

Tail Solubilization Buffer (TSB)

- 1 mL TSB per 1 cm tail fragment
- prepare fresh
- for 10 mL, combine in 15 mL tube:
    - 7 mL ultrapure water
    - 1 mL 10x SET
        1% SDS, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0 final
    - 2 mL 0.5 M NaCl
        100 mM final
- invert to mix

Proteinase K Reconstitution Buffer

- for 100 mL, combine:
    - 40 mL ultrapure water
    - 2 mL 0.5 M Tris-HCl, pH 7.4
        10 mM final
    - 4 mL 0.5 M $CaCl_2$
        20 mM final
    - 50 mL glycerol
        50% final
- stir to mix
- adjust pH to 7.4
- adjust to final volume with ultrapure water
- prepare aliquots in 50 mL tubes
- store at -20°C

20 mg/mL Proteinase K

- reconstitute at 20 mg/mL in Proteinase K Reconstitution Buffer
- allow to hydrate for ~10 mins. at room temp.
- store at -20°C

Tail Salts

- for 100 mL:
    - pre-heat 90 mL of ultrapure water to 30°C with stirring
- gradually add the following salts until completely dissolved:
    - 24.6 g NaCl
        4.21 M final
    - 4.7 g KCl
        0.63 M final
- continue to add:
    - 2 mL 0.5 M Tris-HCl, pH 8.0
        10 mM final
- cool to room temp.
- adjust pH to 8.0
- adjust to final volume with ultrapure water
- store at room temp.

10x SDS, EDTA, Tris (SET), pH 8.0

- for 100 mL, combine:
    - 60 mL ultrapure water
    - 10 g SDS
        10% final
- stir to dissolve for ~1 hr at 30°C
- continue to add:
    - 10 mL 0.5 M EDTA, pH 8.0
        50 mM final
    - 20 mL 0.5 M Tris-HCl, pH 8.0
        100 mM final
- stir to mix
- adjust final pH to 8.0
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at room temp.

5.1.4. Cell Culture / In Vitro

Freezing Medium

10% DMSO + 90% FBS

- prepare fresh
- for 10 mL, combine:
    - 1 mL DMSO, cell culture grade
    - 9 mL FBS
        - heat-inactivated
- invert to mix

10x Erythrosine B

- for 100 mL, combine:
    - 80 mL PBS
    - 1.5 g erythrosine B

1.5% final

- stir to dissolve
- adjust to final volume with PBS
- prepare aliquots in 50 mL tubes
- store at 4°C

1x Erythrosine B

- for 100 mL, combine:
    - 80 mL PBS
    - 10 mL 10x Erythrosine B

0.15% final

- stir to mix
- adjust to final volume with PBS
- aliquot 10 mL per 15 mL tube
- store at 4°C

1% Sodium Deoxycholate

for endotoxin removal

- for 25 mL, combine:
    - 25 mL water, cell culture grade
    - 250 mg sodium deoxycholate
- vortex to dissolve
- filter (0.22 μm PES)
- store at 4°C

Jurkat / Raji Wash Medium (WM)

RPMI-1640 without supplements

293 Wash Medium (WM)

DMEM without supplements

complete RPMI (cRPMI)

RPMI-1640 + 10 mM HEPES, 2 mM L-glutamine, 100 I.U./mL penicillin and 100 µg/mL streptomycin

- for 515 mL, combine:
    - 500 mL bottle RPMI-1640
    - 5 mL 1 M HEPES
        - stored at 4°C
    - 5 mL 200 mM L-glutamine
        - stored at -20°C
    - 5 mL 10,000 I.U./mL penicillin - 10,000 µg/mL streptomycin
        - stored at -20°C
- invert to mix
- store at 4°C for ≤ 2 months

complete DMEM (cDMEM)

DMEM + 10 mM HEPES, 2 mM L-glutamine, 100 I.U./mL penicillin and 100 µg/mL streptomycin

- for 515 mL, combine:
    - 500 mL bottle DMEM
    - 5 mL 1 M HEPES
        - stored at 4°C
    - 5 mL 200 mM L-glutamine
        - stored at -20°C
    - 5 mL 10,000 I.U./mL penicillin - 10,000 µg/mL streptomycin
        - stored at -20°C
- invert to mix
- store at 4°C for ≤ 2 months

Jurkat / Raji Growth Medium (GM)

cRPMI + 10% FBS

- for 565 mL, combine:
    - 515 mL bottle cRPMI
    - 50 mL FBS
        - heat-inactivated
- invert to mix
- store at 4°C for ≤ 2 months

293 Growth Medium (GM)

cDMEM + 10% FBS

- for 565 mL, combine:
    - 515 mL bottle cDMEM
    - 50 mL FBS
        - heat-inactivated
- invert to mix
- store at 4°C for ≤ 2 months

Transfection Medium (TM)

RPMI-1640 / DMEM + 10 mM HEPES, 2 mM L-glutamine and 10% FBS (without penicillin-streptomycin)

- for 560 mL, combine:
    - 500 mL bottle RPMI-1640 / DMEM
    - 5 mL 1 M HEPES
        - stored at 4°C
    - 5 mL 200 mM L-glutamine
        - stored at -20°C
    - 50 mL FBS
        - heat-inactivated
- invert to mix
- store at 4°C for ≤ 2 months

Starvation Medium

cRPMI / cDMEM + 0.2% FBS

- for 516 mL, combine:
    - 515 mL bottle cRPMI / cDMEM
    - 1 mL FBS
        - heat-inactivated
- invert to mix
- store at 4°C for ≤ 2 months

Cell Line Nucleofector Solution V

for use with Jurkat Amaxa Nucleofection Protocol

- for 2.75 mL, combine:
    - 2.25 mL Cell Line Nucleofector Solution V
    - 0.5 mL Supplement 1
- mix gently
- store at 4°C for ≤ 3 months

T Cell Medium (TCM)

cRPMI + 10% FBS + 0.05 mM 2-ME

- for 565.5 mL, combine:
    - 515 mL bottle cRPMI
    - 50 mL FBS
        - heat-inactivated
    - 500 µL 1,000x 2-ME
- invert to mix
- store at 4°C for ≤ 2 months

Buffer 1

for CD4+ purification kit

- for ~250 mL, combine:
    - 200 mL PBS
    - 250 µL FBS
        0.1% final
        - heat-inactivated
    - 1 mL 0.5 M EDTA, pH 8.0
        2 mM final
- stir to mix
- adjust final pH to 7.4
- adjust to final volume with PBS
- filter (0.22 µm PES)
- store at 4°C

1,000x 2-Mercaptoethanol (2-ME)

50 mM

- for ~10 mL, combine:
    - 10 mL RPMI-1640
    - 35 µL 14.3 M 2-ME, cell culture grade
- invert to mix
- aliquot 600 µL per microcentrifuge tube
- store at -20°C

ACK Lysis Buffer

- for 1 L, combine:
  - 8.29 g NH_4Cl
    0.15 M final
  - 1 g $KHCO_3$
    10 mM final
  - 37.2 mg Na_2EDTA
    0.1 mM final
  - 800 mL ultrapure water
- stir to dissolve
- adjust pH to 7.4
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at room temp.

Mouse T Cell Nucleofector Solution

for use with Mouse T Cell Amaxa Nucleofection Protocol

- for 2.75 mL, combine:
  - 2.25 mL Mouse T Cell Nucleofector Solution
  - 0.5 mL Mouse T Cell Solution Supplement
- mix gently
- store at 4°C for ≤ 3 months

Mouse T Cell Nucleofector Medium

for use with Mouse T Cell Amaxa Nucleofection Protocol

- for 107 mL, combine:
  - 100 mL Mouse T Cell Nucleofector Medium
    supplied with kit
  - 1 mL 200 mM L-glutamine
    2 mM final
    - stored at -20°C
  - 5 mL FBS, heat-inactivated
    5% final
  - 1 mL Medium Component A
    supplied with kit
- mix gently
- store at 4°C
- prior to use, supplement with Medium Component B as follows:
  - remove aliquot from medium above supplemented with L-glutamine, FBS and Component A
  - add 10 μL Medium Component B per 1 mL

200 μg/mL PMA

- reconstitute in DMSO
- store at -20°C

10 mM Ionomycin (IM)

- reconstitute at 7.5 mg/mL in DMSO
- store at -20°C

100x PMA/IM

- prepare fresh in microcentrifuge tube
- for 50 μL, combine:
    - 40 μL RPMI-1640
    - 5 μL 200 μg/mL PMA
        1/10 dilution of stock
    - 5 μL 10 mM Ionomycin
        1/10 dilution of stock
- pipet up and down to mix

murine rIL-2

500 ng/mL in TCM

- quick-spin vial
- reconstitute at 100 μg/mL in water (cell culture grade)
- dilute 1:200 in TCM to prepare 500 ng/mL working solution
    5 ng/mL final
- store at -20°C

murine rIL-4

1 μg/mL in TCM

- quick-spin vial
- reconstitute at 100 μg/mL in water (cell culture grade)
- dilute 1:100 in TCM to prepare 1 μg/mL working solution
    10 ng/mL final
- store at -20°C

murine rIL-12

100 ng/mL in TCM

- quick-spin vial
- reconstitute at 200 μg/mL in PBS (cell culture grade)
- dilute 1:2,000 in TCM to prepare 100 ng/mL working solution
    1 ng/mL final
- store at -20°C

5.1.5. Protein Biochemistry

10% Ammonium Persulfate (APS)

- prepare fresh in microcentrifuge tube
- for 500 μL, combine:
    - 50 mg APS
    - 500 μL ultrapure water
- vortex to dissolve
- keep on ice

TEMED

- aliquot 500 μL per microcentrifuge tube
- store at 4°C

5 mg/mL Leupeptin

- reconstitute in ultrapure water
- aliquot 50 μL per microcentrifuge tube
- store at 4°C

1 mg/mL Pepstatin A

- reconstitute in ethanol (molecular biology grade)
- aliquot 500 μL per microcentrifuge tube
- store at -20°C

100 mM PMSF

- reconstitute at 17.4 mg/mL in isopropanol (molecular biology grade)
- aliquot 500 μL per microcentrifuge tube
- store at -20°C

100 mM NaO

- prepare fresh in microcentrifuge tube
- for 1 mL, combine:
    - 18 mg NaO
    - 1 mL ultrapure water
- vortex to dissolve
- keep on ice

NP-40 Lysis Buffer

- for 100 mL, combine:
    - 80 mL ultrapure water
    - 0.9 g NaCl
        150 mM final
- stir to dissolve
- continue to add:
    - 4 mL 0.5 M Tris-HCl, pH 7.4
        20 mM final
    - 80 μL 0.5 M EDTA, pH 8.0
        0.4 mM final
    - 1 mL NP-40
        1% final
- stir to mix
- adjust pH to 7.4
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

Complete Lysis Buffer (CLB)

- prepare fresh in microcentrifuge tube
- for ~1 mL, combine:
    - 1 mL NP-40 Lysis Buffer
    - 1 μL 5 mg/mL Leupeptin
        5 μg/mL final
    - 5 μL 1 mg/mL Pepstatin A
        5 μg/mL final
    - 10 μL 100 mM PMSF
        1 mM final
    - 10 μL 100 mM NaO
        1 mM final
- vortex to mix
- keep on ice

2x Laemmli Sample Buffer (LB)

- for 10 mL, combine in small beaker:
    - 2.5 mL 0.5 M Tris-HCl, pH 6.8
        125 mM final
    - 2 mL glycerol
        20% v/v final
    - 0.4 g SDS
        4% final
    - 0.3 g DTT
        0.2 M final
- stir to dissolve at room temp.
- continue to add while mixing:
    - 2 μL Bromophenol Blue
        0.02% final
- adjust to final volume with ultrapure water (in 15 mL tube)
- invert to mix
- aliquot 500 μL per microcentrifuge tube
- store at -20°C

30% Acrylamide

- prepare inside chemical fume hood; wear gloves and face mask
- for 100 mL, combine:
    - 60 mL ultrapure water
        - pre-heat to 30°C
    - 29 g acrylamide
    - 1 g N,N'-methylenebisacrylamide
- stir to dissolve
- cool to room temp.
- adjust pH to ≤ 7.0
- adjust to final volume with ultrapure water
- filter (0.4 μm PES)
- wrap bottle in aluminum foil, protect from light
- store at 4°C

4x (1.5 M) Running Gel Buffer, pH 8.8

- for 100 mL, combine:
    - 80 mL ultrapure water
    - 18.7 g Tris base
    - 0.4 g SDS
- stir to dissolve
- adjust pH to 8.8
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

4x (0.5 M) Stacking Gel Buffer, pH 6.8

- for 100 mL, combine:
    - 80 mL ultrapure water
    - 6.1 g Tris base
    - 0.4 g SDS
- stir to dissolve
- adjust pH to 6.8
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

6% Acrylamide Gel Buffer

for running gel and plug

- prepare fresh
- for 10 mL, combine in 15 mL tube:
    - 5.5 mL ultrapure water
    - 2.5 mL 4x Running Buffer
        1x final
    - 2 mL 30% Acrylamide
        6% final
- vortex to mix

7.5 % Acrylamide Gel Buffer

for running gel and plug

- prepare fresh
- for 10 mL, combine in 15 mL tube:
    - 5.0 mL ultrapure water
    - 2.5 mL 4x Running Buffer
        1x final
    - 2.5 mL 30% Acrylamide
        7.5% final
- vortex to mix

9% Acrylamide Gel Buffer

for running gel and plug

- prepare fresh
- for 10 mL, combine in 15 mL tube:
    - 4.5 mL ultrapure water
    - 2.5 mL 4x Running Buffer
        1x final
    - 3.0 mL 30% Acrylamide
        9% final
- vortex to mix

Stacking Gel Buffer

- prepare fresh
- for 5 mL, combine in 15 mL tube:
    - 3 mL ultrapure water
    - 1.25 mL 4x Stacking Buffer
        1x final
    - 670 µL 30% Acrylamide
        4% final
- vortex to mix

5x Running Buffer

- for 1 L, combine:
    - 800 mL ultrapure water
    - 15.1 g Tris base
    - 72 g glycine
    - 5 g SDS
- stir to dissolve
- do not adjust pH
    will be ~8.3 when diluted to 1x
- adjust to final volume with ultrapure water
- transfer to glass bottle
- store at 4°C

1x Running Buffer

- prepare in graduated cylinder
- for 1 L, combine:
    - 800 mL ultrapure water
    - 200 mL 5x Running Buffer
- transfer to glass bottle
- store at 4°C

Western Transfer Buffer (WTB), pH 8.0

- for 1 L, combine:
    - 800 mL ultrapure water
    - 3 g Tris base
        25 mM final
    - 14.4 g glycine
        192 mM final
- stir to dissolve
- continue to add:
    - 50 mL methanol, ACS grade
        5% final
- stir to mix
- adjust pH to 8.0 using 10 N NaOH
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at 4°C

Coomassie Stain

50% methanol, 10% acetic acid + 0.05% Brilliant Blue R-250

- for 200 mL, combine:
    - 100 mL methanol
    - 0.1 g Brilliant Blue R-250
    - mix until completely dissolved
- continue to add:
    - 20 mL acetic acid
- adjust to final volume with ultrapure water
- prepare aliquots in 50 mL tubes
    reuse each aliquot several times

Ponceau Dye

- for 100 mL, combine:
    - 80 mL glacial acetic acid
    - 2 g Ponceau dye
        2% final
- stir to dissolve
- adjust to final volume with glacial acetic acid

TBST, pH 7.4

- for 2 L, combine:
    - 1.8 L ultrapure water
    - 2.5 g Tris base
        10 mM final
    - 17.5 g NaCl
        150 mM final
- stir to dissolve
- continue to add:
    - 2 mL Tween 20
        0.1% final
- stir to mix
- adjust pH to 7.4 with 10 N HCl
- adjust to final volume with ultrapure water
- filter sterilize (0.22 *μ*m PES)
- aliquot to 1 L bottles
- store at 4°C

Blocking Buffer

- for 100 mL, combine:
    - 80 mL TBST
        - pre-heat to 30°C
    - 5 g BSA
        5% final
- stir to dissolve
- adjust to final volume with TBST
- aliquot to 50 mL tubes
- store at -20°C

Blotting Solution

- prepare fresh
- for 15 mL, combine in 15 mL tube:
    - 7.5 mL Blocking Buffer
        2.5% BSA final
    - 7.5 mL TBST
- invert to mix

Stripping Buffer, pH 6.8

- for 100 mL, combine:
    - 60 mL TBST
    - 7.9 g Tris base
    - 20 g SDS
- stir to dissolve
- continue to add:
    - 7 mL β-mercaptoethanol
- adjust pH to 6.8
- adjust to final volume with TBST
- store at room temp.

5.1.6. Microscopy / FACS

10% (w/v) Sodium Azide (NaN_3)

- for 50 mL, combine:
    - 5 g NaN_3
    - 40 mL ultrapure water
- stir to dissolve
- adjust to final volume with ultrapure water
- store at 4°C

FACS Buffer

0.5% BSA/PBS + 0.02% (w/v) NaN_3

- for 1 L, combine:
    - 800 mL ultrapure water
    - 100 mL 10x PBS
        - pre-heat to 37°C
- heat to 30°C
- continue to add:
    - 5 g BSA
- stir to dissolve
- continue to add:
    - 2 mL 10% (w/v) NaN_3
- cool to room temp.
- adjust pH to 7.4
- adjust to final volume with ultrapure water
- store at 4°C

4% Paraformaldehyde (PFA) / PBS

- work inside chemical fume hood
- for 100 mL, combine:
    - 90 mL PBS
        - heat to 60°C
    - 4 g paraformaldehyde
- for 500 mL:
    - 450 mL PBS
        - heat to 60°C
    - 20 g paraformaldehyde
- stir to dissolve completely
- cool to room temp.
- adjust pH to 7.0 by adding 1 M NaOH
- adjust to final volume with ultrapure water
- aliquot to 15 mL tubes / microcentrifuge tubes
- store at -20°C for ≤ 1 year

2% Paraformaldehyde (PFA) / PBS

- prepare by diluting 4% paraformaldehyde 1:2 (v/v) in PBS
- aliquot to 15 mL tubes / microcentrifuge tubes
- store at -20°C for ≤ 1 year

0.01% (w/v) Poly-L-Lysine (pLL) / H_2O

- for 10 mL, combine in 50 mL tube:
    - 1 mL 0.1% (w/v) poly-L-lysine
        Sigma, Cat. # P8920; 100 mL
    - 9 mL ultrapure water
- invert tube to mix
- store at room temp.

Microsphere Storage Buffer

0.05% BSA/PBS (0.5 mg/mL)

Microsphere Blocking Buffer

0.1% BSA/PBS (1 mg/mL)

Permeabilization Buffer

0.1% (v/v) Triton X-100 / PBS

- for 100 mL, combine:
    - 100 mL PBS
    - 100 µL Triton X-100
        Sigma, Cat. # X-100
- stir to dissolve
- store at 4°C

Blocking Buffer

1% BSA/PBS

- for 100 mL, combine:
    - 90 mL PBS
    - 1 g BSA
        1% final
- stir to dissolve
- adjust to final volume with PBS
- store at 4°C

5.1.7. ELISA

Coating Buffer

0.1 M sodium carbonate, pH 9.5

- for 250 mL, combine:
    - 1.8 g $NaHCO_3$
        84.8 mM final
    - 398 mg Na_2CO_3
        150 mM final
    - 200 mL ultrapure water
- stir to dissolve
- adjust pH to 9.5
- adjust to final volume with ultrapure water
- filter (0.22 μm PES)
- store at 4°C for ≤ 4 weeks

Blocking Buffer

1% BSA / PBS, pH 7.4

- for 250 mL, combine:
    - 200 mL PBS
        - pre-heat to 30°C with stirring
    - 2.5 g BSA, Fraction V
- stir until completely dissolved
- adjust to final volume with PBS
- filter (0.22 μm PES)
- store at 4°C for ≤ 4 weeks

Reaction Buffer

0.1% BSA / PBS, pH 7.4

- for 250 mL, combine:
    - 200 mL PBS
        - pre-heat to 30°C with stirring
    - 250 mg BSA, Fraction V
- stir until completely dissolved
- adjust to final volume with PBS
- filter (0.22 μm PES)
- store at 4°C for ≤ 4 weeks

Wash Buffer

0.05% Tween-20 / PBS, pH 7.4

- prepare in large beaker
- for 2 L, combine:
    - 200 mL 10x PBS
    - ~1.6 L ultrapure water
    - 1 mL Tween-20
- stir to mix
- adjust pH to 7.4
- adjust to final volume with ultrapure water
- filter (0.22 μm PES)
- transfer to 1 L bottles
- store at 4°C for ≤ 4 weeks

Stop Solution

1 M H_3PO_4 / H_2O

- for 100 mL, combine in graduated cylinder:
    - 6.8 mL 14.7 M H_3PO_4
        1 M final
    - ultrapure water to final volume
- transfer to glass bottle
- store at room temp.

5.1.8. ICCS

FACS Buffer

see 'General' section

PBS

see 'General' section

2% Paraformaldehyde / PBS

see 'General' section

Saponin, 10% (w/v)

- for 10 mL, combine in 15 mL tube:
    - 1 g saponin
        Sigma, Cat. # S-7900
    - 8 mL PBS, pH 7.4
- place tube in 37°C water bath to dissolve
- adjust to final volume with PBS
- filter (0.22 μm)
- aliquot 1 mL per microcentrifuge tube
- store at -20°C
    once thawed, the 10% solution is stable for several months at 4°C

Permeabilization Buffer

0.5% BSA + 0.1% saponin + 0.02% NaN_3 in PBS

- for 100 mL, combine:
    - 80 mL FACS Buffer
    - 1 mL 10% (w/v) saponin
- stir to mix
- adjust to final volume with FACS Buffer
- store at 4°C for ≤ 6 months

Blocking Buffer

5% BSA + 0.1% saponin in PBS

- for 10 mL, combine in 15 mL tube:
    - 500 mg BSA
    - 8 mL Permeabilization Buffer
- place tube in 37°C water bath to dissolve
- adjust to final volume with Permeabilization Buffer
- centrifuge for 30 min. at 15,000 g
    to sediment solids
- transfer supernatant to new tube
- store at 4°C for ≤ 6 months

5.1.9. ELISPOT

1x PBS, pH 7.4

- see 'General' section for instructions

Wash Buffer

- see 'ELISA' section for instructions

Blocking Buffer

1% BSA / PBS + 0.05% Tween-20, pH 7.4

- for 250 mL, combine:
    - 225 mL Wash Buffer (see above)
        - pre-heat to 30°C with stirring
    - 2.5 g BSA, Fraction V
- stir until completely dissolved
- adjust to final volume with Wash Buffer
- filter sterilize, 0.22 μm PES
- store at 4°C

1.5 M Sodium Acetate, pH 5.2

- for 100 mL, combine:
    - 20.4 g sodium acetate
        CH_3COONa
    - 80 mL ultrapure water
- stir until completely dissolved
- adjust pH to 5.2 using acetic acid (not HCl)
- adjust to final volume with ultrapure water
- filter sterilize (0.22 μm PES)
- store at room temp.

AEC / DMF Solution

- prepare fresh
- combine in glass scintillation vial:
    - 1 tablet AEC (3-amino-9-ethyl-carbazole)
        Sigma, Cat. # A6926-50TAB
    - 1 mL DMF (N, N, -dimethylformamide)
        Sigma, Cat. # D4551
- agitate to dissolve completely

Substrate Solution

- prepare fresh
- combine in 50 mL tube:
    - 3.33 mL 1.5 M sodium acetate, pH 5.2 (see above)
    - ultrapure water to 50 mL
    - 825 μL AEC / DMF (see above)
    - 25 μL 30% H_2O_2
- invert tube to mix

5.1.10. OVA System / Asthma

0.2 mg/mL OVA/PBS

for i.p. sensitization

- prepare fresh in 15 mL Falcon tube
- remove OVA powder from refrigerator
  equilibrate to room temp. before opening
- cut 3"x3" sheet of weigh paper into 1/4 size and tare on analytical balance
- weigh 2-3 mg OVA, grade V
  Sigma, Cat. # A-5503
- reconstitute in sterile PBS at 0.2 mg/mL
  nominal concentration by gross mass
- invert tube to dissolve; do not vortex

OVA/Alum, 2:1 (v/v)

- *for i.p. sensitization*
- *20 µg OVA / 2 mg alum / 150 µL i.p. per mouse*

- prepare fresh in 50 mL Falcon tube
- for 20 mice (3 mL), combine:
  - 2 mL 0.2 mg/mL OVA/PBS
    - *0.133 mg/mL final*
    - *prepared fresh (see above)*
  - 1 mL 40 mg/mL Imject alum
    - *13.33 mg/mL final*
    - *Pierce / Thermo Scientific, Cat. # 77161*
- incubate on tube rotator for ≥ 2 hours at room temp. to adsorb OVA to alum

0.5 mg/mL OVA/PBS

- *for i.n. challenge*
- *10 µg OVA / 20 µL i.n. per mouse*
- prepare fresh in 15 mL tube
- remove OVA powder for refrigerator
  equilibrate to room temp. before opening
- cut 3"x3" sheet of weigh paper into 1/4 size and tare on analytical balance
- weigh 2-3 mg OVA, grade V
  Sigma, Cat. # A-5503
- reconstitute in sterile PBS at 0.5 mg/mL
  nominal concentration by gross mass
- invert tube to dissolve -- do not vortex
- aliquot to microcentrifuge tubes for each challenge day
- store single-use aliquots at -20°C for ≤ 1 week

1% OVA/PBS

- for aerosol challenge
- 10 mg/mL OVA/PBS
- prepare fresh for each challenge day in 50 mL tube
- remove OVA powder from refrigerator
    equilibrate to room temp. before opening
- weigh ~200 mg OVA, grade V
    Sigma, Cat. # A-5503
- reconstitute at 10 mg/mL in sterile PBS
    nominal concentration by gross mass

100 mg/mL Collagenase D

for lung tissue dissociation
- equilibrate lyophilizate to room temp.
- reconstitute in PBS
- allow to hydrate for ~10 min.
- aliquot 250 μL per microcentrifuge tube
- store at -20°C

10 mg/mL DNase I

for lung tissue dissociation
- reconstitute in PBS
- aliquot to microcentrifuge tubes
- store at -20°C

10 mg/mL OVA

for re-stimulation in vitro
- prepare in 15 mL tube
- remove OVA powder from refrigerator
    equilibrate to room temp. before opening
- cut 3"x3" sheet of weigh paper into 1/4 size and tare on analytical balance
- weigh ~20 mg OVA, grade V
    Sigma, Cat. # A-5503
- reconstitute at 10 mg/mL in sterile PBS
    nominal concentration by gross mass
- invert tube to dissolve completely -- do not vortex
- prepare 120 μL aliquots in microcentrifuge tubes
- store single-use aliquots at -80°C

100 mg/mL Ketamine HCl

- Ketaset III
  Fort Dodge Animal Health, NDC 0856-4403-01
- supplied at 100 mg/mL (10x stock)
- store at room temp. in locked cabinet

20 mg/mL Xylazine HCl

- AnaSed
  Lloyd Laboratories, Cat. # 4811
- supplied at 20 mg/mL (20x stock)
- store at room temp. in locked cabinet

Ketamine / Xylazine Cocktail

for 25 mg mouse,
- 80 mg/kg ketamine and 8 mg/kg xylazine
- 2 mg ketamine / 0.2 mg xylazine / 200 µL i.p. per mouse

- prepare fresh
- for 15 mice (3 mL), combine in 50 mL tube:
  - 300 µL 100 mg/mL ketamine
    see above; 10 mg/mL final
  - 150 µL 20 mg/mL xylazine
    see above; 1 mg/mL final
  - 2.55 mL sterile PBS
- keep at room temp.

5% Methacholine (MCh)

- 50 mg/mL methacholine in PBS (Sigma, Cat. # A2251)
- for AHR assay: 0, 3, 6, 12 and 24 mg/mL methacholine doses

- for ≥ 5 mL:
  - prepare in 15 mL tube
  - remove powder from -20°C and equilibrate to room temp. before opening
  - weigh ≥ 250 mg
  - dissolve in PBS at 50 mg/mL
- prepare 10 mL of each dose in 15 mL tubes
  - dilute to 24 mg/mL in PBS
    - add 4.8 mL of 50 mg/mL stock
    - add 5.2 mL of PBS
  - invert to mix
  - prepare 1:2 serial dilutions at 12, 6 and 3 mg/mL in PBS
    - dilute serially 1:2 in PBS
- store at room temp.

5.2. Protocols

5.2.1. General

Pipet Tip Sterilization

for routine bench work – do not use for tissue culture work (requires filter tips)

Equipment:

- autoclave
- lab oven

Materials:

- pipet tips, bulk packaging
  - 10 µL
    VWR, Cat. # 82028-528
  - 200 µL
    Fisher, Cat. # 02-681-134
  - 200 µL gel-loading
    Fisher, Cat. # 02-707-181
  - 1 mL
    VWR, Cat. # 82028-564
- empty tip boxes
- autoclave tape
- gloves

Protocol:

1) fill empty pipet tip boxes with tips from bulk package as needed
2) adhere autoclave tape across opening of box
   to indicate whether or not box has been autoclaved
3) autoclave for 30 min. on dry cycle
4) dry tips overnight in lab oven
5) transfer to shelf for storage

Absorbance Measurement Using Spectrophotometer

for routine absorbance measurement

Equipment:

- UV-VIs spectrophotometer
  - *Beckman*
  - *instrument is most sensitive for detecting absorbance readings in range from 0.1 - 1.0*
- quartz cuvette
  - *keep clean with Kimwipes*
- dI water squirt bottle
- waste container
- P1000 micropipettor and tips
- microcentrifuge rack

Materials:

- microcentrifuge tubes
  - *for preparing dilutions*
- gloves
  - *to protect samples from DNases and proteases*
- ultrapure water
- solution for blank
  - *matched to sample buffer*
- sample
  - DNA
    - plasmid DNA, purified
    - primer stock solution
  - protein
  - peptide
- Kimwipes
- paper and pen
  - *for recording readings*

Protocol:

- *DNA absorbs optimally at 260 nm*
  - *depending on purity of DNA preparation, contaminating bacterial proteins may also be present in solution which can be detected optimally by absorbance at 280 nm*
    - *a 260 / 280 ratio of 1.8 or greater indicates a pure DNA preparation*
- *protein / antibody absorbs optimally at 280 nm*
  - *calculate antibody concentration in mg/mL from A_{280} according to Beer's Law as follows: $(A_{280} / \varepsilon) \times 10 = mg/mL$*
- *peptide bond absorbs optimally at 214 nm*

1) prepare dilutions of stock solution in microcentrifuge tubes
    1.1) for 1:100 dilution, combine 10 μL of stock + 990 μL of ultrapure water
    1.2) for 1:200 dilution, combine 5 μL of DNA stock + 995 μL of ultrapure water
2) initialize spectrophotometer
    2.1) turn on UV lamp
    2.2) allow ~5 min. for lamp to warm-up / stabilize
3) for plasmid DNA:
    3.1) select 'DNA/Oligo Quant' > dsDNA program
    3.2) enter sample ID and dilution factor for each sample
4) for DNA primers:
    4.1) select 'DNA/Oligo Quant' > 'Oligo DNA Short' program
    4.2) enter sample ID, nucleotide sequence and dilution factor for each sample
5) for protein:
    5.1) select 'Protein' program
6) for peptide:
    6.1) select fixed wavelength program
7) blank instrument
    7.1) add 1 mL of blank solution to cuvette
    7.2) place cuvette inside compartment
    7.3) close lid
    7.4) select 'blank'
    7.5) remove cuvette and discard contents
    7.6) dry by inverting on top of Kimwipes
8) read samples
    8.1) add 1 mL of sample to cuvette
    8.2) place cuvette inside compartment
    8.3) close lid
    8.4) read sample
    8.5) record concentration
        - correct for dilution factor
        - for DNA samples, record 260/280 ratio
    8.6) remove cuvette and discard contents
9) wash cuvette
    9.1) fill cuvette with dI water from squirt bottle (wash)
    9.2) discard contents
    9.3) repeat for a total of 3 washes
    9.4) dry by inverting on top of Kimwipes
    9.5) repeat blank (see above) after each sample
10) exit spectrophotometer
    10.1) print report of results (if printer is available)
    10.2) exit program

10.3) turn off UV lamp

11) for accuracy, use reading(s) for dilutions within sensitive detection range

average readings if possible

Absorbance Measurement Using NanoDrop

for measurement of valuable and/or low concentration stock solutions – consumes only 2 μL of sample volume measured neat (without prior dilution required)

Equipment:

- NanoDrop spectrophotometer
  - *ND-1000*
  - *instrument is most sensitive for detecting absorbance readings in range from 0.1 - 1.0*
  - PC with NanoDrop application software installed
- waste container
- P2 micropipettor and tips
- microcentrifuge rack

Materials:

- gloves
  - *to protect samples from DNases and proteases*
- ultrapure water
- solution for blank
  - *matched to sample buffer*
- sample
  - DNA
  - protein
- Kimwipes
- paper and pen
  - *for recording readings*

Protocol:

- *DNA absorbs optimally at 260 nm*
  - *depending on purity of DNA preparation, contaminating bacterial proteins may also be present in solution which can be detected optimally by absorbance at 280 nm*
    - *a 260 / 280 ratio of 1.8 or greater indicates a pure DNA preparation*
- *protein / antibody absorbs optimally at 280 nm*
  - *calculate antibody concentration in mg/mL from A_{280} according to Beer's Law as follows: $(A_{280} / \varepsilon) \times 10 = mg/mL$*

1) initialize NanoDrop
   - 1.1) launch NanoDrop application on connected PC
   - 1.2) follow software prompts
   - 1.3) open sampling arm
   - 1.4) dry / clean sample pedestal using Kimwipe
   - 1.5) add 1.5 μL of ultrapure water to sample pedestal
   - 1.6) close sampling arm

1.7) select 'OK'
1.8) dry / clean sample pedestal using Kimwipe

2) choose DNA / protein program
3) blank instrument
3.1) follow software prompts
3.2) open sampling arm
3.3) add 2 μL of blank solution to pedestal
3.4) close sampling arm
3.5) select 'blank'
3.6) dry / clean sample pedestal using Kimwipe

4) read DNA / protein sample
4.1) follow software prompts
4.2) open sampling arm
4.3) add 2 μL of sample to pedestal
4.4) close sampling arm
4.5) select 'measure'
4.6) record concentration
for DNA samples, record 260/280 ratio
4.7) dry / clean sample pedestal using Kimwipe
4.8) close sampling arm

5) exit NanoDrop

Antibody Purification from Ascites Fluid

Equipment:

- fingertight stop plug 1/16", black
  - *to seal top of column*
  - *GE Healthcare Life Sciences, Cat. # 11-0003-55*
- stop plug female, 1/16", black
  - *to seal bottom of column*
  - *GE Healthcare Life Sciences, Cat. # 11-0004-64*
- 1/16" male / luer female, red
  - *to connect syringe to top of column*
  - *GE Healthcare Life Sciences, Cat. # 18-1112-51*
- union 1/16" female / M6 male, red
  - *connect directly to bottom of column*
  - *GE Healthcare Life Sciences, Cat. # 18-1112-57*
- union M6 female / 1/16" male, red
  - *connect to union M6 male at bottom of column*
  - *GE Healthcare Life Sciences, Cat. # 18-3858-01*

Materials:

- 5 mL syringe
- HiTrap Protein G HP column, 1 mL
  GE Healthcare Life Sciences, Cat. # 17-0404-03
  - stored at 4°C
  - equilibrate to room temp. prior to use
- binding buffer, 10x
  GE Healthcare Life Sciences, Cat. # 17-1128-01
  - stored at 4°C
  - equilibrate to room temp prior to use
- elution buffer, 10x
  GE Healthcare Life Sciences, Cat. # 17-1128-01
  - stored at 4°C
  - equilibrate to room temp. prior to use
- neutralizing buffer, 1x
  GE Healthcare Life Sciences, Cat. # 17-1128-01
  - stored at 4°C
  - equilibrate to room temp. prior to use
- 15 mL tube
  for collection of sample flow through
- microcentrifuge tubes
  for collection of fractions numbered from 1-20
- ascites fluid
  - stored at -20°C
- 0.45 μm filter

- 20% ethanol (molecular biology grade) in ultrapure water, 10 mL
- parafilm

Protocol:

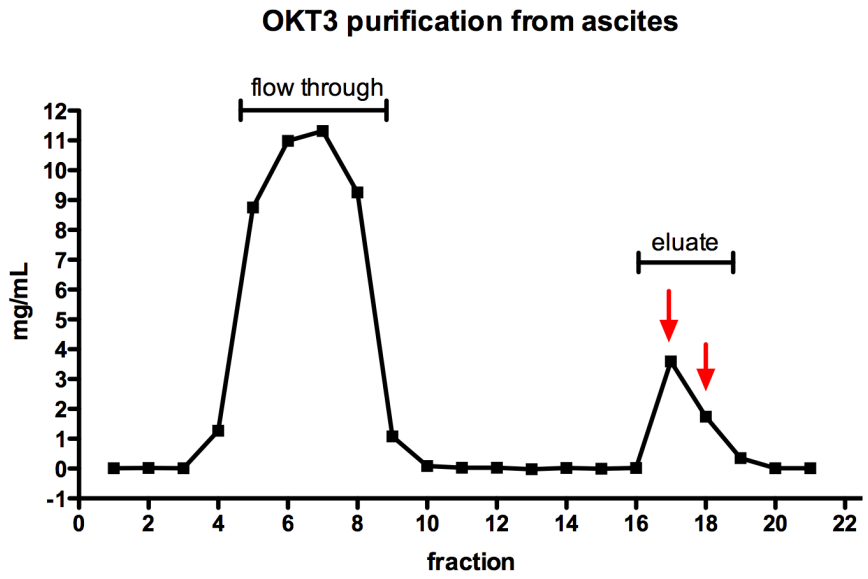


Figure 27: Purification of OKT3 antibody from mouse ascites fluid. Red arrows indicate fractions containing purified antibody.

1) buffer preparation
    1.1) dilute 10x binding buffer as follows: 2.5 mL 10x binding buffer + 22.5 mL ultrapure water (25 mL total volume)
    1.2) dilute 10x elution buffer as follows: 0.5 mL 10x elution buffer + 4.5 mL ultrapure water (5 mL total volume)
    1.3) aliquot 75 μL of neutralization buffer per microcentrifuge tube numbered from 1-20
2) prepare ascites for antibody (IgG) purification
    2.1) filter fluid through 0.45 μm filter to remove particulates
    2.2) dilute ascites fluid 1:1 in 1x binding buffer
3) assemble column apparatus; listed from top to bottom:
    3.1) 5 mL syringe
    3.2) 1/16" male / luer female, red
    3.3) HiTrap Protein G HP column, 1 mL
    3.4) union 1/16" female / M6 male, red
    3.5) union M6 female / 1/16" male, red
4) wash column with 5 mL of ultrapure water (to waste) at a flow rate of 1 drop / sec
5) equilibrate column with 3 mL of binding buffer (to waste)

6) apply 1-5 mL of ascites (filtered through 0.45 μm filter and diluted 1:1 in binding buffer) to column
    6.1) collect sample flow through in 15 mL tube
7) wash column with 7 mL binding buffer
    7.1) collect 1 mL fractions in numbered microcentrifuge tubes containing 75 μL neutralization buffer per tube
8) elute with 5 mL elution buffer
    8.1) collect 1 mL fractions in numbered microcentrifuge tubes containing 75 μL neutralization buffer per tube
9) after use, clean column with 10 mL of 20% ethanol
    9.1) pass ~ 8 mL through column to waste
    9.2) while fully saturated with ethanol solution, close both ends of column with stoppers
    9.3) seal stoppers with parafilm and store column at 4°C after use
    9.4) column can be used repeatedly for designated antibody purification
        9.4.1) do not use same column for purification of different antibodies
10) determine antibody concentration of each fraction
    see supporting protocol
11) identify and combine fraction(s) containing highest concentration of antibody (≥ 0.5 mg/mL)
12) proceed with dialysis protocol

Dialysis of Purified Antibody

Equipment:

- autoclave
- 2 L capacity glass flask
- deli fridge
- stir plate
- stir bar
- weight attached to string
- rubber stopper attached to string

Materials:

- 10x PBS, 1 L
- dialysis tubing, molecular weight cutoff 12-14,000 daltons
  Fisher, Cat. # 08-667B
  - stored at 4°C
- antibody
  - purified from ascites
- clips for dialysis tubing
- parafilm
- syringe
- needle
- 50 mL tube
- glycerol, molecular biology grade
- microcentrifuge tubes

Supporting Protocols:

- Antibody Purification from Ascites Fluid
- Absorbance Measurement using NanoDrop

Protocol:

1) sterilize 2 L capacity glass flask
   1.1) cover opening with foil and secure using autoclave tape
   1.2) autoclave for 30 min. on dry cycle
2) sterilize PBS
   2.1) dilute 10x PBS to 1x with ultrapure water
   2.2) adjust pH to 7.4
   2.3) sterilize PBS by passing through 0.22 μm filter
   reuse filter for additional rounds
3) fill flask to top with sterile PBS
4) prepare dialysis tubing
   4.1) cut ample length of dialysis tubing
   4.2) rinse with distilled water prior to use

4.3) tie knot to secure bottom end of tubing
5) add purified antibody to tubing at top end
6) tie knot to secure top end of tubing
avoid trapping air below knot
7) trim excess tubing as needed
8) attach bottom end of tubing to weight using clip
to keep tubing completely submerged in PBS
9) attach top end of tubing to rubber stopper
10) add tubing with attached weight to flask filled with PBS
11) secure tubing with rubber stopper at top of flask
12) allow buffer exchange overnight (~16 hrs.) at 4°C with gently stirring
1st exchange
13) discard buffer
14) replace PBS and allow buffer exchange for ~8 hrs. at 4°C with gently stirring
2nd exchange
15) discard buffer
16) replace PBS and allow buffer exchange for ~16 hrs. at 4°C with gently stirring
3rd / final exchange
17) discard buffer
18) remove weight and rubber stopper from tubing
19) carefully remove antibody solution from interior of tubing using needle attached to syringe
keep inside 50 mL tube for recovery if spilled
20) dispense antibody solution to sterile tube
21) dilute antibody solution 1:1 (v/v) in glycerol
22) mix well
23) determine antibody concentration according to Beer's Law as follows:
see supporting protocol
$(A_{280} / \varepsilon) \times 10 = mg/mL$
where $\varepsilon = 14$
24) aliquot antibody solution to labeled microcentrifuge tubes
25) store at -20°C

5.2.2. DNA

Agar Plate Preparation

- agar is added to 2xYT medium and dissolves in the autoclave
- antibiotic is then added and liquid medium is poured into petri dishes before agar solidifies

Equipment:

- 2 L capacity flask
- autoclave
- thermometer
- bunsen burner
- refrigerator
- insulated gloves
- ultrafine Sharpie marker

Materials:

- 2x YT, 1 L
  - *see 'Buffers and Solutions'*
  - *prepare in 2 L flask*
- agar
- ultrapure water
- autoclave tape
- foil
- 1,000x penicillin
  - stored at -20°C
- 500x kanamycin
  - stored at -20°C
- petri dishes
- lab tape

Protocol:

1) add 15 g of agar to 1 L of 2x YT
2) shake flask to mix
3) cover opening with foil
   adhere using autoclave tape
4) place flask inside autoclave-safe container
   add tap water to base
5) autoclave for 30 min. on wet cycle with slow exhaust
   takes 1 hr. total time
6) remove from autoclave
7) place thermometer in water at base of container to monitor temperature of medium
8) add antibiotic to medium when temperature cools to 55°C
   antibiotic is unstable when added at higher temperatures
9) swirl flask to mix antibiotic

use insulated gloves to handle flask

10) arrange petri dishes on lab bench
    keep plastic sleeve for later storage
11) remove lids from dishes – set aside
12) swirl flask and pour ~30 mL to each open petri dish while medium is still liquid / hot
    use insulated gloves to handle flask
13) briefly flame surface of medium using bunsen burner
14) replace lids on dishes
15) leave dishes on lab bench overnight
    to allow agar to solidify
16) label bottom of each plate with type of antibiotic
17) stack plates inside plastic sleeve for storage
    add plates upside down to prevent contamination
18) secure opening of plastic sleeve with tape
19) label sleeve with type of antibiotic and date of preparation
20) store in refrigerator

Preparation of DH5α Aliquots

Equipment:

- styrofoam container
- tube rack
  for holding microcentrifuge tubes
- -80°C freezer
- bucket with ice
  for thawing DH5α stock

Materials:

- dry ice
- 95% ethanol
- microcentrifuge tubes
- DH5α E.coli
  - *sub-cloning efficiency*
  - *chemically-competent*
  - stored at -80°C

Protocol:

1) prepare dry ice ethanol bath
   for quick-freezing aliquots
   1.1) add pieces of dry ice to styrofoam container
   1.2) slowly add 95% ethanol to container
   1.3) add tube rack
   partially immerse in ethanol
2) thaw DH5α stock on ice
3) label microcentrifuge tubes
4) aliquot 50 μL to each tube
   immediately place in tube rack to quick-freeze
5) transfer directly from dry ice ethanol bath to -80°C freezer

Transformation of DH5α

Equipment:

- 37°C warm room
- 42°C water bath
- thermometer
- -80°C freezer
- refrigerator
- bunsen burner
- glass / metal rod
  for spreading on agar plates
- glass container with 70% ethanol
  for sterilizing rod
- 37°C shaker
- lab timer
- microcentrifuge
- bucket with ice

Materials:

- plasmid DNA, purified
- ligation reaction
- microcentrifuge tubes (1 per reaction)
  for DNA
- DH5α aliquots, chemically competent (1 per reaction)
  - stored at -80°C
- LB / 2xYT growth medium
  - stored at 4°C
- glass culture tubes with caps (1 per reaction)
  for culture medium
- 1,000x ampicillin stock aliquots
  - stored at -20°C
- 500x kanamycin stock aliquots
  - stored at -20°C
- agar plates (1 per reaction)
  see supporting protocol
  - stored at 4°C
- microcentrifuge tubes (1 per reaction)
  for concentrating DH5α
- parafilm

Supporting Protocols:

- Ligation Reaction
- Preparation of DH5α Aliquots
- Agar Plate Preparation

Protocol:

1) aliquot 1-2 μL of plasmid DNA or 15-20 μL of ligation reaction into separate microcentrifuge tubes for each transformation reaction
2) remove DH5α aliquot from -80°C freezer and thaw on wet ice
3) transfer thawed DH5α cells to each tube containing DNA
4) chill the cell/DNA mixture on wet ice for 30 min.
   continue with preparations below while waiting
5) transfer 1 mL of growth medium to each culture tube
6) label each tube
7) prepare agar plates
   7.1) remove from refrigerator
      to equilibrate to room temp.
   7.2) thaw antibiotic stock aliquot
   7.3) add 1x ampicillin / kanamycin to each plate
      - estimate 30 mL volume agar per plate
      - e.g., add 30 μL of 1,000x ampicillin stock per plate
   7.4) spread evenly on surface of each plate using rod
   7.5) equilibrate plates to 37°C inside warm room
   7.6) place plates upside down on shelf
      to prevent condensation from accumulating on agar surface
8) heat shock cell/DNA mixture for 20 seconds at 42°C
   perform serially for each reaction
9) immediately return to ice for 2 min.
10) transfer heat-shocked cells to labeled culture tubes containing 1 mL of growth medium
11) incubate / shake culture tubes for 1 hr at 255 rpm (37°C)
12) concentrate DH5α
   12.1) transfer 1 mL of culture to each microcentrifuge tube
   12.2) centrifuge for 5 min. at 5,000 rpm
   12.3) remove 50 μL of supernatant using P200 micropipettor and tip
      retain for resuspending cells
   12.4) discard remaining supernatant
   12.5) resuspend cell pellet in 50 μL of retained supernatant
13) transfer 50 μL of concentrated cell suspension to each agar plate
14) spread cells evenly on agar surface using rod
   flame / decontaminate before each plate
15) incubate plates overnight in 37° warm room
   keep plates upside down
16) inspect plates for colonies
   incubate longer if needed
17) seal plates with parafilm
18) store in bacterial refrigerator for ≤ 2 weeks

19) proceed with Mini / Mega Prep protocol

Mini Prep

small scale (50 µg yield) DNA purification

Equipment:

- 37°C shaker
- 37°C water bath
- microcentrifuge
- vacuum pump
- vacuum manifold
- -20°C freezer

Materials:

- agar plate with transformed colonies
  - freshly prepared or stored for ≤ 2 weeks at 4°C
- toothpicks / pipet tips
  for inoculating starter culture
- LB medium
- glass culture tubes w/ caps (1 per MiniPrep)
- 1,000x ampicillin stock
  - stored at -20°C
- 500x kanamycin stock
  - stored at -20°C
- microcentrifuge tubes (1 per MiniPrep)
  for pelleting cells
- Resuspension Buffer (Buffer #1)
  - stored at 4°C
- Lysis Buffer (Buffer #2)
  - stored at room temp.
- Neutralization Buffer (Buffer #3)
  - stored at 4°C
- Wizard MiniColumns (1 per MiniPrep)
- syringe barrels (1 per MiniPrep)
  for attachment to MiniColumns
- Promega MaxiPrep resin
  - warm in 37°C water bath if precipitate visible
- Column Wash
  - stored at 4°C
- microcentrifuge tubes (1 per MiniPrep)
  for drying resin
  - caps removed
- microcentrifuge tubes(1 per MiniPrep)
  for eluting DNA
  - caps removed

- microcentrifuge tubes (1 per MiniPrep)
  for storage of purified DNA

Supporting Protocols:

- Transformation of DH5α
- Absorbance Measurement using NanoDrop

Protocol:

proceed independently for each MiniPrep

1) prepare starter culture
   1.1) add 5 mL of LB medium to each culture tube
   1.2) add 1x antibiotic
   1.3) pick a colony and transfer to culture tube
   to inoculate
   1.4) incubate / shake overnight at 255 rpm (37°C)
2) pellet transformed DH5α
   proceed depending on optical density (growth) of starter culture
   2.1) transfer 1 mL from starter culture to microcentrifuge tube
   2.2) centrifuge for 5 min. at 5,000 rpm
   2.3) discard supernatant
   2.4) repeat 3x in same tube
   2.5) optional: retain 1 mL of starter culture to use for Mega Prep culture (keep at 4°C)
3) prepare lysate
   3.1) resuspend pellet in 500 μL of Resuspension Buffer (#1)
   3.2) add 500 μL of Lysis Buffer (#2)
   3.3) invert tube 5x to mix
   3.4) wait for solution to become clear (5 min.)
   3.5) add 500 μL of Neutralization Buffer (#3)
   3.6) invert 5x to mix
   3.7) wait for 5 min.
   3.8) centrifuge bacterial lysate for 5 min. at 14,000 rpm
   3.9) retain supernatant
4) prepare MiniColumn
   4.1) label MiniColumn on lip
   4.2) attach MiniColumn to syringe barrel
   4.3) attach column-barrel to port on vacuum manifold
   4.4) add 500 μL of MaxiPrep resin to each barrel
   4.5) fill designated barrel with lysate supernatant
5) filter liquid through MiniColumn
   5.1) with one port open on vacuum manifold, turn on vacuum pump and increase pressure to '30' mark on gauge

5.2) open vacuum port for each column-barrel until liquid has completely passed through resin
5.3) close vacuum port
5.4) add 2 mL of column wash to each column-barrel
5.5) open vacuum port to allow column wash to completely pass through resin
5.6) apply vacuum for an additional 30 seconds

6) dry MiniColumn
6.1) detach syringe barrel from MiniColumn
6.2) secure MiniColumn inside microcentrifuge tube with lid removed
6.3) centrifuge for 1 min. at 14,000 rpm
6.4) remove MiniColumn from microcentrifuge tube
6.5) discard tube and residual column wash

7) elute DNA
7.1) secure MiniColumn inside new microcentrifuge tube with lid removed
7.2) add 50 μL of cell culture grade water to each MiniColumn
7.3) centrifuge for 1 min. at 14,000 rpm
7.4) remove MiniColumn from microcentrifuge tube
7.5) discard column and save tube containing eluted DNA
7.6) transfer purified DNA to new microcentrifuge tube
7.7) label tube for storage

8) measure DNA concentration and purity (260 / 280 ratio) using NanoDrop
see supporting protocol – use NanoDrop to conserve limited DNA yield from MiniPrep

9) store DNA at -20°C

Mega Prep

large scale DNA purification

Equipment:

- 37°C shaker
- autoclave
- ultra-centrifuge
    - rotor for large centrifuge tubes
        - equilibrate to 4°C
    - rotor for small centrifuge tubes
        - equilibrate to 4°C
- large centrifuge tubes (3 per MegaPrep)
    for pelleting cells following expansion
    - autoclave before use
- small centrifuge tubes (4 per MegaPrep)
    for preparing lysate
    - autoclave before use
- funnels
    for filtering lysate supernatant
- graduated cylinders
    for filtering lysate supernatant
- small centrifuge tubes (4 per MegaPrep)
    for precipitating DNA
    - autoclave before use
- 37°C water bath
- vacuum pump
- vacuum manifold
- centrifuge
- -20°C freezer

Materials:

- option 1: fresh starter culture
    - agar plate with transformed colonies
        - freshly prepared or stored for ≤ 2 weeks at 4°C
    - toothpicks / pipet tips
        for inoculating starter culture
    - LB medium
    - glass culture tubes w/ caps (1 per MegaPrep)
- option 2: starter culture from MiniPrep
    - 1 mL culture reserved from MiniPrep
        - stored at 4°C
    - 2xYT medium
- 1,000x ampicillin stock
    - stored at -20°C

- 500x kanamycin stock
    - stored at -20°C
- 2 L flask containing 1 L of LB medium (1 per MegaPrep)
    for expanding starter cultures
- bleach
    for decontamination
- buffers for DNA isolation
    - Resuspension Buffer (Buffer #1)
        - stored at 4°C
    - Lysis Buffer (#2)
        - stored at room temp.
    - Neutralization Buffer (#3)
        - stored at 4°C
    - Column Wash
        - stored at 4°C
- Whatman #1 filter paper
    for filtering lysate supernatant
- 100% isopropanol, molecular biology grade
    - keep on ice
- TE buffer
- Wizard MegaColumns (1 per MegaPrep)
- MaxiPrep resin
    Promega, Cat. # A7401
    - warm in 37°C water bath if precipitate visible
- 70% ethanol (molecular biology grade) in ultrapure water (10 mL per MegaPrep)
- 50 mL centrifuge tubes
    for drying resin (1 per MegaColumn)
- 50 mL centrifuge tubes
    for collecting eluted DNA (1 per MegaColumn)
- ultrapure water
    for elution
- 5 mL syringe
    for filtering DNA solution
- 0.22 μm filter
    for filtering DNA solution
- microcentrifuge tubes
    for storage of DNA aliquots

Supporting Protocols:

- Transformation of DH5α
- Absorbance Measurement Using NanoDrop

Protocol:

proceed independently for each MegaPrep

1) option 1: fresh starter culture
    1.1) add 2 mL of LB medium to culture tube
    1.2) add 1x antibiotic
    1.3) pick a colony and transfer to culture tube
        to inoculate
    1.4) incubate / shake for 6-8 hrs. at 255 rpm (37°C)
2) option 2: starter culture from MiniPrep
    2.1) add 1 mL of 2xYT to 1 mL of reserved MiniPrep culture
    2.2) incubate / shake for 1-2 hrs. at 255 rpm (37°C)
3) expand starter culture
    3.1) add 1x antibiotic to 1 L of 2xYT
    3.2) add starter culture (from either option 1 or 2 above) to 2 L flask
    3.3) incubate / shake overnight at 255 rpm (37°C)
4) pellet transformed DH5α
    proceed depending on optical density / growth of culture
    4.1) distribute culture to 3 large centrifuge tubes
        do not overfill to prevent leakage while inside ultra-centrifuge
    4.2) centrifuge for 20 min. at 5,000 g (4°C)
    4.3) discard supernatant to bleach container
        for decontamination
    4.4) optional: keep pellet at 4°C overnight
5) prepare lysate
    5.1) resuspend pellet from each large tube in 10 mL of Resuspension Buffer (#1)
    5.2) combine cell suspension from each tube
    5.3) distribute cell suspension to 4 small centrifuge tubes (10 mL / tube)
    5.4) add 10 mL of Lysis Buffer (#2)
    5.5) invert tube 5x to mix
    5.6) wait for solution to become clear (5 min.)
    5.7) add 10 mL of Neutralization Buffer (#3)
    5.8) invert 5x to mix
    5.9) wait for 5 min.
        5.9.1) keep on ice
        5.9.2) mix once per minute
    5.10) centrifuge bacterial lysate for 20 min. at 14,000 g (4°C)
    5.11) retain supernatant
6) filter lysate supernatant

6.1) prepare Whatman filter paper / funnels / cylinders apparatus to filter lysate supernatant
rate limiting step – setup a few filter apparatuses to operate in parallel
6.2) filter lysate supernatant through Whatman paper and collect in graduated cylinders
6.3) combine filtered lysate supernatant into large graduated cylinder and determine total volume

7) precipitate DNA
7.1) add 0.7x volume of ice-cold isopropanol
final isopropanol will be 41% (v/v)
7.2) distribute to 4 small centrifuge tubes
40 mL maximum volume per tube
7.3) centrifuge for 20 min at 14,000 g (4°)
7.4) discard supernatant from each tube while taking care to avoid disturbing DNA pellet
7.5) optional: store DNA pellet at 4°C overnight

8) resuspend / combine DNA pellet from each tube
8.1) add 4 mL of TE buffer to tube #1
8.2) resuspend DNA pellet from tube #1
pellet may not be visible
8.3) transfer DNA solution to tube #2
8.4) repeat resuspend / transfer steps for tube #3 and #4

9) prepare MegaColumn
9.1) add 5 mL of MaxiPrep DNA purification resin to DNA solution
9.2) swirl tube to mix
9.3) label MegaColumn on rim
9.4) attach to port on vacuum manifold
9.5) transfer DNA-resin slurry to MegaColumn
9.6) add 25 mL of column wash above slurry

10) filter liquid through MegaColumn
10.1) with one port open on vacuum manifold, turn on vacuum pump and increase pressure to '30' mark on gauge
10.2) open vacuum port for MegaColumn until liquid has completely passed through resin
10.3) add an additional 25 mL of column wash to MegaColumn and filter as before

11) wash MegaColumn with ethanol
11.1) add 5 mL of 70% ethanol (molecular biology grade) to MegaColumn
11.2) filter through MegaColumn as before
11.3) repeat for a total of 2 washes (10 mL total volume)

12) dry MegaColumn

12.1) remove MegaColumn from vacuum manifold and place inside 50 mL centrifuge tube
12.2) centrifuge for 5 min. at 2,500 rpm
12.3) return MegaColumn to vacuum manifold and apply vacuum for an additional 5 min
to completely dry resin

13) elute DNA
13.1) add ultrapure water to small beaker (need > 3 mL)
13.2) heat in microwave oven for ~ 3 min
13.2.1) adjust temperature to 65-70°C
13.3) place MegaColumn inside new 50 mL centrifuge tube
13.4) add 2 mL of heated water to MegaColumn
13.5) wait for 1 min.
13.6) centrifuge for 5 min. at 2,500 rpm
eluted DNA contained in water at bottom of tube
13.7) add an additional 1 mL of heated water to each MegaColumn
13.8) wait for 1 min.
13.9) centrifuge for 5 min. at 2,500 rpm

14) filter DNA solution
to sterilize
14.1) label microcentrifuge tubes for storage of DNA aliquots
14.2) attach syringe barrel to 0.22 μm filter
14.3) add DNA solution to syringe barrel
14.4) using plunger, pass DNA solution through filter
collect in labeled tubes

15) measure DNA concentration and purity (260 / 280 ratio) using NanoDrop or spectrophotometer

16) store DNA at -20°C

Ethanol Precipitation

small-scale purification and/or concentration of DNA preparations

Equipment:

- bucket with ice
- vortex
- refrigerated microcentrifuge
- biosafety cabinet / TC hood

Materials:

- DNA solution for precipitation
- 3.0 M sodium acetate, pH 5.2
- microcentrifuge tubes
- cell culture grade water
- 100% ethanol, molecular biology grade
    - keep on ice
- 70% ethanol, molecular biology grade
    - prepared fresh using cell culture grade water
    - keep on ice

Protocol:

perform all steps using microcentrifuge tubes

1) estimate volume of DNA solution to be purified
2) add 3.0 M sodium acetate to DNA solution for final concentration of 0.3 M
    - Na+ neutralizes phosphate backbone of DNA and favors precipitation
    - e.g., add 40 µL of 3.0 M sodium acetate to 360 µL of DNA solution (total volume should not exceed 400 µL)
3) mix well
4) add 2 volumes of ice-cold 100% ethanol
    - precipitates DNA from solution
    - e.g., to 400 µL volume above, add 800 µL
5) mix well
6) keep on ice for 1 hour
    to precipitate DNA
7) centrifuge for 15 min. at 12,000 g (4°C)
    to recover precipitated DNA
8) carefully aspirate supernatant
    do not disturb DNA pellet
    8.1) retain supernatant as precaution
9) gently resuspend DNA pellet in 1 mL of ice-cold 70% ethanol
    to dissolve precipitated salts

10) centrifuge for 15 min. at 12,000 g (4°C)
    to recover precipitated DNA
11) carefully aspirate supernatant
    do not disturb DNA pellet
    11.1) retain supernatant as precaution
12) lay tube flat inside tissue culture hood
    - to allow ethanol to evaporate
    - do not proceed until tube is completely dry
13) dissolve DNA pellet in small volume of cell culture grade water (or buffer of choice)
    13.1) rinse walls of tube to dissolve precipitated DNA
14) proceed to determine DNA concentration and purity using spectrophotometer or NanoDrop
    see supporting protocols

Restriction Enzyme Digestion Reaction

for small-scale analytical DNA gel

Materials:

- cell culture grade water
- plasmid DNA
  - stored at -20°C
- 10x (1 mg/mL) BSA
  diluted from supplied 100x (10 mg/mL) stock in cell culture grade water
- 10x reaction buffer
  - enzyme specific
  - for double-digestion, check for most compatible buffer
- restriction enzyme(s)
  - stored at -20°C
- 6x DNA loading dye

Supporting Protocol:

- Agarose Gel Electrophoresis

Protocol:

1) create table for each reaction and calculate amounts / volumes of all reagents below
   - prepare non-cut (negative control) reaction for each plasmid
   - for double-digest reaction, prepare single-cut control reaction for each restriction enzyme alone
2) set up each reaction in separate microcentrifuge tube (10 μL total volume per reaction)
   use DNAse-free tubes and pipet tips
   2.1) add ultrapure water
   2.2) add 1μg plasmid DNA
   2.3) add 1μL of 10x BSA (1x final)
   2.4) add 1μL of 10x buffer to each tube (1x final)
   2.5) add 1μL of restriction enzyme(s) to each tube (1 unit final)
   for double-digestion, add 1 unit of each restriction enzyme
   2.6) mix each tube using pipettor and tip
   change tips for each tube
3) incubate in 37°C water bath for 1 hr.
4) remove from water bath and keep on ice
5) add 2μL of 6x DNA loading dye to each tube
6) mix each tube using pipettor and tip
7) prepare agarose gel for analysis
8) resolve fragments by DNA gel electrophoresis

Agarose Gel Electrophoresis

Equipment:

- microwave oven
- gel caster
- gel comb
- horizontal gel electrophoresis apparatus
- power supply
- black and red wires
  for connection to power supply
- UV light source
  to monitor separation progress during electrophoresis
- gel imaging apparatus
- memory card, compatible with gel imaging apparatus
- solid waste container for ethidium bromide
  to discard contaminated pipet tips, gloves, etc.
- liquid waste container for ethidium bromide
  to discard contaminated TAE buffer

Materials:

- agarose, electrophoresis grade
- ethidium bromide
  - protect from light
- DNA sample
- microcentrifuge tubes
- 0.1-10 kb DNA ladder, 1 μg/mL
  NEB, Cat. # N3200L
  - stored at 4°C
- 6x loading dye
  - stored at 4°C
- TAE buffer

Protocol:

1) prepare 0.8% agarose gel in flask
   1.1) for small / analytical gel: 0.4 g agarose + 50 mL TAE
   14 wells total
   1.2) for large gel: 1.2 g agarose + 150 mL TAE
   1.3) microwave for 3 min. at 50% power
   1.3.1) if needed, increase time to dissolve completely
   1.4) allow to cool slightly
   1.5) add 1 μL of ethidium bromide to gel
   1.6) mix well
2) cast gel
   2.1) assemble caster components

2.2) pour gel
2.3) insert gel comb
2.4) allow to gel to cool / solidify completely

3) prepare DNA ladder for loading
3.1) aliquot 5 μL of DNA ladder to microcentrifuge tube
3.2) add 1 μL of 6x loading dye (1x final)

4) prepare DNA samples for loading
4.1) aliquot DNA for electrophoresis to microcentrifuge tube
4.2) add 6x loading dye to 1x final
e.g., add 2 μL of 6x loading dye to 10 μL DNA sample

5) load gel
5.1) remove solidified gel from caster
keep gel on top of plastic base
5.2) place gel and base in electrophoresis apparatus
5.3) add TAE buffer
wells should be completely submerged
5.4) carefully remove comb from gel
5.5) slowly load ladder / DNA samples to designated wells
- take care not to damage wells
- 12 μL maximum sample volume per well of small gel
5.5.1) add 6 μL of ladder to designated wells

6) run gel
6.1) connect electrophoresis apparatus to power supply using wires
6.2) set limits for power supply as follows:
500 V, 200 mA, 100 W
constant voltage: 100 V
6.3) press 'run'
6.4) allow DNA to separate 30 min - 1 hr
6.5) monitor separation progress using UV light source

7) acquire gel image
7.1) turn on UV light
7.2) capture digital image
7.3) save image to memory card
7.4) analyze using Image J software

DNA Sequencing Reaction Preparation

Equipment:

- -20°C freezer

Materials:

- template DNA
  - plasmid DNA, purified
  - PCR product
- primer stock solution
- cell culture grade water
- microcentrifuge tube

Supporting Protocols:

- Mini / Mega Prep
- Absorbance Measurement using Spectrophotometer
- Agarose Gel Electrophoresis

Protocol:

1) empirically determine concentration of template DNA using spectrophotometer
   see supporting protocol
2) characterize DNA for sequencing on gel prior to submitting for sequencing
3) empirically determine concentration of primer stock solution using spectrophotometer
   see supporting protocol
4) dilute primer for sequencing reaction to 6.4 μM (= 6.4 pmol/μL) in cell culture grade water
5) prepare sequencing reaction in microcentrifuge tube (12 μL final volume) as follows:
   5.1) add 1 μL of 6.4 μM primer (6.4 pmol)
   5.2) add 0.5-1 μg of purified plasmid or PCR product
   5.3) adjust to final volume with cell culture grade water
6) keep at -20°C prior to submitting for sequencing
   request chromatograms to be including with sequencing results

PCR Reaction

Equipment:

- thermal cycler

Materials:

- 1.1x (22 units/mL) Platinum Blue PCR Supermix
  - *Invitrogen, Cat. # 12580-015*
  - *contains Taq DNA polymerase, anti-Taq DNA polymerase antibody, Mg2+, dNTPs, glycerol and blue tracking dye*
  - stored at -20°C
- template DNA
- forward primer
- reverse primer
- cell culture grade water
- PCR tubes with caps

Supporting Protocols:

- Absorbance Measurement Using Spectrophotometer

Protocol:

modify as needed

1) empirically determine concentration of template DNA
   see supporting protocol
2) prepare PCR reaction in PCR tube (50 μL final volume)
   for multiple reactions, prepare master mix of common components and aliquot to each tube
   2.1) add 1 unit (45 μL) of Platinum Blue PCR SuperMix
   2.2) add 10 pmols of forward primer (0.2 μM final concentration)
   2.3) add 10 pmols of reverse primer (0.2 μM final concentration)
   2.4) add 1 ng of plasmid DNA
   2.5) adjust to final volume with cell culture grade water
3) cap tubes
4) load in thermal cycler
5) incubate for 2 min. at 94°C
   to denature template DNA and active polymerase
6) amplify for 25-30 cycles as follows:
   6.1) denature for 30 sec. at 94°C
   6.2) anneal for 30 sec. at 55°C
   6.3) extend for 1 min. per kb of template at 72°C
   6.4) hold at 4°C
7) analyze PCR product on gel

Gel Band Purification

for purification of DNA fragment for sub-cloning / ligation

Equipment:

- UV light source
- dark room
- 55°C water bath
- tube rotator
- microcentrifuge

Materials:

- plasmid DNA
- straight-edge razors
- 1.5 mL microcentrifuge tubes, DNAse-free
- UltraClean 15 UltraSalt
  Mo Bio Laboratories, Cat. # 121003002
  - stored at room temp.
- UltraClean 15 UltraBind
  Mo Bio Laboratories, Cat. # 121003001
  - stored at room temp.
- UltraClean 15 UltraWash
  Mo Bio Laboratories, Cat. # 121003003
  - stored at 4°C

Supporting Protocols:

- Restriction Enzyme Digestion Reaction
- Agarose Gel Electrophoresis

Protocol:

1) digest plasmid DNA with restriction enzyme pair to yield fragment of interest (see supporting protocol for details)
2) resolve DNA fragment on 0.8% agarose gel
3) excise fragment using straight-edge razor (make perpendicular cuts around band)
   use UV light source in dark room to visualize
4) transfer gel slice to microcentrifuge tube
5) fill tube with Ultra Salt solution
6) melt gel slice in 55°C water bath
7) add 10 μL of Ultra Bind slurry
8) rotate / mix for 5 min.
9) centrifuge for 10 seconds at 12,000 rpm in microcentrifuge
10) aspirate / discard supernatant
11) resuspend pellet in 1 mL of Ultra Wash solution
12) soak for 1 min.

13) centrifuge for 10 seconds at 12,000 rpm in microcentrifuge
14) aspirate / discard supernatant
15) centrifuge for 10 seconds at 12,000 rpm in microcentrifuge
16) aspirate / discard remaining supernatant
17) resuspend pellet in 20 μL of ultrapure water
    to elute DNA
18) centrifuge for 10 seconds at 12,000 rpm in microcentrifuge
19) transfer supernatant (contains eluted DNA) to new microcentrifuge tube
    take care to avoid transferring pellet / slurry
20) remove 5 μL aliquot of eluted DNA and analyze on gel to confirm purification of desired fragment
21) estimate DNA amount / concentration using semi-quantitative ladder
    presence of carryover salts from purification affects 260 nm absorbance – do not determine concentration using NanoDrop
22) store remaining DNA from purification at 4°C
23) proceed immediately with PCR amplification and/or ligation reaction

5' Phosphate Removal

incubation with antarctic phosphatase removes 5' phosphates from vector DNA fragment and prevents self-ligation during ligation reaction with insert DNA

Equipment:

- 37°C water bath
  for enzyme reaction
- 65°C water bath
  for heat-inactivation of phosphatase
- lab timer

Materials:

- vector / backbone fragments
  - purified from gel
    see supporting protocol
- Antarctic Phosphatase, 5 units/μL
  New England BioLabs, Cat. # M0289S
  - stored at -20°C
- 10x Antarctic Phosphatase Reaction Buffer
  supplied with enzyme
  - stored at -20°C
- cell culture grade water
- microcentrifuge tube

Supporting Protocol:

- Gel Band Purification

Protocol:

1) prepare phosphatase reaction in microcentrifuge tube (50 μL final reaction volume) as follows:
   1.1) add up to 1 μg of vector DNA fragment
   1.2) add 10x Antarctic Phosphatase Reaction Buffer to 1x final
   1.3) add 1 unit of antarctic phosphatase enzyme
   1.4) adjust to final volume with cell culture grade water
2) incubate for 30 min. at 37°C
3) heat-inactivate for 5 min. at 65°C
4) proceed immediately with ligation reaction
   no need for additional purification

Ligation Reaction

for sub-cloning insert DNA to vector backbone

Equipment:

- 16°C water bath
- 65°C water bath
- lab timer

Materials:

- vector backbone fragment
  - purified from gel
    see supporting protocol
  - prepared fresh
- insert fragment
  - purified from gel
    see supporting protocol
  - prepared fresh
- T4 DNA ligase
  - stored at -20°C
- 10x T4 DNA ligase buffer
  - stored at -20°C
- cell culture grade water

Supporting Protocols:

- Gel Band Purification
- 5' Phosphate Removal

Protocol:

1) determine length (# of base pars) of vector and insert fragments from map / analytical gel
2) determine approximate DNA concentration of vector and insert fragments in reference to semi-quantitative DNA ladder from analytical gel (see supporting protocol)
3) remove 5' phosphates from vector backbone fragment (see supporting protocol)
4) calculate amount (ng) of insert DNA fragment needed for 2:1 molar ratio of insert to vector
   4.1) solve for X in the following ratio:
   100 ng vector fragment / # bp = X ng of insert fragment / # bp
   4.2) multiply X by 2
5) prepare ligation reaction in microcentrifuge tube (20 μL final volume) as follows:
   5.1) add 1 unit of T4 DNA ligase

5.2) add 2 μL of 10x T4 DNA ligase buffer (1x final)
5.3) add 100 ng of vector fragment
5.4) add calculated amount of insert fragment (2:1 molar ratio of insert to vector)
5.5) adjust to final volume with cell culture grade water

6) incubate overnight at 16°C
7) heat inactivate enzyme for 10 min. at 65°C
8) analyze 5 μL of ligation reaction on analytical gel to determine efficiency of ligation
9) transform 15 μL of DNA ligation reaction to DH5α cells and select clones for further expansion

Preparation of Mouse Tail DNA

purification of mouse tail DNA for amplification by PCR

Equipment:

- vortex
- refrigerated microcentrifuge
- 55°C water bath
- -20°C freezer

Materials:

- ruler
- razor blades
- microcentrifuge tubes, 2.0 mL
  Fisher, Cat. # 05-408-146
- tail solubilization buffers
  see 'Buffers & Solutions > Mouse Tail Digestion'
  - TSB
  - Proteinase K, 20 mg/mL
  - Tail Salts
- ethanol, 100%, molecular biology grade
  - keep ice-cold
- water, cell culture grade
- ethanol, 80%
  - diluted in cell culture grade water
  - keep ice-cold
- TE Buffer (10:1), pH 8.0
  1 mM Tris-HCl, 0.1 mM EDTA

Protocol:

process in parallel for each mouse

1) solubilize tail fragment
   1.1) remove 1 cm from tip of tail using razor blade
   use new razor blade for each mouse
   1.2) transfer tail fragment to pre-labeled microcentrifuge tube
   1.3) add 2 mL of TSB + 0.2 mg/mL Proteinase K (added fresh) to each tube
   i.e., add 20 µL of 20 mg/mL Proteinase K to 2 mL of TSB
   1.4) incubate tubes overnight at 55°C
   1.5) allow tubes to cool to room temp.
   1.6) optional: store at 4°C indefinitely
2) precipitate protein and SDS
   2.1) transfer solubilized tail fragment to 15 mL tube
   2.2) add 0.4 volume of Tail Salts to each tube

i.e., add 0.8 mL to 2 mL of TSB

2.3) vortex well

2.4) incubate for 1-4 hours at 4°C

salts precipitate protein, potassium precipitates SDS

2.5) centrifuge for 10 minutes at maximum speed using microcentrifuge (4°C)

DNA contained in supernatant

3) precipitate DNA

3.1) remove 80 μL of supernatant and transfer to new microcentrifuge tube

3.2) optional: store at 4°C indefinitely

3.3) add 2 volumes of ice-cold 100% ethanol

i.e., add 160μL

3.4) transfer to -20°C freezer for ≥ 4 hours to precipitate DNA

3.5) centrifuge for 10 minutes at 14,000 rpm in a refrigerated microcentrifuge

DNA contained in pellet

3.6) carefully discard supernatant

4) wash DNA pellet

to remove precipitated salts

4.1) gently resuspend DNA pellet in 500 μL of ice-cold 80% ethanol

4.2) centrifuge for 10 minutes at maximum speed in refrigerated microcentrifuge

4.3) remove supernatant completely

4.4) allow to air dry

5) resuspend DNA in 50μL of TE Buffer

6) store at -20°C indefinitely

7) proceed with PCR amplification

7.1) use 4μL of template DNA for each PCR reaction

Itk KO Diagnosis by PCR

for identification of Itk$^{-/-}$ versus WT C57BL/6

Equipment:

- thermal cycler

Materials:

- PCR tubes (1 per mouse / reaction)
- water, cell culture grade
- 10x High Fidelity PCR buffer
- 10 mM dNTP mixture
- 50 mM $MgSO_4$
- 10 μM Itk exon 2 forward primer
  5' GGC, TCC, ATT, GAA, CTC, TCC, AG 3'
- 10 μM neomycin forward primer
  5' CTT, GAC, GAG, TTC, TTC, TGA, GGG, GA 3'
- 10 μM Itk intron 4 reverse primer
  5' GGG, ATG, CCA, GAA, TCA, ACT, TTG, GTA, G 3'
- template DNA from WT and Itk KO mice
  - purified from tail biopsy
- Platinum Taq High Fidelity DNA polymerase

Supporting Protocols:

- Preparation of Mouse Tail DNA
- Agarose Gel Electrophoresis

Protocol:

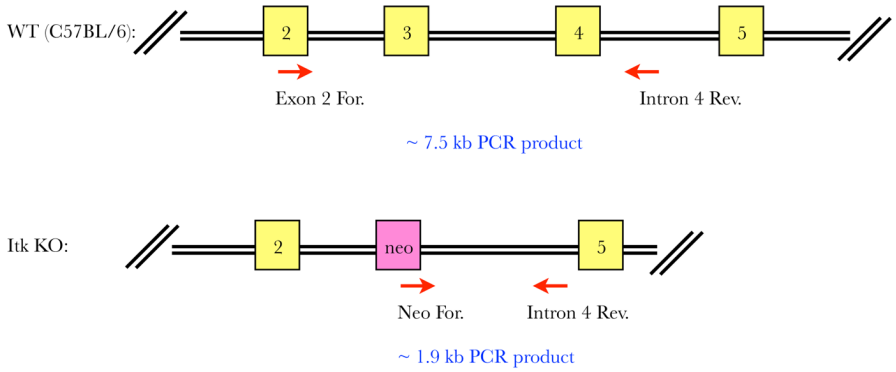


Figure 28: Diagram of Itk introns and exons for C57BL/6 WT and Itk KO mice. The numbered boxes in diagram indicate Itk exons. Itk KO mice contain the neomycin cassette in place of exon 3, intron 3 and exon 4. In a multiplex PCR reaction containing exon 2 forward, neomycin forward and intron 4 reverse primers, the majority products of ~7.5 kb or 1.9 kb are indicative of WT or Itk KO genomic DNA, respectively. A similar PCR reaction from a heterozygote would be predicted to have both PCR products present.

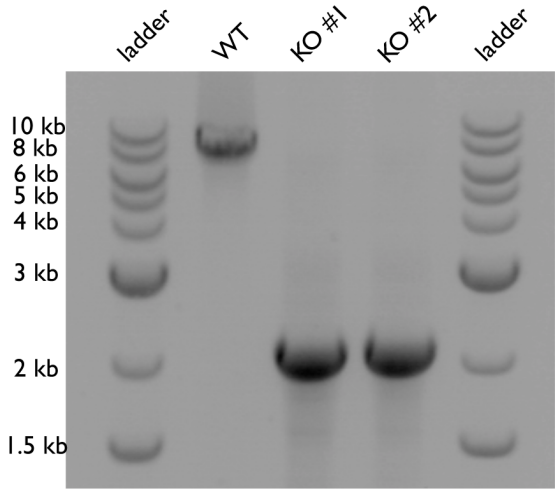


Figure 29: Image of DNA gel for characterization of WT and Itk-/- mice by PCR analysis. Representative data of expected band sizes corresponding to PCR products from WT and Itk KO mice, respectively.

1) setup one multiplex PCR reaction as follows for each mouse tail biopsy sample to be diagnosed

		50 µL final volume / rxn	
component	*[stock]*	*vol / rxn*	*[final]*
PCR water	*n/a*	*35.8 µL*	*n/a*
High Fidelity PCR buffer	*10x*	*5 µL*	*1x*
dNTP mixture	*10 mM*	*1 µL*	*0.2 mM*
$MgSO_4$	*50 mM*	*2 µL*	*2 mM*
Itk exon 2 forward primer	*10 µM*	*1 µL*	*0.2 µM*
neomycin forward primer	*10 µM*	*1 µL*	*0.2 µM*
Itk intron 4 reverse primer	*10 µM*	*1 µL*	*0.2 µM*
template DNA	*n/a*	*3 µL*	*n/a*
Platinum Taq High Fidelity DNA polymerase	*5 U/µL*	*0.2 µL*	*0.02 U/µL (1 unit)*

2) mix samples well and attach caps
3) run on thermal cycler with the following conditions:

1) lid 104°C; wait > auto
2) 93°C for 2 min.
3) 93°C for 1 min. (for template denaturation)
4) 58°C for 1 min. (for primer annealing)
5) 65°C for 8 min. (for strand extension)
6) GOTO 2; REP 40 (for amplification)
7) HOLD 4°C
8) END

4) prepare analytical agarose gel
see supporting protocol
5) resolve samples from WT (C57BL/6) and Itk KO by electrophoresis
6) analyze bands according to diagram above

5.2.3. Cell Culture

Serological Pipet Decontamination

treatment for reusable glass serological pipets

Equipment:

- dI water supply
- pipet washer / rinser
  Nalgene
- pipet plugger
  cotton stuffing machine
- pipet canisters
- autoclave
- lab oven with high temperature thermometer
- forceps
- reusable glass serological pipets
  - stored in 10% bleach solution
    for decontamination

Materials:

- autoclave tape

Procedure:

1) remove excess autoclave tape from pipet canisters
   to prevent fumes while inside lab oven
2) remove cotton plug from serological pipets using forceps
3) segregate 5, 10 and 25 mL pipets
4) rinse at least 3x using dI water using pipet washer / rinser
   removes debris and bleach from pipets
5) place inside labeled canisters according to size
6) stuff cotton in top of pipets using cotton plugging machine
7) return to respective canisters
8) apply autoclave tape across cap of canister
9) autoclave for 30 min. on dry cycle
10) bake in lab oven at 180°C for 4 hrs
    to dry and destroy endotoxin
11) allow to cool

FBS Heat Inactivation

for complement inactivation

Equipment:

- 37°C water bath and thermometer
- biosafety cabinet (TC hood)
  for preparing sterile aliquots
- 55°C water bath and thermometer
- lab timer
- -20°C freezer
  for storage of aliquots

Materials:

- fetal bovine serum (FBS), 0.5 - 1 L bottle
  - thawed at room temp. overnight or in 37°C water bath
- 50 mL tubes, sterile and endotoxin free

Protocol:

1) add dI water to water bath if needed
2) equilibrate water bath to 55°C
3) place bottle of FBS in water bath
   do not submerge cap below water
4) incubate for a total of 30 min. at 55°C
   4.1) after every 10 min., remove bottle
   4.2) invert to mix
   4.3) return to water bath
5) place inside biosafety cabinet
6) allow bottle to rest for 2-3 hr.
   to allow particulate matter to settle to bottom of bottle by gravity
7) aliquot 40 mL of heat-inactivated FBS to each 50 mL tube
   allows space in tube for expansion upon freezing
8) label each tube with 'FBS' and date
9) keep aliquots at -20°C until use

Thawing Cell Lines

Equipment:

- liquid nitrogen log book
- liquid nitrogen vessel
- protective eyewear
  for removing cryovial from liquid nitrogen
- biosafety cabinet (TC hood)
- squirt bottle with 70% ethanol
- 37°C water bath
- refrigerated centrifuge
- humidified, 37°C, 5% CO_2 incubator

Materials:

- cryovial containing cell line
  select low passage number clone with good viability prior to thawing
  - preserved in liquid nitrogen
- 15 mL centrifuge tubes
- wash medium, cell line specific
  - see 'Buffers & Solutions'
  - designate separate bottle for each cell line to prevent cross-contamination

Supporting Protocol:

- Freezing Cell Lines

Protocol:

use aseptic technique throughout

1) consult log book for location, history and characteristics of vial prior to removal from liquid nitrogen vessel
2) add 10 mL of wash medium to 15 mL centrifuge tube
3) remove cryovial from liquid nitrogen
   3.1) wear protective eyewear to prevent injury caused by projectile caps
   3.2) confirm identification written on vial with that indicated in the log book
4) immediately thaw vial in 37°C water bath with gentle agitation
5) wipe surface of vial with ethanol prior to bringing inside TC hood
6) wash cells
   6.1) transfer cell suspension to 15 mL tube containing wash medium
   6.2) invert tube to mix / wash
   6.3) centrifuge at 1,000 rpm for 5 min. (4°C)

6.4) discard supernatant

7) notate log book to reflect removal of vial
8) proceed immediately with cell maintenance protocol for specific cell line

Freezing Cell Lines

Equipment:

- liquid nitrogen log book
- -80°C freezer
- refrigerated centrifuge
- liquid nitrogen vessel

Materials:

- cell culture
  - expanded
    - *low passage number*
    - *logarithmic growth phase*
- wash medium
- freezing medium
  - *90% FBS + 10% DMSO*
- cryovials
- colored caps for cryovials
- freezer box with cotton lining
- bucket with crushed dry ice

Supporting Protocols:

- Cell Line Maintenance
- Cell Counting

Protocol:

1) wash cells
   - 1.1) remove culture from incubator
   - 1.2) transfer to centrifuge tube(s)
   - 1.3) centrifuge for 5 min. at 1,000 - 1,500 rpm (4°C)
   - 1.4) aspirate supernatant completely
   - 1.5) resuspend in wash medium
2) perform cell count
   - 2.1) centrifuge for 5 min. at 1,000 - 1,500 rpm (4°C)
   - 2.2) aspirate supernatant completely
   - 2.3) resuspend in volume suitable for counting
3) resuspend in freezing medium
   - 3.1) add wash medium to tube
   - 3.2) centrifuge for 5 min. at 1,000 - 1,500 rpm (4°C)
   - 3.3) aspirate supernatant completely
   - 3.4) resuspend pellet in freezing medium at 5-10x10^6 cells/mL
4) transfer to cryovials
   - 4.1) label cryovials

4.2) apply colored cap to each vial
choose unique color / color combination for future identification in liquid nitrogen storage box
4.3) aliquot 0.5-1 mL of cell suspension to labeled cryovials
4.4) seal caps tightly
4.5) place vials in center of cotton-lined freezer box
4.6) place box in -80°C freezer overnight

5) transfer to liquid nitrogen
5.1) remove vials and place in bucket with crushed dry ice
5.2) transfer vials to liquid nitrogen vessel storage box
5.2.1) choose location in advance

6) record entry of vials in log book

Cell Counting

Equipment:

- light microscope, upright
  with 40x objective
- P-200 pipettor
- P-20 pipettor
- lab timer
- hemacytometer
  - 9 large squares (arranged in 3x3 grid)
  - large central square sub-divided into 5x5 grid (used for counting cells)
  - chamber depth: 0.1 mm
  - surface area: 1 mm^2 per large square
  - volume: 0.1 mm^3 = 10-4 cm^3 = 10^{-4} mL per large square
  - store in petri dish with cover when not in use
    for protection from breakage
- manual tally counter
- calculator

Materials:

- cell suspension for counting
- petri dish with cover
- coverslip
- erythrosine
  for viability determination
- PBS
  for diluting sample before counting, if needed
- Kimwipes
- 96-well plate
- 70% ethanol squirt bottle

Protocol:

1) resuspend cells well to disrupt clumps
2) remove 30 μL aliquot from cell suspension
3) add to 96-well plate designated for cell counting
4) count live / dead cells
   4.1) count in 5x5 grid in center of hemacytometer
   4.2) mix cell suspension with 30 μL (equal volume) of erythrosine
   dye is actively excluded from healthy / live cells and is taken up by unhealthy / dead cells
   4.3) slowly add 10 μL of cell suspension to each side of hemacytometer with coverslip in place
   avoid air bubbles

4.4) allow 1 min. for cells to settle
4.5) for accuracy, aim to count 100 - 500 total cells in large central square (sub-divided into grid)
use 40x objective

5) if too many cells to count:
5.1) remove another aliquot
5.2) dilute in PBS
note dilution factor used
5.3) repeat count as described above

6) if too few cells:
6.1) allow cells to proliferate further before repeating count (if possible)
6.2) alternatively, concentrate sample prior to repeating count
6.2.1) remove volume from cell suspension
6.2.2) centrifuge
6.2.3) discard supernatant
6.2.4) resuspend in smaller volume
note concentration factor used
6.3) repeat count as described above

7) for precision, count on both sides of hemacytometer
repeat count if numbers differ by > 10%

8) calculate concentration of cell suspension in cells/mL
8.1) calculate number of live / dead cells per large (counting) square
8.1.1) average independent counts on either side of hemacytometer
8.2) multiply average number of live / dead cells by 10,000
 # cells / 0.1 mm^3 x 10,000 = # cells / mL
8.3) multiply each value by 2
correction factor for 1:2 dilution in erythrosine
8.4) multiply / divide each value by additional dilution / concentration factor, respectively
to correct for additional dilution or concentration of original cell suspension prior to counting, if applicable

Doubling Time Calculation

Equipment:
- calculator

Materials:
- lab notebook

Supporting Protocol:
- Cell Counting

Protocol:

Doubling time is a unique property of each cell line and should be monitored along with viability as an indication of culture health.

Doubling time calculation assumes that cells have been seeded in an appropriate culture vessel at a known concentration and incubated for a known amount of time until subsequently resuspended and counted to determine the final concentration.

Formula for doubling time (T) is as follows: $T = t / n$
where $n = \ln (T/T_0) / \ln (2)$
and ln = natural logarithm where $e^{\ln(x)} = x$

1) record N_0
   initial concentration
   ex: 0.3×10^6 cells/mL
2) record N
   final concentration
   ex: 1.5×10^6 cells/mL
3) record t
   time between N_0 and N
   ex: 72 hours
4) calculate 'n' where $n = \ln (T/T_0) / \ln (2)$
   ex:
   $n = \ln (1.5 \times 10^6 / 0.3 \times 10^6) / \ln (2)$
   $n = \ln (5) / \ln (2)$
   $n = 1.609 / 0.693$
   $n = 2.322$
5) calculate 'T' where $T = t / n$
   ex:
   $T = 72 / 2.322$
   $T = 31$ hours

Jurkat / Raji Cell Line Maintenance

- for non-adherent cell lines (e.g., Jurkat, Raji, etc.)
- all work related to culturing cells should be performed using proper aseptic technique in a certified biosafety cabinet

Equipment:

- refrigerated centrifuge
- humidified, 37°C, 5% CO_2 incubator
- biosafety cabinet (TC hood)
  certified for BSL 2 designation
- inverted light microscope
- waste container
  for decontamination of discarded culture
  - containing 10% bleach when full

Materials:

- Jurkat cell line
  - human leukemic T cell line
  - various sub-lines available (e.g., Jurkat E6.1 available from ATCC)
  - freshly thawed from liquid nitrogen
- Raji cell line
  - human Burkitt's lymphoma B cell line
  - available from ATCC
  - freshly thawed from liquid nitrogen
- Wash Medium (WM)
  see 'Buffers & Solutions'
- Growth Medium (GM)
  see 'Buffers & Solutions'
- T-25 flasks
  25 cm^2 surface area / 10 mL culture volume
- T-75 flasks
  75 cm^2 surface area / 50 mL culture volume
- T-150 flasks
  150 cm^2 surface area / 100 mL culture volume
- 1, 5, 10 and 25 mL serological pipets
  sterile and endotoxin-free

Supporting Protocols:

- Thawing Cell Lines
- Cell Counting
- Freezing Cell Lines

Protocol:

- progressive color change of phenol red in medium from red to orange to yellow indicates active cell metabolism and thus cell survival and proliferation
- periodically examine cells using inverted light microscope for presence of grape-like clusters formed due to homotypic adhesions among dividing cells
- in addition, periodically examine cells visually and using microscope for presence of fungal or bacterial contamination, respectively
- healthy culture conditions are intended to maintain cells in logarithmic phase of growth curve (typically in the range between 105-106 cells/mL with a doubling time of approximately 24 hours)
- If working with more than one cel line, reduce the risk of cross-contamination by:
- maintaining each cell line independently,
- sterilizing surfaces with 70% ethanol before and after work and,
- designating separate bottles of medium for each cell line

1) establish starter culture
- passage 0
- growth in small clusters is important for survival and further growth
- maintain starter culture in small volume and do not resuspend cells until needed
1.1) immediately after removal from liquid nitrogen, resuspend pellet in 5 mL of JGM
1.2) label 25 cm^2 flask with initials, cell line, clone, date and passage number
1.3) transfer cell suspension to flask
1.4) place inside incubator overnight in vertical position
1.4.1) loosen cap to allow gas exchange if not using flask with vented cap
1.5) monitor color of growth medium for signs of growth
1.6) continue to incubate in vertical position until medium becomes red-orange
1.7) once cell growth has been noted, add 5 mL of JGM to flask and place flask in horizontal position
do not resuspend cells
1.8) continue to incubate in horizontal position until medium becomes orange-yellow is consistent with expected cell density as judged visually using inverted light microscope
2) perform cell count
2.1) resuspend cells to form single-cell suspension
2.2) remove small aliquot of culture for cell counting and viability determination
see 'Cell Counting' protocol for details
2.3) once density of culture reaches ~10^6 cells/mL, proceed by maintaining cells

if cell density remains well below 10^6 cells/mL, return flask to incubator and continue to culture until target density is achieved

3) maintain original culture

- as cells continue to proliferate, healthy culture conditions are maintained by re-seeding flask while replacing nutrients by adding fresh medium

- continue to re-use original 25 cm^2 flask for multiple passages

- use original culture as source to seed additional flasks (see below)

3.1) re-seed 25 cm^2 flask with 10^6 cells in 10 mL of final culture volume

e.g., add 1 mL of culture at 10^6 cells/mL to 9 mL of fresh JGM

3.1.1) retain remaining culture volume to seed additional flasks

or discard if not required

3.2) label flask with consecutive passage number

3.3) continue to maintain culture in same manner for 10-20 passages

3.4) calculate and record doubling time between successive passages

see 'Cell Counting' protocol for details

4) expanding cells

- in addition to maintaining the culture, one must also expand cells by seeding into larger flask sizes to generate sufficient cell numbers for re-freezing and for use in experiments

- to maintain genetic characteristics of original culture, seed flasks to generate cells for re-freezing at first passage

4.1) seed flask(s) of various sizes with cell number needed for density of 10^5 cells/mL upon addition of JGM to final culture volume

proceed by expanding to progressively larger sized flask

4.1.1) for 75 cm^2 flask, add $5x10^6$ cells in 50 mL of final culture volume

4.1.2) for 150 cm^2 flask, add 10^7 cells in 100 mL of final culture volume

Jurkat Serum Starvation

to reduce basal activation of signaling proteins prior to use in experiments

Equipment:

- biosafety cabinet (TC hood)
  certified for BSL 2 designation
- humidified, 37°C, 5% CO_2 incubator
- waste container
  for decontamination of discarded culture
  - containing 10% bleach when full

Materials:

- Jurkat cells
  logarithmic growth phase
  - cultured to ~10^6 cells/mL
- Raji cells
  logarithmic growth phase
  - cultured to ~10^6 cells/mL
- 50 mL centrifuge tubes
- Starvation Medium
  cRPMI + 0.2% FBS

Protocol:

1) remove cells from culture to use for experiment when concentration reaches ≤ 10^6 cells/mL
   logarithmic growth phase
2) resuspend cells
3) centrifuge for 5 min. at 1,000 rpm (4°C)
4) discard supernatant
5) resuspend pellet in Starvation Medium
6) return cells to flask
7) place flask in incubator overnight (16-18 hrs.)

HEK293 Cell Line Maintenance

for adherent cell lines (e.g., HEK293, Cos, etc.)

Equipment:

- refrigerated centrifuge
- humidified, 37°C, 5% CO_2 incubator
- inverted light microscope
- waste container
  for decontamination of discarded culture
  - containing 10% bleach when full

Materials:

- 10 cm^2 plate
  for adherent cell lines (e.g., HEK293)
- 293 Growth Medium
  cDMEM + 10% FBS
- PBS, cell culture grade
- trypsin-EDTA
- transfer pipets
- 15 mL centrifuge tube

Supporting Protocol:

- Thawing Cell Lines

Protocol:

- protocol assumes 10 cm diameter (60 cm^2) cell culture plate
- scale volumes proportionally for various-size culture vessels
- seed number of plates required for experiment in advance

1) trypsin treatment
   - proceed when cells achieve ≤ 90% confluency
   - discard plate after trypsin treatment (do not reuse plate)
   1.1) gently wash cells with 5 mL PBS
   to remove calcium and magnesium prior to incubation with trypsin
   1.2) decant
   1.3) repeat wash with PBS
   1.4) decant
   1.5) add 500 μL tryspin
   tilt plate to cover entire surface area
   1.6) incubate with trypsin for 5 min. inside 37°C incubator
   to detach cells
2) quench trypsin
   2.1) add 10 mL of 293 Growth Medium

2.2) transfer cell suspension to 15 mL centrifuge tube
2.3) centrifuge for 5 min. at 1,500 rpm (4°C)
2.4) decant supernatant

3) wash cells
3.1) resuspend in 10 mL of PBS
3.2) centrifuge for 5 min. at 1,500 rpm (4°C)
3.3) decant supernatant

4) seed new culture plate
set up multiple plates at different starting densities in order to have continuous supply of plates available for transfection
4.1) resuspend pellet in 293 Growth Medium to yield concentrated cell suspension
- adjust volume based on size of cell pellet
- pipet up and down numerous times to break apart cell clumps
- start by resuspending in small volume and increase volume if needed
4.2) add 15 mL of 293 Growth Medium to plate
4.3) add concentrated HEK293 cell suspension drop-wise
4.4) adjust cell number in order to achieve ≤ 10% confluency
determine optimal starting density using inverted light microscope
4.5) place inside TC incubator overnight

5) examine under microscope for proper cell attachment, morphology, and growth
- viable cells extent projections for attachment to plate surface
- actively dividing cells retract from plate
- dead / unhealthy cells remain unattached and can be removed by replacing medium if needed

HEK293 Serum Starvation

for adherent cell lines (e.g., HEK293, Cos, etc.)

Equipment:

- biosafety cabinet (TC hood)
  certified for BSL 2 designation
- humidified, 37°C, 5% CO_2 incubator
- waste container
  for decontamination of discarded culture
  - containing 10% bleach when full

Materials:

- plate of adherent cells
- Starvation Medium
  cDMEM + 0.2% FBS

Protocol:

1) discard / decant 293 Growth Medium
2) wash 2x with PBS
   to remove remaining growth medium
3) replace with equal volume of Starvation Medium
4) place plate in incubator overnight (16-18 hrs.)

HEK293 Transfection with Lipofectamine

Equipment:

- refrigerated centrifuge
- humidified, 37°C, 5% CO_2 incubator
- inverted light microscope

Materials:

- plasmid DNA
- Lipofectamine 2000 reagent or equivalent
  Invitrogen / Life Technologies
  - Cat. # 11668-027; 0.75 mL
  - Cat. # 11668-019; 1.5 mL
- HEK293
  - seeded in 10 cm diameter plate
  - cultured to ~90% confluency
- 293 Growth Medium
  cDMEM + 10% FBS
- 293 Transfection Medium
  DMEM supplemented with HEPES, L-Glutamine + 10% FBS (without antibiotics)
- 293 Wash Medium
  - without supplements and serum-free
  - for formation of DNA-lipid complexes prior to transfection
- PBS, cell culture grade
- trypsin-EDTA

Supporting Protocol:

- HEK293 Cell Line Maintenance

Protocol:

- protocol assumes 10 cm diameter (60 cm^2) cell culture plate
- scale volumes proportionally for various-size culture vessels based on surface area
- during transfection with Lipofectamine, internalized penicillin-streptomycin may be toxic to cells; thus it is important to seed HEK293 in medium without antibiotics at least 1 day prior to transfection

1) process cells as follows
   refer to 'HEK293 Cell Line Maintenance' protocol
   1.1) treat with trypsin
   1.2) quench trypsin
   1.3) wash cells
2) seed new plate(s) for transfection

2.1) resuspend pellet in 293 Transfection Medium to yield concentrated cell suspension
- adjust volume based on size of cell pellet
- start by resuspending in small volume and increase volume if needed
2.1.1) pipet up and down numerous times
to break apart cell clumps
2.2) add 15 mL of 293 Transfection Medium to plate
2.3) add concentrated HEK293 cell suspension drop-wise
2.4) adjust cell number in order to achieve ~90% confluency within ~24 hours
- determine optimal starting density using inverted light microscope
- may incubate longer until ≤ 90% confluency is achieved
2.5) place inside TC incubator overnight

3) prepare DNA-lipid complexes
prepare using DMEM without supplements or FBS
3.1) dilute Lipofectamine
3.1.1) mix Lipofectamine stock well prior to use
3.1.2) add 60 μL of Lipofectamine to 1.5 mL of DMEM basal medium
3.1.3) incubate for ≥ 5 min. and < 25 min. at room temp. prior to combining with diluted DNA
3.2) dilute 24 μg of plasmid DNA in 1.5 mL of DMEM basal medium
3.3) combine diluted Lipofectamine and diluted DNA solutions (total volume 3 mL)
3.4) mix gently and incubate for > 20 min. and < 6 hrs. at room temp.
for formation of DNA-lipid complexes

4) add 3 mL of DNA-lipid complexes to cells
in addition to 15 mL of 293 Transfection Medium
5) optional: replace medium after 4-6 hours
6) place inside incubator for 18-48 hrs. prior to assessing for expression

Jurkat Bio-Rad Electroporation

low transfection efficiency / high cell yield – for biochemical analyses

Equipment:

- Bio-Rad electroporator
- refrigerated centrifuge
- biosafety cabinet (TC hood)
- 37°C, 5% CO_2 humidified incubator

Materials:

- plasmid DNA
  - purified and endotoxin-free (preferred)
- splenocytes ($80x10^6$ cells per transfection)
- Jurkat cells ($20x10^6$ cells per transfection)
  - cultured to ≤ 10^6 cells/mL
  - seed sufficient number of flasks in advance according to experimental design
  - retain culture supernatant for preparation of conditioned medium
- Sharpie marker, ultrafine point
- Kimwipes
- electroporation cuvettes, 4 mm gap
- 50 mL centrifuge tubes
- Jurkat Wash Medium
  RPMI-1640
- Jurkat Transfection Medium (JTM)
  RPMI-1640 + 10 mM HEPES, 2 mM L-glutamine + 10% FBS
- bucket with ice
- microcentrifuge tubes
- 25 cm^2 flasks (1 per transfection)
- transfer pipets

Supporting Protocols:

- Jurkat Cell Line Maintenance
- Cell Counting

Protocol:

1) perform cell count
2) prepare post-transfection culture medium
   2.1) add 10 mL of growth medium to 25 cm^2 flask
   for Jurkat cells:
   - 10% conditioned medium
   - 10% FBS
   - 80% complete RPMI

2.2) label each flask
2.3) equilibrate in incubator
3) aliquot plasmid DNA
3.1) thaw plasmid DNA stock solution to room temperature
3.2) label microcentrifuge tube
3.3) aliquot 20 μg of plasmid DNA per tube
perform in TC hood
3.4) keep on ice
4) label cuvettes on caps
4.1) equilibrate on ice
5) wash cells 2x
5.1) transfer cell culture to centrifuge tube(s)
5.2) centrifuge cells at 1,000-1,500 rpm for 5 min. (4°C)
5.3) discard supernatant
5.4) wash pellet in 10 mL of RPMI-1640
5.5) repeat
6) resuspend cells for transfection
6.1) centrifuge cells at 1,000-1,500 rpm for 5 min. (4°C)
6.2) aspirate supernatant
remove completely
6.3) for each transfection reaction, resuspend $20x10^6$ cells in 0.5 mL of ice-cold Wash Medium
7) mix DNA and cells
7.1) transfer 500 μL of cell suspension to each microcentrifuge tube containing DNA
7.2) pipet gently up and down to mix cells and DNA
7.3) transfer 500 μL of DNA / cell suspension to ice-cold cuvette
avoid air bubbles
7.3.1) keep on ice
7.4) gently tap cuvette to bring DNA / cell suspension to bottom
8) electroporate
8.1) perform serially for each transfection
8.2) set to deliver 250 mV
display reads '0.250'
8.3) remove cuvette from ice and dry electrodes using Kimwipe
8.4) place cuvette in electroporator
8.5) press both buttons simultaneously to deliver charge
8.6) immediately return cuvette to ice following electroporation
9) culture cells post-transfection
9.1) remove T-25 flask(s) from incubator in advance
9.2) transfer transfected cells to flask containing culture medium
9.3) return to incubator for 48 hours
for gene expression

Jurkat Amaxa Nucleofection

high transfection efficiency / low cell yield – for analysis by microscopy and/or FACS

Equipment:

- Nucleofector II
  Amaxa
- refrigerated centrifuge
- 37°C, 5% CO_2 humidified incubator

Materials:

- Jurkat clone E6.1 (ATCC)
  - 1-5x10^6 cells per nucleofection reaction
  - seed sufficient number of flasks in advance according to experimental design
  - cultured to ≤ 10^6 cells/mL
- plasmid DNA, purified and endotoxin-free (preferred)
- Jurkat Wash Medium
  RPMI-1640
- Jurkat Transfection Medium (JTM)
  RPMI-1640 + 10 mM HEPES, 2 mM L-glutamine + 10% FBS
- Cell Line Nucleofector Kit V
  - Amaxa, Cat. # VCA-1003
  - cuvettes
  - Cell Line Nucleofector Solution V
    see 'Buffers & Solutions' for details
  - transfer pipets
- 12-well culture plates (Corning, Cat. # 3513)
- 15 mL / 50 mL centrifuge tubes
- microcentrifuge tubes

Supporting Protocols:

- Jurkat Maintenance
- Cell Counting

Protocol:

1) perform cell count
2) pre-warm supplemented Nucleofection Solution V to room temperature
   supplied with kit
3) prepare post-nucleofection culture medium
   3.1) for each nucleofection, add 1.5 mL of Jurkat Transfection Medium per well of 12-well plate
   3.2) label each well on top of plate

3.3) equilibrate plate for ≥ 30 min. inside incubator

4) aliquot plasmid DNA
   4.1) thaw plasmid DNA stock solution to room temperature
   4.2) label each microcentrifuge tube
   4.3) for each nucleofection, aliquot 2-5 μg of DNA per tube

5) label caps of Amaxa cuvettes for each nucleofection
   supplied with kit

6) wash cells 2x
   transfer cell culture to centrifuge tube(s)
   6.1) centrifuge cells at 1,000 rpm for 5 min. (4°C)
   6.2) discard supernatant
   6.3) wash pellet in 10 mL of RPMI-1640

7) resuspend cells for nucleofection
   7.1) centrifuge cells at 1,000 rpm for 5 min. (4°C)
   7.2) aspirate supernatant – remove completely
   7.3) for each nucleofection reaction, resuspend 1-5x10^6 cells in 100 μL of supplemented Nucleofector Solution V
   7.4) complete nucleofection protocol within 15 min.

8) mix DNA and cells
   8.1) transfer 100 μL of cell suspension to each microcentrifuge tube containing DNA
   8.2) pipet gently up and down to mix cells and DNA
   8.3) transfer 100 μL of DNA / cell suspension to labeled cuvette -- avoid air bubbles
   8.4) gently tap cuvette to bring DNA / cell suspension to bottom

9) nucleofection
   perform serially for each reaction
   9.1) plug Nucleofector II into power supply and turn device on
   9.2) select program 'X-001'
      - additional programs include 'X-005' and 'S-018'
      - assess each for optimal combination of transfection efficiency and viability
   9.3) place cuvette in device
   9.4) press 'X' button to deliver charge
   9.5) verify that device displays 'OK' following successful nucleofection

10) culture cells post-nucleofection
   10.1) remove 12-well plate from incubator in advance
   10.2) for each nucleofection, remove 0.5 mL of growth medium from designated well of plate
   10.3) gently add 0.5 mL of growth medium to cuvette
   10.4) using supplied transfer pipet, gently resuspend cells in cuvette
   10.5) transfer cell suspension to designated well of plate

10.6) label plate with time of final nucleofection
10.7) return plate to incubator for 24-48 hours
for gene expression

Mouse T Cell Amaxa Nucleofection

high transfection efficiency / low cell yield – for analysis by microscopy and/or FACS

Equipment:

- Nucleofector II
- refrigerated centrifuge
- 37°C, 5% CO_2 humidified incubator

Materials:

- 5-10x10^6 murine lymphocytes
  do not perform RBC lysis
- 0.5-1x10^6 purified T cells
  do not perform RBC lysis
- plasmid DNA
  - purified and endotoxin-free (preferred)
- Mouse T Cell Nucleofector Kit
  Amaxa, Cat. # VPA-1006
  - cuvettes
  - Mouse T Cell Nucleofector Solution
    see 'Buffers & Solutions' for details
  - Mouse T Cell Nucleofector Medium
    see 'Buffers & Solutions' for details
  - transfer pipets
- 12-well culture plates
  Corning, Cat. # 3513
- microcentrifuge tubes

Supporting Protocols:

- In Vivo / Ex Vivo > Splenocyte Preparation
- Cell Counting

Protocol:

1) isolate murine lymphocytes from spleen and/or thymus
   do not perform RBC lysis
2) perform cell count
3) pre-warm supplemented Mouse T Cell Nucleofector Solution to room temperature
   supplied with kit
4) prepare post-nucleofection culture medium
   4.1) for each nucleofection, add 1 mL of Mouse T Cell Nucleofector Medium per well of 12-well plate
   4.2) label each well on top of plate
   4.3) equilibrate plate for ≥ 30 min. inside incubator

5) aliquot plasmid DNA
    5.1) thaw plasmid DNA stock solution to room temperature
    5.2) label each microcentrifuge tube
    5.3) for each nucleofection, aliquot 4 μg of DNA per tube
6) label caps of Amaxa cuvettes for each nucleofection
    supplied with kit
7) resuspend cells for nucleofection
    complete nucleofection protocol within 15 min.
    7.1) centrifuge cells at 1,500 rpm for 5 min. (4°C)
    7.2) aspirate supernatant
        remove completely
    7.3) for each nucleofection reaction, resuspend recommended cell number in 100 μL of supplemented Mouse T Cell Nucleofector Solution
8) mix DNA and cells
    8.1) transfer 100 μL of cell suspension to each microcentrifuge tube containing DNA
    8.2) pipet gently up and down to mix cells and DNA
    8.3) transfer 100 μL of DNA / cell suspension to labeled cuvette
        avoid air bubbles
    8.4) gently tap cuvette to bring DNA / cell suspension to bottom
9) nucleofection
    perform serially for each reaction
    9.1) plug Nucleofector II into power supply and turn device on
    9.2) select program 'X-001'
    9.3) place cuvette in device
    9.4) press 'X' button to deliver charge
    9.5) verify that device displays 'OK' following successful nucleofection
10) culture cells post-nucleofection
    10.1) remove 12-well plate from incubator in advance
    10.2) for each nucleofection, remove 0.5 mL of growth medium from designated well of plate
    10.3) gently add 0.5 mL of growth medium to cuvette
    10.4) using supplied transfer pipet, gently resuspend cells in cuvette
    10.5) transfer cell suspension to designated well of plate
    10.6) label plate with time of final nucleofection
    10.7) return plate to incubator for 24-48 hours
        for gene expression

5.2.4. In Vivo / Ex Vivo

Mouse Euthanasia Using CO_2

CO_2 displaces O_2 and results in death by affixation

Equipment:

- mouse cage with wire rack
- -20°C freezer
  for storage of mouse carcasses

Materials:

- mice to be euthanized
- dry ice
- insulated blanket, disposable
  to wrap cage
- paper towels
- plastic bag
  for mouse carcasses

Protocol:

1) remove food and water from cage containing mice to be euthanized
2) place piece of dry ice on top of folded paper towel above wire rack
3) wrap cage inside insulated blanket
4) allow 5-10 mins. or until mice are unconscious / dead
5) remove each mouse from cage and perform cervical dislocation
   in case CO_2 was not effective

Anesthesia Using Isoflurane

short term anesthesia delivery in glass jar

Equipment:

- fume hood
- glass jar with lid
  for delivery of anesthetic prior to cervical dislocation

Materials:

- mice to be anesthetized
- isoflurane
- cotton / surgical gauze
  to absorb isoflurane

Protocol:

- proceed for each mouse individually
- work with isoflurane inside chemical fume hood

1) absorb isoflurane onto cotton / surgical gauze
2) place inside jar with lid
3) place mouse inside anesthesia chamber
4) observe until just unconscious
   for survival, monitor carefully to prevent overdose
5) optional: sacrifice anesthetized mouse by cervical dislocation

Mouse Tail Biopsy

used for DNA purification and characterization by PCR

Equipment:

- fume hood
- bunsen burner
- beaker with 70% ethanol
  for sterilizing surgical instruments
- scalpel, with sharpened blade
- forceps
- ruler

Materials:

- microcentrifuge tubes (1 per mouse / specimen)
  - labeled with mouse ID
- ultrafine point Sharpie marker
- paper towels
- Kimwipes

Supporting Protocol:

- Anesthesia Using Isoflurane

Protocol:

proceed serially for each mouse

1) sterilize blade of scalpel
   1.1) soak in 70% ethanol
   1.2) flame using bunsen burner
   1.3) allow to cool
2) place mouse inside isoflurane jar until just motionless
   see supporting protocol
3) remove mouse from jar
   proceed quickly
4) cut 1 cm length segment from tip of tail
5) transfer tail segment to labeled tube
6) seal wound site
   6.1) soak blade of scalpel in 70% ethanol
   6.2) heat in flame for 10 seconds
   6.3) expose heated scalpel blade to wound site for 1-2 seconds
   to stop bleeding
7) return mouse to cage

Retroorbital Blood Collection

can be survival for repeated sampling

Equipment:

- microcentrifuge
- -20°C / -80°C freezer

Materials:

- mice
- paper towels
- Natelson blood collecting tubes
  VWR, Cat. # 14705-020
  Fisher, Cat. # 02-668-15
- surgical gauze
- microcentrifuge tubes
- lab timer
- mouse cage with wire top

Supporting Protocol:

- Anesthesia Using Isoflurane

Protocol:

work with one mouse at a time

1) place mouse inside isoflurane jar until just motionless
   see supporting protocol
2) remove mouse from jar and lay face down on top of paper towels
3) using first finger and thumb, pull skin away form eyeball until protruding from socket
4) insert tip of Natelson blood collecting tube at 45° angle into anterior corner of socket behind eyeball
5) rotate blood collecting tube clockwise and counterclockwise until blood begins to enter tube
6) once blood enters tube, adjust angle downward to facilitate flow by capillary action
7) after collecting sufficient amount of blood, dispense into microcentrifuge tube by blowing into top of tube
8) continue to collect blood as before for a total of 300-500 μL per mouse
   use new tubes as needed
9) after blood draw is complete, apply slight pressure to eyeball with surgical gauze
10) return mouse to new cage and allow to recover
11) proceed with additional mice

12) to collect serum, allow blood to clot for ≥ 2 hr. at room temp.
13) centrifuge for 3,000 rpm for 15 min. at 4°C
14) transfer serum (top phase) to new microcentrifuge tube
15) store serum samples at -20°C or -80°C

References:

(138)

Anesthesia Machine Set Up

isoflurane mixed with oxygen for consistent and safe anesthesia

Equipment:

- chemical fume hood
  - *place isoflurane vaporizer and induction chamber inside chemical fume hood to protect from isoflurane vapors*
- anesthesia machine
  - tubing
  - oxygen E-tank
  - pressure regulator / flowmeter
  - isoflurane vaporizer
  - induction chamber

Materials:

- isoflurane
- transfer pipet

Protocol:

1) connect anesthesia machine according to diagram below

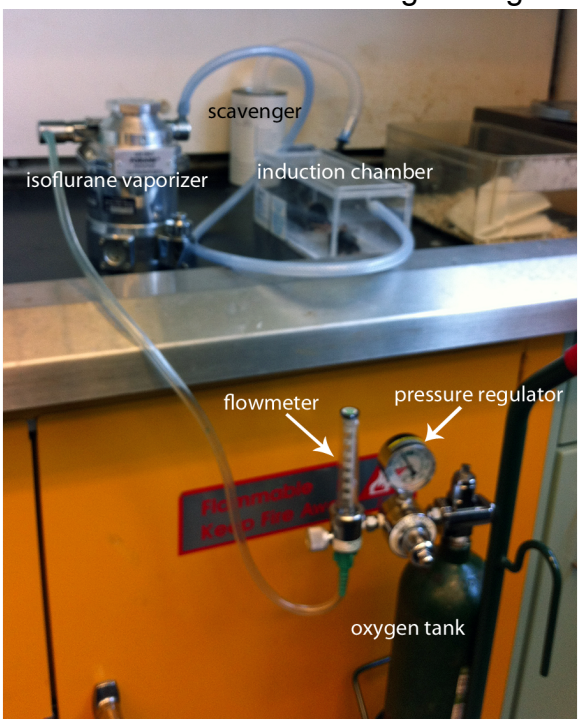


Figure 30: Isoflurane vaporizer placed inside chemical fume hood.

2) fill reservoir of isoflurane vaporizer with isoflurane as needed
3) check hoses for any obstructions

4) adjust O_2 flow rate to 1 liter/minute
5) adjust level of isoflurane to 3.5%

RBC Lysis

erythrocyte lysis

Equipment:

- refrigerated centrifuge
- vortex
- lab timer

Materials:

- primary lymphocyte
  - freshly isolated
- ACK lysis buffer
  - keep at room temp.
- Wash Medium / RPMI-1640
  - keep at 4°C
- FACS Buffer
  - keep at 4°C

Supporting Protocol:

- Splenocyte Preparation

Protocol:

1) for each tube, resuspend cells in 0.2-1 mL of ACK lysis buffer
2) incubate for 5 min. at room temp.
3) vortex occasionally
4) fill tube with Wash Medium / FACS Buffer
   to quench / wash
5) centrifuge for 5 min. at 1,500 rpm (4°C)
6) discard supernatant
7) visually inspect pellet for efficient lysis of RBCs
   removal of red layer
8) if needed, repeat for removal of red layer

References:

(139)

Splenocyte Preparation

Equipment:

- bucket with ice
- refrigerated centrifuge
- forceps (2)
- surgical scissors (2)
- beaker with 70% ethanol
  for sterilizing surgical instruments
- 70% ethanol squirt bottle

Materials:

- mice
- 15 mL tubes
- T Cell Medium (TCM)
  RPMI-1640 + 10% FBS + HEPES + L-Glutamine, + 2-ME + Pen/Strep
- paper towels

Supporting Protocols:

- Anesthesia Using Isoflurane
- Tissue Dissociation
- RBC Lysis
- Cell Culture > Cell Counting

Protocol:

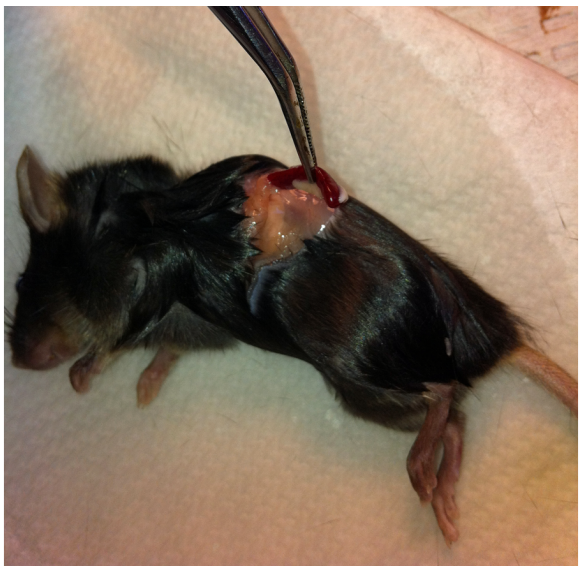


Figure 31: Splenectomy.

1) add 10 mL of ice-cold TCM to 15 mL tube
   - *prepare inside TC hood*
   - *prepare separately for each mouse*
   1.1) keep on ice
2) sacrifice mouse using isoflurane followed by cervical dislocation
   see supporting protocol
3) place mouse on stack of paper towels with left side facing up
4) soak fur on left side with 70% ethanol solution
5) lift loose skin and make single incision through skin on left side above spleen
6) firmly pull apart skin on either side of incision to expand opening
   spleen should be visible below peritoneal membrane
7) lift peritoneal membrane and make single incision through membrane
8) excise spleen using sterile surgical scissors and forceps to separate from connective tissue and blood vessels
9) place spleen in 15 mL tube containing ice-cold medium
10) proceed with Tissue Dissociation protocol
    see supporting protocol
11) perform RBC lysis
    see supporting protocol
    11.1) resuspend cells from single spleen in 0.5 mL of ACK lysis buffer
12) resuspend splenocytes in 5 mL of TCM
13) perform cell count
    see supporting protocol
14) optional: keep cells on ice overnight

References:

(139)

Mouse Dissection

opening of chest cavity to harvest thymus, mediastinal lymph nodes, and/or lungs

Equipment:

- light source for dissection
- surgical scissors (2)
  - *sharp end (1)*
  - *blunt end (1)*
- forceps (1)
- squirt bottle containing 70% ethanol
- bucket with ice

Materials:

- dissection board
- needles
  for securing mouse to dissection board
- paper towels

Supporting Protocol:

- Anesthesia Using Isoflurane

Protocol:

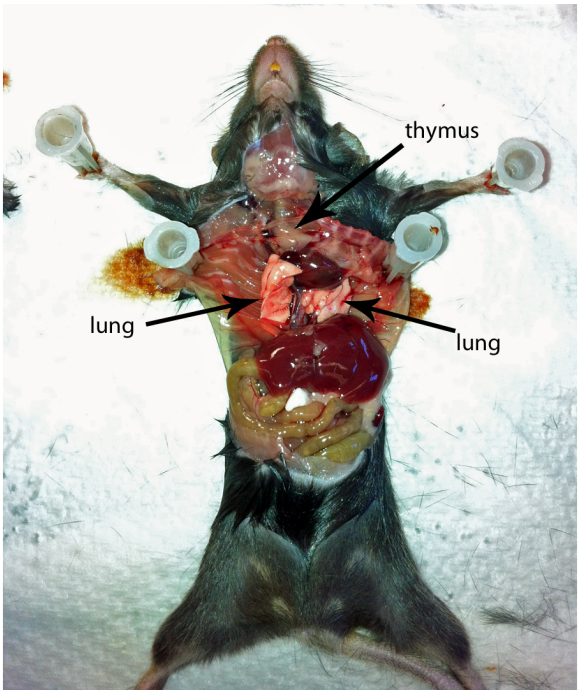


Figure 32: Mouse dissection.

1) sacrifice mouse using isoflurane followed by cervical dislocation
   see supporting protocol
2) place mouse supine on top of dissection board
3) extend front and rear paws and secure using needles
4) soak fur with 70% ethanol solution
5) using forceps, lift loose skin just below sternum
6) make single longitudinal incision through skin at midline
7) separate skin above chest cavity by extending flat edge of blunt scissors towards lower jaw
8) separate skin below rib cage by extending flat edge of blunt scissors laterally on both sides
9) make longitudinal incision through skin above chest cavity
10) make two lateral incisions through skin below rib cage on both sides
11) make single incision through peritoneal membrane just below sternum
12) make two lateral incisions through peritoneal membrane below rib cage on both sides
13) make single longitudinal incision through sternum to penetrate chest cavity
14) make two lateral incisions through membrane surrounding chest cavity below rib cage on both sides
15) continue longitudinal incision through rib cage towards anterior
16) separate rib cage using pins to open chest cavity (optional)

Tissue Dissociation

to prepare single-cell suspension from primary tissue

Equipment:

- bucket with ice
- refrigerated centrifuge

Materials:

- T Cell Medium (TCM)
  RPMI-1640 + 10% FBS + HEPES + L-Glutamine, + 2-ME + Pen/Strep
- tissue specimen in medium
  - keep on ice
- 70 μm nylon cell strainers (1 per specimen)
- 50 mL centrifuge tubes (1 per specimen)
- transfer pipets (1 per specimen)
- syringes (1 per specimen)

Protocol:

1) place 70 μm cell strainer on top of 50 mL tube
2) using transfer pipet, wet surface of cell strainer with medium
3) collect tissue on top of strainer
4) using blunt end of syringe plunger, manually dissociate tissue against top of strainer
5) pass 10 mL of TCM through strainer to collect cells in 50 mL tube
   5.1) rinse blunt end of syringe plunger to recover additional cells
6) centrifuge splenocytes for 5 min. at 1,500 rpm (4°C)
7) discard supernatant

References:

(139)

CD4+ Negative Enrichment

for CD4 negative isolation from splenocytes or DLN cells

Equipment:

- bucket with ice
- refrigerated centrifuge
- tube rotator
- magnetic particle concentrator
    - *Invitrogen/Dynal, MPC-S*
    - *for use with microcentrifuge tubes*

Materials:

- splenocytes or DLN cells
    - *see supporting protocol*
    - do not lyse RBCs
    - count WBCs
        - *see supporting protocol*
    - keep on ice
    - reserve aliquot of ~10^6 cells
        - *for CD4 staining prior to purification*
- Buffer 1
    - *see 'Buffers & Solutions'*
    - keep on ice
- 15 mL centrifuge tube (1 per reaction)
    - *for cells*
- FBS
    - heat-inactivated
- mouse CD4 negative isolation kit
    - *Invitrogen/Dynal, 114.16D*
    - antibody mix
        - *0.4 mL total volume*
    - mouse depletion Dynabeads
        - *400x10^6 beads/mL stock*
        - *4 mL total volume*
- microcentrifuge tubes (2 per reaction)
    - *for beads (1)*
    - *for purified cells (1)*
- T Cell Medium (TCM)
    - *cRPMI + 10% FBS*

Supporting Protocols:

- Splenocyte Preparation
- Lung Inflammation / Asthma > DLN Preparation
- Cell Culture > Cell Counting

Protocol:

- for purification of 50x10^6 WBCs

- scale volumes proportionally (with some noted exceptions) from 10-100x10^6 cells depending on cell number

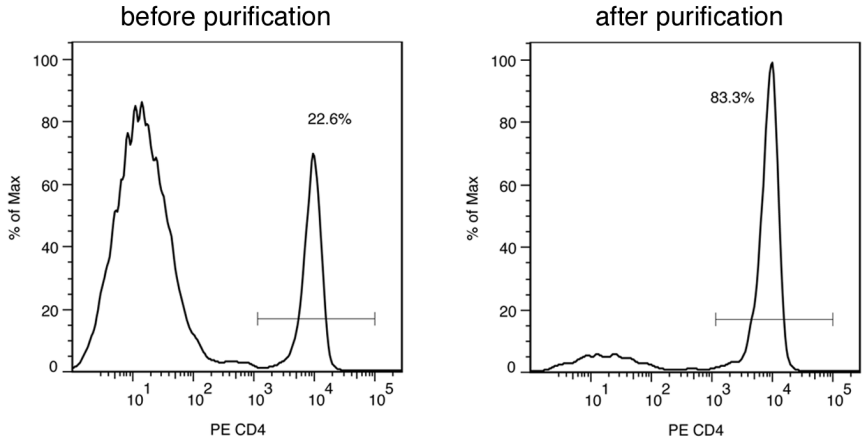


Figure 33: Purification of CD4+ cells.

Splenocytes were purified for CD4+ cells using manufacturer's kit and aliquots of cells obtained before and after purification were stained for surface expression of CD4.

1) prepare cells
    1.1) centrifuge for 5 min. at 1,500 rpm (4°C)
    1.2) discard supernatant
    1.3) resuspend cells in 0.5 mL of Buffer 1
        100x10^6 cells/mL
    1.4) transfer cell suspension to 15 mL Falcon tube
2) incubate with antibody mix
    2.1) add 100 µL of FBS
        20% FBS final
    2.2) add 20 µL of antibody mix
        - increase up to 5x for greater purity
        - do not use less than 20 µL
    2.3) mix well
    2.4) incubate for 20 min. on ice
3) wash magnetic beads
    3.1) resuspend beads using P1000 and tip
    3.2) transfer 200 µL of bead suspension to microcentrifuge tube
        - increase up to 2.5x for greater purity
        - do not use less than 200 µL or more than 500 µL
    3.3) add 1 mL Buffer 1
    3.4) resuspend with P1000 and tip to wash
    3.5) place tube in magnet for 1min

3.6) remove/discard supernatant
3.7) remove tube from magnet
3.8) resuspend beads in equal volume (200 μL) of Buffer 1

4) wash cells
4.1) add 10 mL of Buffer 1 (wash)
4.2) centrifuge for 5 min. at 1,500 rpm (4°C)
4.3) aspirate / discard supernatant using pipet tip
4.4) resuspend cells in 800 μL of Buffer 1

5) incubation with magnetic beads
5.1) transfer 800 μL of cell suspension to microcentrifuge tube containing 200 μL of pre-washed beads
1 mL final volume
5.2) tumble on rotator for 15 min at room temp.
5.3) remove tube from rotating wheel
do NOT resuspend cells

6) CD4+ negative isolation
6.1) place microcentrifuge tube containing beads/cells in magnet for 2 min.
6.2) transfer supernatant containing negatively-isolated CD4+ cells to new microcentrifuge tube

7) replace medium
7.1) centrifuge for 5 min. at 1,500 rpm (4°C)
7.2) aspirate supernatant
7.3) resuspend pellet in volume of TCM for counting
7.4) count cells
reserve aliquot of ~10^6 cells for CD4 staining post-purification
7.5) adjust to desired cell concentration in TCM

8) proceed with downstream application

Mononuclear Cell Isolation Using Density Gradient

for isolating / improving viability of lymphocytes and other mononuclear cells above histopaque layer – RBCs, granulocytes and dead cells contained in pellet

Equipment:

- centrifuge

Materials:

- source of lymphocytes for mononuclear cell isolation
  Jurkat cell culture, whole blood (anti-coagulated), splenocytes, DLN cells, lung cells, etc.
- 15 / 50 mL centrifuge tubes
- Histopaque-1077
  - density of 1.077 g/mL
  - Sigma, Cat. # 10771; 100 mL
  - stored at 4°C
- transfer pipets
- PBS, cell culture grade
- T Cell Medium (TCM)
  cRPMI + 10% FBS

Supporting Protocol:

- Cell Culture > Cell Counting

Protocol:

1) add 3 mL of Histopaque to 15 mL tube
2) equilibrate to room temp.
3) slowly layer equal volume (3 mL) of cell source for isolation above histopaque
   use transfer pipet to add cells drop-wise against wall of tube held at an angle
4) centrifuge for 30 min. at 400 g / ~1,500 rpm (room temp.)
   - do not centrifuge at 4°C during isolation step to avoid clumping / poor recovery
   - do not leave cells on histopaque for > 30 min. to avoid toxicity
5) add 10 mL of PBS to new 15 mL tube
   for washing isolated mononuclear cells
6) using transfer pipet, remove (opaque) layer of isolated mononuclear cells above histopaque
7) transfer isolated mononuclear cells to new tube containing PBS (wash)
8) centrifuge for 5 min. at 1,500 rpm (4°C)
9) discard supernatant

10) resuspend pellet in volume of TCM for counting
11) count live / dead cells and determine viability
12) adjust to desired cell concentration in TCM
13) proceed with downstream application

Intraperitoneal (i.p.) Injection

for delivery of reagents to abdominal cavity

Equipment:

- empty cage with wire top
- biohazardous sharps container

Materials:

- mice
- solution for injection
  - equilibrate to room temp. prior to injection
- 1 mL syringes
  BD, Cat. # 309602
- 27G 1/2" needles
  BD, Cat. # 305109
- Kimwipes
- Sharpie marker, ultra-fine point

Protocol:

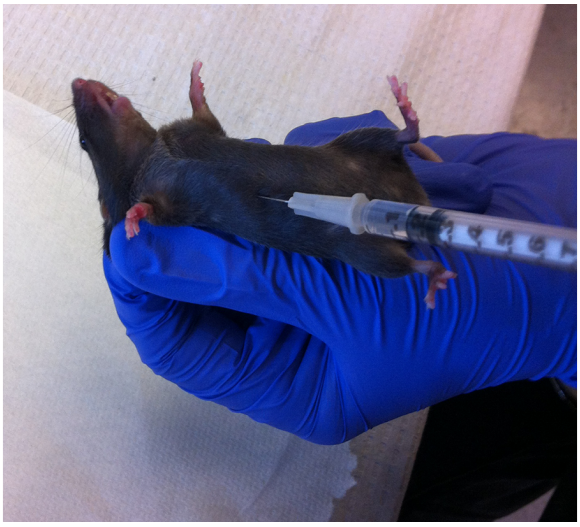


Figure 34: Demonstration of i.p. injection into upper right quadrant of abdomen.

1) prepare needle / syringe for injection(s):
   proceed in parallel for mice receiving same treatment
   1.1) attach needle to syringe
   align tapered end of needle with number/calibrations on syringe barrel
   1.2) mark calibrations on syringe barrel for desired number of injections
   injection volume = 150 µL per mouse

1.3) remove protective cap from needle
1.4) submerge needle in solution and withdrawal plunger to fill syringe beyond desired total volume
1.5) position syringe with needle upright and flick barrel to collect air bubbles at top
1.6) depress plunger to remove air and adjust to desired final volume

2) restrain mouse
- proceed serially for each mouse
- reverse left / right hand for left-handed individual
2.1) scruff mouse from behind with left hand
2.2) grasp tail with right hand
2.3) flip left hand so that abdomen of mouse is facing up
2.4) secure tail using small finger of left hand against palm

3) perform injection
3.1) using right hand, insert needle into upper-right quadrant of abdomen (see above)
- tapered end of needle facing up
- 45° angle to abdomen
- insert needle just below skin
3.2) steadily depress plunger to deliver 150 μL into peritoneal cavity
3.3) slowly remove needle in reserve path

4) following injection, place mouse in separate cage and repeat for additional mice receiving similar treatment as needed
5) discard used needle / syringe in biohazardous sharps container
6) observe injected mice for acute signs of complication from injection

References:

(140, 141)

Intranasal (i.n.) Treatment

- for delivery of cost-limiting reagents to respiratory tract
- allows for controlled dosing

Equipment:

- 20 μL pipettor
- bucket with ice
- dI water squirt bottle
- lab timer

Materials:

- mice
- solution for inhalation
- Kimwipes
- gel-loading pipet tips
- paper towels

Supporting Protocol:

- Anesthesia Machine Set Up

Protocol:

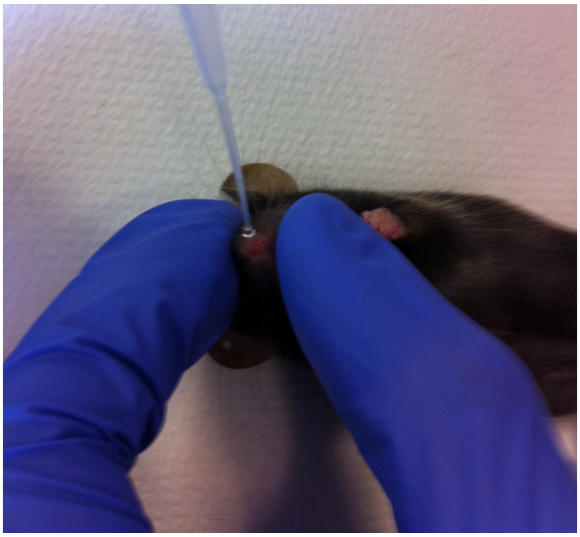


Figure 35: Demonstration of i.n. administration of liquid droplet.

1) set up anesthesia machine
   see supporting protocol
2) anesthetize small cohort of 3-5 mice (1 cage of mice) in parallel
   2.1) place mice inside chamber for ≥ 3 minutes
   2.2) observe until mice remain unconscious and motionless
   increase exposure time and/or % isoflurane to effect

3) position mouse for intranasal treatment
    perform serially for each mouse in cohort
    3.1) remove mouse from chamber
        proceed quickly between subsequent steps until completion of intranasal treatment
    3.2) place supine on top of paper towel on lab bench
    3.3) using forefinger and thumb of left hand, apply slight opposing pressure to upper and lower jaw
        - to close mouth and to promote inhalation versus ingestion of bolus
        - reverse left / right hand for left-handed individual
4) perform intranasal treatment
    4.1) deliver 20 μL bolus of solution for inhalation applied externally as droplets to alternating nares
        observe inhalation of each droplet prior to applying subsequent droplets
    4.2) continue to apply slight opposing pressure to upper and lower jaw until complete inhalation of bolus
    4.3) to stimulate breathing, tap chest cavity and/or squeeze hind paw
5) following complete inhalation, place mouse on top of folded paper towel inside cage with abdomen facing down
6) challenge each additional mouse of cohort inside anesthesia chamber
    limit isoflurane exposure to ≤ 15 min. for any given mouse in cohort
7) observe mice for recovery following treatment
8) remove paper towel from cage
9) clean interior of induction chamber with water
    do not use ethanol

References:

(136, 137)

Aerosol Treatment

- for delivery of non-cost-limiting reagents to respiratory tract
- provides consistent delivery from mouse to mouse

Equipment:

- ultrasonic nebulizer
  Ultra-NEB99; Devilbiss / Sunrise Medical, Model # 099HD
- induction chamber
- tubing (2)
- medicine cup

Materials:

- mice
- solution for inhalation
- paper towels

Protocol:

combine cohort of ~8 mice inside induction chamber

1) fill transducer compartment of nebulizer with tap water between 'min' and 'max' calibrations
2) dispense solution for inhalation inside medicine cup
3) connect components for nebulizer according to diagram below

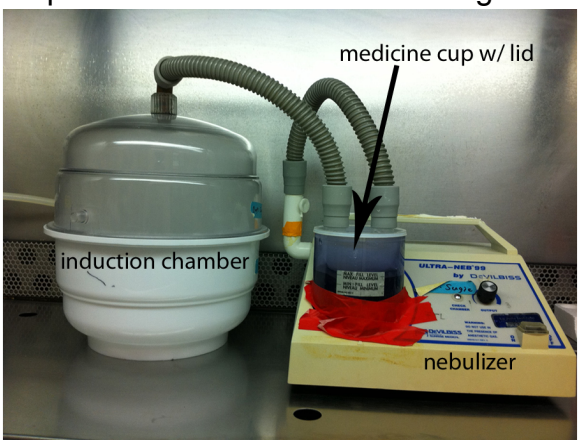


Figure 36: Nebulizer setup for delivery of aerosolized material to mice.

4) place mice inside induction chamber
5) connect tubing (2) as indicated in diagram below
   5.1) connect tubing on nebulizer from fan to medicine cup
   5.2) connect tubing from nebulizer to induction chamber
6) turn on power for nebulizer
7) adjust airflow control knob on front of nebulizer to generate dense aerosol fog

between 12 to 3 o'clock position

8) set timer for desired exposure to aerosol
9) return mice to respective cage(s)
10) carefully invert nebulizer to discard water from transducer compartment

do not detach from unit

11) use folded paper towels to wick excess water from compartment
12) clean induction chamber, medicine cup and hoses with tap water

5.2.5. Peptide

Lyophilized Stocks

Equipment:

- -20°C freezer
- desiccator jar w/ lid

Materials:

- drierite
- parafilm

Protocol:

- peptides are most stable as lyophilized powder and can be stored for extended periods (> 1 year) at -20°C inside desiccator jar containing drierite
- do not reconstitute until needed for series of experiments
- peptides can be shipped as lyophilized powder at ambient temperature or with refrigerant pack for 2nd day delivery
- unless stated otherwise, mass indicated on label represents total mass including salts
- where indicated, linear peptides have been modified at the N-terminus with FITC or biotin
- all linear peptides contain an amide group at the C-terminus (amidiation)

for reference, the composition and molecular weight of selected peptides are as follows:

abbreviation:	*sequence:*	*g/mol*
R9-QQP	*RRRRRRRRRQQPPVPPQRPMA-NH2*	*2,750.27*
FITC-R9-QQA	*FITC-RRRRRRRRRQQAAVAAQRAMA-NH2*	*3,122.62*
FITC-R9-PQM	*FITC-RRRRRRRRRPQMPAPQRPQPV-NH2*	*3,252.81*
Biotin-QQP	*Biotin-QQPPVPPQRPMA-NH2*	*1,570.89*
QQP	*QQPPVPPQRPMA-NH2*	*1,344.59*
QQA	*QQAAVAAQRAMA-NH2*	*1,214.40*

1) remove peptide vial(s) from packaging
   - do not sign for package if external evidence of water damage
   - inner packaging should be free of moisture
   - vial should be enclosed in secondary plastic bag containing desiccant
2) seal vial cap with parafilm if not done so already
3) for each peptide, verify that label on tube matches accompanying packing slip / documentation
4) review HPLC data sheet and confirm that purity is ≥ 95%
5) review LCMS data sheet and confirm that all major peaks are consistent with predicted molecular weight of peptide
6) store peptide vial inside desiccator jar containing drierite

6.1) close lid securely

7) store at -20°C

8) record information for each peptide including description, mass, and desiccator location in lab notebook and/or electronic database

- *physical description*
- *mass*
- *freezer location*

Reconstitution / Stock Solutions

Equipment:

- analytical balance
- -80°C freezer
- refrigerated centrifuge

Materials:

- lyophilized peptide stock
    - stored inside desiccator jar at -20°C
- screw cap aliquot tubes or microcentrifuge tubes, sterile
- 50 mL centrifuge tube(s)
- weighing paper, 3" x 3"
- cell culture grade water
- metal spatula
- parafilm

Supporting Protocol:

- Lyophilized Stocks

Protocol:

- many peptides are very hygroscopic

- to reduce exposure with moisture due to condensation, allow ample time for peptide to equilibrate to ambient temperature before opening cap of vial and exposure to air

- lyophilized peptide should be dissolved in cell culture grade water unless factors such as solubility and/or pH require a different solvent (e.g., DMSO) to be used

*- do *not* reconstitute in any solution containing carrier protein (e.g. BSA)*

- nominal stock concentration should be ≥ 0.5 mg/mL

- lower stock concentrations are not recommended due to the loss of free peptide caused by binding to the plastic tube

- stock solutions can be stored for ≤ 2 months at -80°C

1) calculate approximate amount of peptide needed for series of experiments
2) when needed for experiment, remove desiccator jar from -20°C
3) equilibrate to room temp. for ≥ 30 min. before opening lid of desiccator jar

4) remove peptide vial from desiccator and allow to equilibrate to ambient temperature for an additional ≥ 30 min. before opening cap of vial
5) spin vial to collect lyophilize at bottom (if needed)
    5.1) place peptide vial inside secondary 50 mL centrifuge tube
    5.2) spin for 5 min. at 2,000 rpm (4°C)
6) depending on amount of peptide in tube as well as requirements for experiment, weigh aliquot of lyophilizate and transfer to new tube
        for accuracy, do not weigh less than 1.0 mg using analytical balance
    6.1) trim piece of weighing paper to 1/4 size, fold and tare on analytical balance
    6.2) use clean metal spatula to remove small piece of lyophilizate
    6.3) carefully place on weighing paper
    6.4) transfer to designated aliquot tube and label with peptide name, date, and nominal mass
7) re-seal stock vial containing remaining lyophilized peptide with parafilm and return to desiccator
    7.1) store at -20°C
8) dissolve peptide in cell culture grade water at ≥ 0.5 mg/mL
    8.1) add calculated volume of water to vial using micropipettor and tip
    8.2) pipet up and down to dissolve
            do not vortex
    8.3) allow to dissolve / hydrate for 15 min. at ambient temperature
9) store stock solution for ≤ 2 months at -80°C

Relative Concentration Determination

Materials:

- reference peptide stock solution
  - previously characterized and biologically active
  - reconstituted at ≥ 0.5 mg/mL (nominal concentration) in water *without* carrier (e.g., BSA)
  - stored at -20°C
- 'concentration unknown' peptide stock solution
  - reconstituted at ≥ 0.5 mg/mL in water without carrier (e.g., BSA)
- cell culture grade water
  for preparing dilutions and blank
- microcentrifuge tubes

Supporting Protocols:

- Reconstitution / Stock Solution
- General > Absorbance Measurement Using Spectrophotometer

Protocol:

based on peptide bond absorbance maximum at 214 nm (190 nm - 230 nm), one can generate a standard curve of absorbance versus nominal concentration (mg/mL)

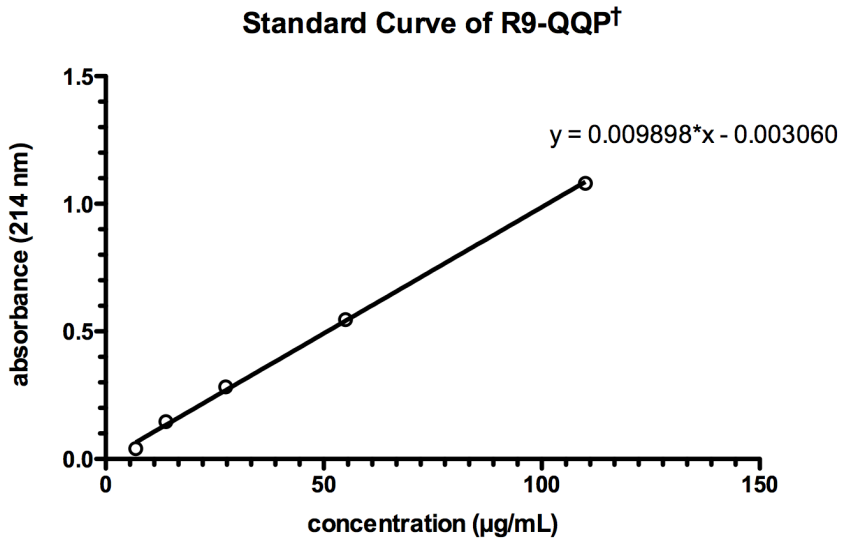
one can use absorbance 214 nm values of peptide stock solutions in the following specific scenarios:

- to compare / standardize the concentration of independently reconstituted stock solutions
- to monitor / standardize the concentration of the same peptide stock solution over time
- to compare / standardize the concentration of different peptides of identical length and composition of amino acids (e.g., sequence specific peptide versus scrambled control peptide)
- to compare / standardize the concentration of different peptides of identical length and similar composition of amino acids – provided differences among amino acids are similar in terms of side group absorbance properties

limitations:

*- only peptide stocks dissolved in water and *without* carrier (e.g., BSA) can be analyzed for absorbance at 214 nm*
- one cannot compare tagged (fluorescently or otherwise) and non-tagged versions of the same peptide
- one cannot compare peptides of different length (i.e., number of amino acids)

1) prepare standard curve of reference peptide stock solution that has been previously characterized in a biological assay



†reconstituted at 2.75 mg/mL (1 mM) in H_20 on 3/17/10

Figure 37: R9-QQP standard curve of absorbance at 214 nm versus nominal concentration of reference peptide stock solution.

- 1.1) prepare range of dilutions (e.g., 1:25, 1:50, 1:100, 1:200, 1:400) in water
  need ≥ 0.5 mL per sample
- 1.2) measure absorbance for each prepared dilution
  see supporting protocol
- 1.3) plot absorbance at 214 nm versus nominal concentration (mg/mL)
- 1.4) determine slope and y-intercept from linear regression analysis according to linear equation (y = mx + b)
  once determined, can be used for future reference

2) measure concentration of 'unknown' peptide stock solution
   - 2.1) prepare dilutions of stock solution (e.g., 1:50 and 1:100) in water
     need ≥ 0.5 mL per sample
   - 2.2) measure absorbance for each prepared dilution
     see supporting protocol

3) calculate concentration of 'unknown' stock solution using linear equation determined from standard curve above

4) as needed, label concentration of 'unknown' stock solution using calculated concentration

to prevent variations in concentration that may result from relying on nominal concentration

Dilutions / Working Solutions

Equipment:

- -20°C freezer

Materials:

- buffer for dilutions (e.g., PBS, RPMI)
  with or without carrier protein
- peptide stock solution
  - stored at -20°C
- tube(s) for preparing dilution, sterile
  optional: can be coated with carrier protein
- aliquot tubes, sterile
  optional: can be coated with carrier protein

Supporting Protocol:

- Reconstitution / Stock Solutions

Protocol:

- peptide stock solution can be diluted in buffer of choice (e.g., PBS, RPMI) with or without carrier protein as required
- prepare dilutions at needed concentration
- do not dilute beyond needed working concentration in order to reduce loss of free peptide caused by binding to plastic tube
- optional: prepare dilutions / aliquots in tubes pre-coated with carrier protein
- working solutions should preferably be used immediately or stored for ≤ 2 weeks at -20°C
- limit freeze-thaw cycles

1) thaw peptide stock solution
   1.1) keep on ice
2) prepare dilution in selected buffer for experiment
3) prepare aliquots as needed
4) use immediately or store for ≤ 2 weeks at -20°C

In Vitro Peptide Transduction

Equipment:

- refrigerated centrifuge
- humidified, 5% CO_2, 37°C incubator
- bucket with ice

Materials:

- R9-peptide stock solution
  - stored at -20 / -80°C
- Jurkat cells (required # according to experimental design)
  e.g., $20x10^6$ cells per condition for biochemistry
- splenocytes (required # according to experimental design)
  e.g., $60\text{-}80x10^6$ cells per condition for biochemistry
  - lyse RBCs
    to minimize uptake of peptide by platelets
- Wash Medium (RPMI-1640)
  serum-free
- 12-well plate
  2 mL volume per well
- 24-well plate
  1 mL volume per well
- Jurkat Growth Medium (JGM)
- T Cell Medium (TCM)

Supporting Protocols:

- Reconstitution / Stock Solutions
  - Dilutions / Working Solutions
- Cell Culture > Jurkat Cell LIne Maintenance
- In Vivo / Ex Vivo > Splenocyte Preparation
  - RBC Lysis

Protocol:

1) prepare peptide
   1.1) thaw peptide stock solution on ice just prior to use
   1.2) dilute stock solution in PBS to desired working concentration (e.g., 0.1-10 mM)
   prepare working solution to allow for accurate pipetting without significantly affecting final volume
   1.3) keep on ice
2) wash cells
   remove serum prior to incubation with peptide:
   - to reduce protease-mediated degradation of peptide
   - to minimize binding of peptide to serum proteins

2.1) centrifuge for 5 min. at 1,000 - 1,500 rpm (4°C)
2.2) aspirate / discard supernatant
2.3) resuspend cells in serum-free RPMI at required concentration according to experimental design

3) incubate with peptide
3.1) transfer required number of cells to designated well(s) of 12 or 24-well plate
3.2) add peptide stock / working solution to designated well(s)
3.3) pipet up and down to mix
3.4) cover plate with lid
to prevent contamination during incubation
3.5) place inside TC incubator for ≥ 30 min.

4) optional: wash cells
to remove excess peptide from medium
4.1) centrifuge for 5 min. at 1,000 - 1,500 rpm (4°C)
4.2) aspirate / discard supernatant
4.3) resuspend cells in complete medium (containing 10% FBS) at required concentration according to experimental design

5) proceed with experiment

Trypsin Treatment for Uptake Studies

to reduce membrane-associated peptide

Equipment:

- refrigerated centrifuge with adaptors for microcentrifuge tubes
- 37°C water bath
- vortex
- bucket with ice

Materials:

- Jurkat cells / primary lymphocytes
  - treated with FITC-R9 peptide
  - protect from light
- 0.05% trypsin - 0.53 mM EDTA in HBSS
  - *with sodium bicarbonate, without calcium and magnesium*
  - *Mediatech, Cat. # 25-051-CI*
  - keep on ice
- PBS (without Ca^{2+} and Mg^{2+})
  - keep on ice
- FACS buffer

Supporting Protocol:

- In Vitro Peptide Transduction

Protocol:

1) remove free peptide
   - 1.1) immediately following peptide treatment, centrifuge for 5 min. at 1,000 - 1,500 rpm (4°C)
   - 1.2) aspirate to discard supernatant completely
   - 1.3) resuspend in 1 mL of ice-cold PBS (wash)
   - 1.4) centrifuge for 5 min. (4°C)
   - 1.5) aspirate / discard supernatant completely
2) reduce membrane-associated peptide
   - 2.1) resuspend pellet in 200 μL of trypsin
   - 2.2) incubate at 37°C for 10 min.
   - 2.3) add 1 mL of FACS buffer to quench
   - 2.4) centrifuge for 5 min. (4°C)
   - 2.5) aspirate / discard supernatant completely
3) additional washes
   - 3.1) resuspend in 1 mL of ice-cold PBS (wash #1)
   - 3.2) centrifuge for 5 min. (4°C)
   - 3.3) aspirate / discard supernatant completely
   - 3.4) resuspend in 1 mL of ice-cold PBS (wash #2)
   - 3.5) centrifuge for 5 min. (4°C)

3.6) aspirate / discard supernatant completely
3.7) resuspend in 1 mL of ice-cold PBS (wash #3)
3.8) aspirate / discard supernatant completely
4) for primary cells, proceed with staining for CD4+ T Cells

FACS Staining for Uptake Studies

for uptake analysis of FITC-conjugated peptide by CD4+ TCR+ lymphocytes

Equipment:

- refrigerated centrifuge
- vortex
- bucket with ice
- lab timer

Materials:

- FACS buffer
  - keep on ice
- FACS tubes
- primary lymphocytes
  - treated with FITC-R9 peptide
  - treated with trypsin
    to reduce membrane-associated peptide
- rat anti-mouse CD16/CD32
  BD, Cat. # 553141
  Mouse BD Fc Block
- Pacific Blue anti-mouse CD4, clone MR4-5
  BioLegend, Cat. # 100531
  ex: 405 nm laser
  em: 450/40 filter
- APC anti-TCRβ, clone H57-597
  BioLegend, Cat. # 109211
  ex: 633 nm laser
  em: 660/20 filter
- 70 μm cell strainers
- 2% paraformaldehyde (PFA) / PBS
  - keep on ice

Supporting Protocol:

Trypsin Treatment for Uptake Studies

Protocol:

- in order to minimize spectral overlap with FITC, lymphocytes are stained with Pacific Blue anti-CD4 and APC anti-TCRβ
- thus, each fluorophore is excited with a different laser as follows (analyze using FACSAria not FACSCanto):
- 405 nm laser for Pacific Blue
- 488 nm laser for FITC
- 633 nm laser for APC

- single-color control samples should be prepared in order to establish optimal voltages for each channel and to compensate for any residual spectral overlap if required

- keep cells on ice throughout

1) prepare samples for FACS staining
    1.1) resuspend peptide and trypsin-treated lymphocytes in required volume of FACS buffer for staining
        calculate volume based on number of FACS staining tubes required for each cell preparation
    1.2) transfer 100 μL to each staining tube
        plan in advance
2) block Fc receptors
    2.1) add 0.5-1 μg of Fc blocking antibody to each tube
    2.2) incubate for 5 min.
        do not wash
3) stain for CD4 and TCRβ
    3.1) add 0.5-1 μg of Pacific Blue anti-mouse CD4 to each tube
    3.2) add 0.5-1 μg of APC anti-mouse TCRβ to each tube
    3.3) incubate for ~45 min. at 4°C
4) wash cells
    4.1) add 2 mL of ice-cold FACS buffer to each tube
    4.2) centrifuge for 5 min. at 1,500 rpm (4°C)
    4.3) discard supernatant
5) fix cells
    5.1) add 200 μL of 2% PFA to each tube
    5.2) vortex to resuspend
    5.3) incubate for 10 min. at 4°C
        protect from light
    5.4) add 2 mL of ice-cold FACS buffer (quench)
    5.5) centrifuge for 5 min. at 1,500 rpm (4°C)
    5.6) discard supernatant
6) to remove debris / cell clumps
    6.1) resuspend in 2 mL of ice-cold FACS buffer
    6.2) pass each sample through 70 μm cell strainer
        6.2.1) collect in separate FACS tubes
    6.3) centrifuge for 5 min. at 1,500 rpm (4°C)
    6.4) discard supernatant
7) resuspend in 300 μL of FACS buffer
8) store in dark at 4°C until FACS analysis
9) analyze FACS data
    9.1) create SSC vs. FSC dot plot
        9.1.1) display all events

9.1.2) create lymphocyte gate
9.2) create SSC vs. APC dot plot
9.2.1) display lymphocyte population
9.2.2) create T cell gate
child of lymphocyte gate
9.3) create Pacific Blue vs. FITC gate
9.3.1) display T cell population
9.3.2) create quadrants to determine % CD4+ FITC+ cells
9.3.3) create CD4+ gate to determine FITC MFI of CD4+ T cells

5.2.6. T Cell Stimulation / Itk Activation

Jurkat α-TCR Antibody Stimulation

for investigating TCR-dependent signaling events in human T cells

Equipment:

- humidified, 5% CO_2, 37°C incubator
- refrigerated centrifuge with tube adaptors
- 37°C water bath
- vortex
- lab timer
- bucket with ice

Materials:

- Jurkat cells, ≤ $20x10^6$ cells
  seed sufficient number of flasks in advance according to experimental design
  - cultured to ≤ 10^6 cells/mL
  - serum starved overnight
- Wash Medium / RPMI-1640
  - prepare aliquot in 50 mL Falcon tube
  - keep on ice
- microcentrifuge tubes
- OKT3, mouse IgG2a anti-human TCR CD3ε
- UPC10, mouse IgG2a isotype control
  used for non-stimulation control
- rabbit α-mouse IgG (RαM), unconjugated
  cross-linking antibody

Supporting Protocols:

- Cell Culture > Jurkat Maintenance
  - Cell Culture > Jurkat Serum Starvation
- Cell Culture > Cell Counting
- Peptide > In Vitro Peptide Transduction

Protocol:

1) count cells
2) pre-treatment with peptide (if included in experimental design)
   see supporting protocol for details
3) prepare cells
   3.1) centrifuge required number of cells for 5 min. at 1,000 rpm (4°C)
   3.2) aspirate supernatant
   3.3) resuspend cells in ice-cold RPMI at required concentration
   0.5 mL volume per reaction

3.4) transfer 0.5 mL per reaction to designated microcentrifuge tube(s)
3.5) incubate on ice for ≥ 10 min.

4) incubation with OKT3 (for stimulation) / UPC10 (for non-stim. control)
keep on ice throughout
4.1) add 2.5 μg of OKT3 / UPC10 (5 μg/mL final)
4.2) vortex to mix
4.3) incubate for 30 min. on ice to allow binding

5) wash cells
5.1) centrifuge for 5 min. at 1,000 rpm (4°C)
5.2) aspirate supernatant using pipet tip
5.3) resuspend pellet in 0.5 mL of ice-cold RPMI

6) incubate with rabbit anti-mouse (RαM) cross-linking antibody
keep on ice throughout
6.1) add 2.5 μg of RαM antibody (5 μg/mL final)
6.2) vortex to mix
6.3) incubate for 15 min. on ice to allow binding

7) stimulate cells
optimal kinetics should be determined empirically for each investigator
7.1) for non-stim. control, keep tube on ice
7.2) place tubes in 37°C water bath for designated time points (e.g., 0.5, 1, 3, 5 min.)
7.3) return tube(s) to ice immediately following incubation at 37°C
7.4) continue with downstream assay (e.g., phosphoflow, lysis)

Primary Lymphocyte α-TCR Antibody Stimulation

for investigating TCR-dependent signaling events in primary mouse T cells

Equipment:

- humidified, 5% CO_2, 37°C incubator
- refrigerated centrifuge with tube adaptors
- 37°C water bath
- vortex
- lab timer
- bucket with ice

Materials:

- primary lymphocytes, ≤ $80x10^6$ cells
  harvest sufficient number of cells according to experimental design
  - freshly isolated
  - rested inside incubator for ≤ 4 hours prior to stimulation
    keep in 50 mL polypropylene tube (rather than polystyrene) to prevent loss of cell yield due to adherence
- Wash Medium / RPMI-1640
  - prepare aliquot in 50 mL Falcon tube
  - keep on ice
- microcentrifuge tubes
- 2C11, armenian hamster α-mouse TCR CD3ε
  BD, Cat. # 553058
  0.5 mg/mL stock
- goat α-armenian hamster IgG (GαAH), unconjugated
  Jackson ImmunoResearch, Cat. # 127-005-099
  1.8 mg/mL stock

Supporting Protocols:

- In Vivo / Ex Vivo > Splenocyte Preparation
  - In Vivo / Ex Vivo > Tissue Dissociation
- Cell Culture > Cell Counting
- Peptide > In Vitro Peptide Transduction

Protocol:

1) count cells
   see supporting protocol
2) pre-treatment with peptide (if included in experimental design)
   see supporting protocol
3) prepare cells
   3.1) centrifuge required number of cells for 5 min. at 1,500 rpm (4°C)
   3.2) aspirate supernatant

3.3) resuspend cells in ice-cold RPMI at required concentration
0.5 mL volume per reaction
3.4) transfer 0.5 mL per reaction to designated microcentrifuge tube(s)
3.5) incubate on ice for ≥ 10 min.

4) incubate with 2C11 (for stimulation)
keep on ice throughout
4.1) add 2.5 μg of 2C11 (5 μg/mL final)
4.2) vortex to mix
4.3) incubate for 30 min. on ice to allow binding

5) wash cells
5.1) centrifuge for 5 min. at 1,500 rpm (4°C)
5.2) aspirate supernatant using pipet tip
5.3) resuspend pellet in 0.5 mL of ice-cold RPMI

6) incubate with goat anti-armenian hamster (GαAH) cross-linking antibody
keep on ice throughout
6.1) add 2.5 μg of GαAH antibody (5 μg/mL final)
6.2) vortex to mix
6.3) incubate for 15 min. on ice to allow binding

7) stimulate cells
optimal kinetics should be determined empirically for each investigator
7.1) for non-stim. control, keep tube on ice
7.2) place tubes in 37°C water bath for designated time points (e.g., 0.5, 1, 3, 5 min.)
7.3) return tube(s) to ice immediately following incubation at 37°C
7.4) continue with downstream assay (e.g., phosphoflow, lysis)

PMA / Ionomycin (IM) Stimulation

for investigating TCR-independent signaling events (positive control for TCR-dependent stimulation)

Equipment:

- humidified, 5% CO_2, 37°C incubator
- refrigerated centrifuge
- 37°C water bath
- vortex
- lab timer
- bucket with ice

Materials:

- Jurkat cells (≤ $20x10^6$ cells)
  seed sufficient number of flasks in advance according to experimental design
  - cultured to ≤ 10^6 cells/mL
  - serum starved overnight
- primary lymphocytes (≤ $80x10^6$ cells)
  harvest sufficient number of cells according to experimental design
  - freshly isolated
  - rested inside incubator for ≤ 4 hours prior to stimulation
    keep in 50 mL polypropylene tube (rather than polystyrene) to prevent loss of cell yield due to adherence
- Wash Medium / RPMI-1640
  - prepare aliquot in 50 mL Falcon tube
  - keep on ice
- microcentrifuge tubes
- 100x PMA/IM
  see Buffers & Solutions
  - keep on ice

Supporting Protocols:

- Cell Culture > Jurkat Maintenance
  - Cell Culture > Jurkat Serum Starvation
- In Vivo / Ex Vivo > Splenocyte Preparation
  - In Vivo / Ex Vivo > Tissue Dissociation
- Cell Culture > Cell Counting

Protocol:

1) count cells
2) prepare cells
   2.1) centrifuge required number of cells for 5 min. at 1,000 / 1,500 rpm (4°C)

2.2) aspirate supernatant
2.3) resuspend cells in ice-cold RPMI at required concentration
0.5 mL volume per reaction
2.4) transfer 0.5 mL per reaction to designated microcentrifuge tube(s)
2.5) incubate on ice for ≥ 10 min.

3) add 5 μL of 100x PMA/IM to designated tubes
4) vortex to mix
5) for non-stim. control, keep tube on ice
6) place stimulation tube(s) in 37°C water bath for 15 min.
7) continue immediately with assay

Indo-1 Assay

for measuring calcium flux in Jurkat cells upon TCR stimulation

Equipment:

- PTI fluorimeter, monochromator-based
  - *excitation: 355 nm*
  - *temperature control (37°C) for cuvette chamber*
  - *emission range: 350 - 550 nm (at 5 nm intervals)*
  - 2 independent PMTs
    for acquisition at 400 nm and 470 nm
- bucket with ice
- refrigerated centrifuge
- humidified, 37°C, 5% CO_2 incubator
- 37°C water bath
- 4-sided cuvette, 3 mL capacity
  - micro stir bar

Materials:

- Jurkat cells ($6x10^6$ cells per cuvette / condition)
- Indo-1 stock, 1,000x
  see Buffers & Solutions
  - stored at -20°C
- RPMI-1640
  - keep on ice
- 1 mM $CaCl_2$ / PBS
  - *calcium imaging buffer*
  - *see Buffers & Solutions*
  - keep on ice
- PBS
- Kimwipes
- OKT3
  anti-TCR antibody
- UPC10
  isotype control antibody
- OKT9
  control antibody
- 30 mM EGTA / 300 mM Tris-HCl, pH 7.4
  see Buffers & Solutions
  - equilibrate to 37°C before use
- 50 mL centrifuge tubes

Supporting Protocols:

- Cell Culture > Cell Counting
- Peptide > In Vitro Peptide Transduction

Protocol:

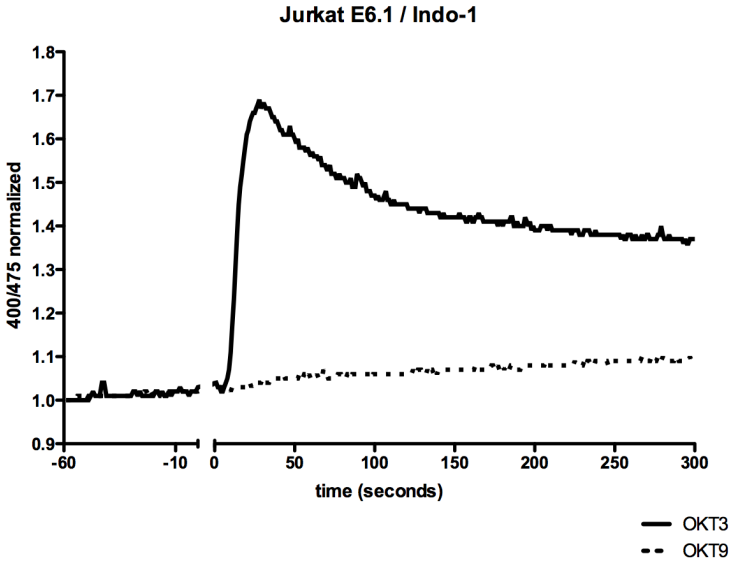


Figure 38: Indo-1 reporter of calcium flux in non-stimulated (OKT9) versus stimulated (OKT3) Jurkat cells as a function of time.

1) load Jurkat cells intracellularly with Indo-1
   - *need > 5x10^6 cells per cuvette / condition*
   - *prepare samples in triplicate*
   - *include non-Indo-1-loaded Jurkat cells to determine background autofluorescence*
   1.1) count cells
      see supporting protocol
   1.2) aliquot required number of cells to 50 mL centrifuge tube(s)
   1.3) centrifuge for 5 min. at 1,000 rpm (4°C)
   1.4) discard supernatant
   1.5) resuspend at 5x10^6 cells/mL in RPMI
   1.6) combine cell suspension into single 50 mL tube
   1.7) add Indo-1 at 1x
      2 µg/mL final
   1.8) place tube upright inside incubate for 30 min.
      1.8.1) loosen cap to allow gas exchange
2) wash cells
   to remove excess Indo-1
   2.1) centrifuge for 5 min. at 1,000 rpm (4°C)
   2.2) discard supernatant
   2.3) resuspend cells in equal volume of RPMI (5x10^6 cells/mL)
   2.4) return to incubator for 30 min. as before
      to allow cells to secrete excess Indo-1

3) optional: peptide treatment
   see supporting protocol
4) replace medium with calcium imaging buffer
   4.1) centrifuge cells for 5 min. at 1,000 rpm (4°C)
   4.2) aspirate / discard supernatant
   4.3) resuspend cells at $5x10^6$ cells/mL in ice-cold 1 mM $CaCl_2$ / PBS
   4.4) aliquot > $5x10^6$ cells to separate tubes for each cuvette / condition
   4.5) centrifuge cells for 5 min. at 1,000 rpm (4°C)
   4.6) keep pellet on ice until just before use
5) equilibrate to 37°C
   5.1) just prior to use, aspirate / discard supernatant
   5.2) resuspend $5x10^6$ cells in 2.5 mL of 1 mM $CaCl_2$ / PBS
      $2x10^6$ cells/mL final
   5.3) equilibrate for 10 min. in 37°C water bath
   5.4) wash cuvette with PBS between samples
   5.5) transfer $5x10^6$ cells / 2.5 mL to a 3 mL capacity 4-sided cuvette
      wash cuvette
   5.6) add micro stir bar
   5.7) place cuvette inside fluorimeter with gentle stirring for 3 min.
      to equilibrate to 37°C
   5.8) optional: add 250 μL of 30 mM EGTA / 300 mM Tris-HCl, pH 7.4
      to chelate extracellular calcium for investigation of intracellular calcium release
6) stimulation / data acquisition
   including non-stimulation control
   6.1) add OKT3 / control antibody directly to cuvette at 10 μg/mL final
   6.2) immediately begin acquiring data at 400 nm and 470 nm
7) data analysis
   7.1) in Excel, average traces from non-Indo-1-loaded cells to determine background autofluorescence
   7.2) subtract background from each experimental sample
   7.3) determine 400/470 nm ratio for each sample
   7.4) plot traces of 400/470 nm ratio for each sample in Prism and compare for each treatment
      non-stimulated, stimulated, peptide-treated and stimulated, etc.

In Vitro Kinase Assay

- use of a heterologous cell line for expression of Itk is important to avoid the possibility of dimerization and trans-phosphorylation via association with endogenous Itk
- since Itk is a substrate for auto-phosphorylation, no additional substrate is required to be added to the reaction for assessing kinase activity

Equipment:

- refrigerated centrifuge
- refrigerated microcentrifuge
- tube rotator
    - keep at 4°C
- 37°C water bath
- bucket with ice

Materials:

- HEK293 transfectants
- protein G sepharose beads
- kinase reaction buffer
    - prepared fresh (same day)
        see 'Buffers and Solutions'
    - keep on ice
- α-Itk antibody
    for immunoprecipitation
- microcentrifuge tubes
- α-phospho-tyrosine antibody
    for immunoblotting

Supporting Protocols:

- Cell Culture > HEK293 Cell Line Maintenance
- Cell Culture > HEK293 Transfection with Lipofectamine
- Protein Biochemistry > HEK293 Lysis
- Protein Biochemistry > Immunoprecipitation
    - Protein Biochemistry > Protein G Sepharose Preparation
- Protein Biochemistry > SDS-PAGE
- Protein Biochemistry > Immunoblotting
- Protein Biochemistry > Transblotting
- Protein Biochemistry > Immunoblotting

Protocol:

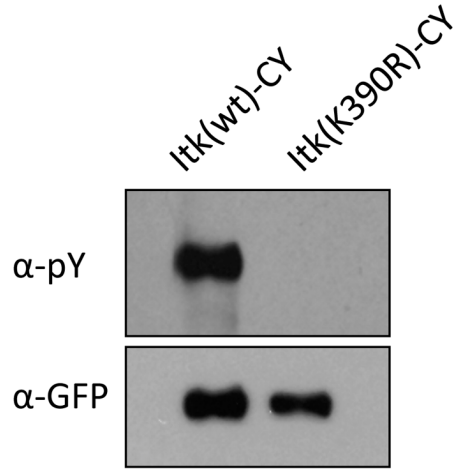


Figure 39: *In vitro* kinase assay of HEK293 cells transfected with Itk(WT)-CY and Itk(K390R)-CY.

Cellular lysates were prepared and incubated with 5 μg of anti-GFP followed by 30 μL of protein G sepharose. Immune complexes were washed, resuspended in kinase reaction buffer for 10 min. at 37°C, resolved by SDS-PAGE and analyzed by immunoblotting.

1) transfect HEK293 with kinase transgene
   see 'Cell Culture > HEK293 Transfection with Lipofectamine'
   1.1) incubate for 24 hours
2) lyse cells for 1 hr. on tube rotator
   see 'Protein Biochemistry > HEK293 Lysis'
3) incubate for 2 hrs. with immunoprecipitation antibody (α-Itk)
   see 'Protein Biochemistry > Immunoprecipitation'
4) incubate for 1 hr. with protein A/G sepharose beads
   see 'Protein Biochemistry > Immunoprecipitation'
5) wash immune complex in kinase reaction buffer
   5.1) gently aspirate supernatant using P1000 micropipette and tip
   5.2) resuspend pellet in 1 mL of ice-cold kinase reaction buffer
   5.3) centrifuge for 10 sec at 14,000 rpm in microcentrifuge
   5.4) gently remove supernatant using P1000 micropipette and tip
6) resuspend immune complex in 100 μL of ice-cold kinase reaction buffer
7) incubate for 10 min. in 37°C water bath
   to activate kinase
8) proceed immediately to resolve by SDS-PAGE
   see 'Protein Biochemistry > SDS-PAGE'

9) transfer to PVDF and detect by immunoblotting using anti-phopho-specific antibody

see 'Protein Biochemistry > Transblotting' and 'Protein Biochemistry > Immunoblotting'

Phosphoflow, Part I

stimulation, fixation and permeabilization

Equipment:

- refrigerated centrifuge
- vortex
- bucket with ice
- lab timer
- -20 / -80°C freezer

Materials:

- Jurkat cells (10^6 cells per staining tube / condition)
  - cultured to ≤ 10^6 cells/mL
  - serum starved overnight
- primary lymphocytes ($2x10^6$ cells per staining tube / condition)
  - freshly isolated
  - rested inside incubator for ≤ 4 hours prior to stimulation
    keep in 50 mL polypropylene tube (rather than polystyrene) to prevent loss of cell yield due to adherence
- FACS tubes, 5 mL
- 100% methanol (ACS grade)
  - keep on ice
- FACS buffer
  - keep on ice

Supporting Protocols:

- Cell Culture > Jurkat Maintenance
  - Cell Culture > Jurkat Serum Starvation
- In Vivo / Ex Vivo > Splenocyte Preparation
  - In Vivo / Ex Vivo > Tissue Dissociation
- Jurkat α-TCR Antibody Stimulation
- Splenocyte α-TCR Antibody Stimulation
- PMA / IM Stimulation

Protocol:

1) stimulate cells
   see supporting protocol(s) for details
2) fix cells
   2.1) immediately following stimulation, add equal volume (0.5 mL) of ice-cold 2% paraformaldehyde to each tube
   2.2) vortex / mix
   2.3) incubate for 10 min. on ice
   2.4) centrifuge for 5 min. at 1,000 / 1,500 rpm (4°C)
   2.5) decant to remove supernatant

retain residual volume

3) permeabilize

perform in microcentrifuge tubes

3.1) vortex to resuspend pellet in residual volume

3.2) add 1 mL of ice-cold methanol (ACS grade) to each tube

add methanol to cells in suspension rather than to undisturbed pellet to reduce clumping

3.3) vortex to mix

3.4) incubate for ≥ 20 min. on ice

4) store at -20°C overnight or at -80°C for several months if needed

References:

(142)

Phosphoflow, Part II

blocking and staining

Equipment:

- refrigerated centrifuge
- vortex
- -20 / -80°C freezer
- flow cytometer for analysis
- bucket with ice
- lab timer

Materials:

- Jurkat cells (10^6) / primary lymphocytes ($2x10^6$)
  cell number per tube / staining condition
  - stimulated
  - fixed
  - permeabilized
  - stored at -20 / -80°C in microcentrifuge tube(s)
- FACS tubes, 5 mL
- FACS buffer
  - keep on ice
- blocking antibody
  select to match species of phospho-specific antibody
  0.22 mg/mL final
  - mouse gamma globulin
    Jackson ImmunoResearch, Cat. # 015-000-002
    11.0 mg/mL
    - dilute 1:10 in FACS buffer to prepare 1.1 mg/mL stock
- phospho-specific staining antibodies
  - determine optimal staining concentration from titration experiment (e.g., 0.3 µg/mL final)
  - protect from light
  - PE mouse α-phosphotyrosine
    BD, Cat. # 558008
  - Alexa Fluor 647 mouse α-ERK1/2 pT202/pY204
    BD, Cat. # 612593
    1.5 µg/mL
  - PE-Cy7 mouse α-ERK1/2 pT202/pY204
    BD, Cat. # 560116
  - Alexa Fluor 647 mouse α-Itk pY511
    BD, Cat. # 558134
    1.5 µg/mL
  - PE mouse α-Itk pY511
    BD, Cat. # 558129
    1.5 µg/mL

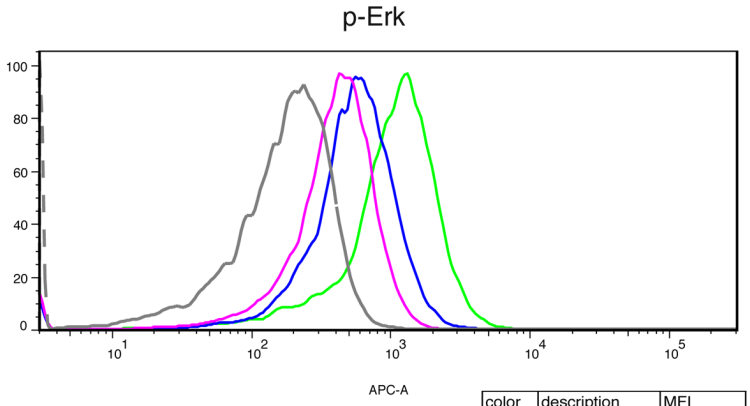
- Alexa Fluor 488 mouse α-PLCγ1 pY783
  BD, Cat. # 557884
- Alexa Fluor 647 mouse α-human PLCγ1 pY783
  BD, Cat. # 557883

- isotype control staining antibodies
  - dilute in FACS buffer to match concentration of phospho-specific antibody
  - protect from light
  - Alexa Fluor 488 mouse IgG1
    Invitrogen, Cat. # MG120
    0.1 mg/mL
  - Alexa Fluor 647 mouse IgG1
    Invitrogen, Cat. # MG121
    0.1 mg/mL
  - PE mouse IgG1
    Invitrogen, Cat. # MG104
    0.1 mg/mL
  - PE-Cy7 mouse IgG1
    Invitrogen, Cat. # MG112
    0.1 mg/mL
- surface marker antibodies
  protect from light
  - PE anti-mouse TCR-β, clone H57-597
    BioLegend, Cat. # 109208
    0.2 mg/mL
  - PB anti-mouse CD4
    BioLegend, Cat. # 100531, clone RM4-5
    0.5 mg/mL

Supporting Protocol:

- Phosphoflow, Part I

Protocol:



color	description	MFI
	isotype	193.6
	non-stim.	452.7
	1" OKT3	639.7
	5" OKT3	1226.4

Figure 40: TCR and time-dependent Erk activation in Jurkat cells.

1) plan experiment
    1.1) determine spectral compatibility among phospho-specific and surface marker antibodies (if required)
    1.2) determine ability of instrument to excite / detect each fluorophore
    1.3) prepare spreadsheet listing staining conditions (including relevant controls) for each tube
        100 µL final volume per tube (80 µL of cell suspension + 20 µL of 5x antibody solution)
    1.4) prepare 5x master mix for tubes receiving common antibodies
        to reduce variation in staining
        1.4.1) dilute in FACS buffer as needed
2) prepare cells
    complete through FACS analysis from this point forward
    2.1) remove cells from freezer
    2.2) vortex microcentrifuge tube to resuspend
    2.3) transfer 1-2x10^6 cells to designated FACS tubes
3) wash cells 3x
    3.1) add 3 mL of FACS buffer (wash)
        volume of FACS buffer should be ≥ 2x methanol volume to prevent precipitation of BSA
    3.2) vortex / mix
    3.3) centrifuge for 5 min. at 1,000 / 1,500 rpm (4°C)
    3.4) decant / discard supernatant
    3.5) repeat for a total of 3 washes
4) block cells

4.1) add 80 μL of FACS buffer to tube
4.2) vortex / resuspend
4.3) transfer 80 μL of cell suspension to new FACS tube
equivalent volumes needed for consistent blocking
4.4) add 20 μL of 1.1 mg/mL blocking antibody to each tube
100 μL final volume per tube
4.5) vortex / mix
4.6) incubate for 30 min - 1 hr. at room temp.

5) wash cells 2x
5.1) add 3 mL of FACS buffer (wash)
5.2) vortex / mix
5.3) centrifuge for 5 min. at 1,000 / 1,500 rpm (4°C)
5.4) decant / discard supernatant
5.5) repeat for a total of 2 washes

6) stain cells
6.1) add 80 μL of FACS buffer to tube
6.2) vortex / resuspend
6.3) transfer 80 μL of cell suspension to new FACS tube
equivalent volumes needed for consistent staining
6.4) add 20 μL of 5x antibody master mix including the following components:
- surface marker antibody (or isotype control), if required
- phospho-specific antibody (or isotype control)
- 100 μL final volume per tube
6.5) vortex / mix
6.6) incubate for 30 min. - 1 hr. at room temp. in dark

7) wash cells 2x
7.1) add 3 mL of FACS buffer (wash)
7.2) vortex / mix
7.3) centrifuge for 5 min. at 1,000 / 1,500 rpm (4°C)
7.4) decant / discard supernatant
7.5) repeat for a total of 2 washes

8) add 300 μL of FACS buffer per tube
9) vortex / mix
10) acquire FACS data
complete within same day
10.1) acquire ≤ 50,000 events per tube
11) analyze FACS data
11.1) calculate ratio of MFI of stimulated to non-stimulated control cells stained with the same phospho-specific antibody

References:

(142)

5.2.7. Protein Biochemistry

Jurkat / Primary Lymphocyte Lysis

proceed immediately following stimulation

Equipment:

- refrigerated centrifuge
- refrigerated microcentrifuge
- tube rotator
    - keep at 4°C
- microcentrifuge tube rack
    for assistance in lysis
- bucket with ice

Materials:

- 15 / 50 mL centrifuge tubes
- Jurkat cells / primary lymphocytes, required number per microcentrifuge tube
    keep on ice
    - stimulated / non-stimulated
    - transfected / non-transfected
- complete NP-40 lysis buffer (CLB)
    - including protease inhibitors
    - keep on ice
- NP-40 lysis buffer
    - keep on ice
- laemmli sample buffer (LB), 2x

Supporting Protocol:

- Cell Culture > Jurkat Maintenance
- Cell Culture > Jurkat Serum Starvation

Protocol:

- perform on lab bench
- keep cells at 4°C during and following lysis

1) centrifuge cells for 5 min. at 1,000 (Jurkat cells) / 1,500 (primary lymphocytes) rpm for 5 min (4°C)
2) aspirate / discard supernatant
3) resuspend pellet in 500 μL of ice-cold complete NP-40 lysis buffer
4) scratch tube against microcentrifuge tube rack to disrupt / lyse cells
5) rotate for at least 1 hour to complete lysis
6) centrifuge for 20 min. at 14,000 rpm in refrigerated microcentrifuge; pellet contains cellular debris, 'lysate' refers to cytoplasmic proteins contained in supernatant
7) for resolving by SDS-PAGE:

7.1) aliquot 10 μL of lysate to new microcentrifuge tube
7.2) add 10 μL of 2x laemmli sample buffer, reduces proteins
7.3) mix well, lysate samples can be stored overnight at 4°C
8) for immunoprecipitation, proceed immediately with protocol

HEK293 Lysis

Equipment:

- refrigerated centrifuge
- refrigerated microcentrifuge
- tube rotator
    - keep at 4°C
- microcentrifuge tube rack
    for assistance in lysis
- bucket with ice

Materials:

- HEK293 transfectants, culture plate(s) of required size and confluency
- PBS, molecular biology grade
    - keep on ice
- transfer pipets
- cell scrapers
- microcentrifuge tube
- complete NP-40 lysis buffer (CLB)
    - including protease inhibitors
    - keep on ice
- laemmli sample buffer (LB), 2x

Supporting Protocols:

- Cell Culture > HEK293 Maintenance
- Cell Culture > HEK293 Transfection

Protocol:

- protocol assumes 10 cm diameter (60 cm^2) cell culture plate
- scale volumes proportionally for various-sized culture vessels based on surface area
- perform on lab bench
- keep cells at 4°C during and following lysis

1) 18-48 hours post-transfection, remove plate(s) from incubator
2) decant medium
3) wash cells 2x with 10 mL of cold PBS
4) aspirate excess PBS
5) add 500 μL of ice-cold complete NP-40 lysis buffer
6) use cell scraper to aid in cell detachment and lysis
7) transfer cell lysate to labeled microcentrifuge tube
8) scratch tube against microcentrifuge tube rack to disrupt / lyse cells
9) rotate for at least 1 hour to complete lysis

10) centrifuge for 20 min. at 14,000 rpm in refrigerated microcentrifuge; pellet contains cellular debris, 'lysate' refers to cytoplasmic proteins contained in supernatant
11) for resolving by SDS-PAGE:
    11.1) aliquot 10 μL of lysate to new microcentrifuge tube
    11.2) add 10 μL of 2x laemmli sample buffer, reduces proteins
    11.3) mix well; lysate samples can be stored overnight at 4°C
12) for immunoprecipitation, proceed immediately with protocol

Protein G Sepharose Preparation

Equipment:

- biosafety cabinet (TC hood)
- microcentrifuge

Materials:

- Protein G Sepharose 4 Fast Flow, 5 mL
  - *GE Healthcare, Cat. # 17-0618-01*
  - *supplied in 20% ethanol storage buffer*
  - store at 4°C
- NP-40 lysis buffer
- microcentrifuge tubes, sterile
- parafilm

Protocol:

work inside TC hood to prevent contamination

1) resuspend stock well
2) aliquot 0.5 mL per microcentrifuge tube
3) wash 3x
   3.1) resuspend in 1 mL of NP-40 (wash #1)
   3.2) centrifuge at 12,000 g (max speed) for 30 seconds
   3.3) aspirate / discard supernatant
4) estimate volume of pellet and resuspend in equal volume of NP-40 lysis buffer to prepare 50% slurry
5) seal cap with parafilm to reduce moisture loss during storage
6) store at 4°C
7) mix well before use

Immunoprecipitation (IP)

Equipment:

- vortex
- tube rotator
  - keep at 4°C
- microcentrifuge

Materials:

- cellular lysate stored in microcentrifuge tube
  - keep on ice
- Complete Lysis Buffer (CLB), 1x
  - keep on ice
- laemmli sample buffer (LB), 2x
- pre-clearing antibody
  normal isotype of species used for IP
- IP antibody
- NP-40, 1x

Supporting Protocol:

- Lysis
- Protein G Sepharose Preparation

Protocol:

proceed immediately following preparation of cellular lysate

1) pre-clear lysate (optional)
   1.1) add 5 μg of species-specific IgG to new microcentrifuge tube
   1.2) add complete volume of lysate
   avoid removing pellet
   1.3) tumble for ~2 hours on tube rotator (4°C)
   if required, incubate overnight
   1.4) add 30 μL of protein sepharose (A/G) to each tube
   sepharose settles quickly – vortex stock prior to use
   1.5) tumble for 1 hour on tube rotator (4°C)
   1.6) centrifuge for 10 sec at 14,000 rpm in microcentrifuge
   - pellet contains non-specific immune complex
   - supernatant contains pre-cleared lysate (retain)
2) incubate with IP antibody
   2.1) add 5 μg of IP antibody to separate microcentrifuge tube
   2.2) add complete volume of lysate; avoid removing pellet
   2.3) tumble for ≥ 2 hours on tube rotator (4°C)
   for convenience, incubate overnight

3) incubate with protein sepharose A/G
    3.1) add 30 μL of protein sepharose A/G to IP sample
        sepharose settles quickly – vortex stock prior to use
    3.2) tumble for ~2 hours on tube rotator (4°C)
        if required, incubate overnight
    3.3) centrifuge for 10 sec at 14,000 rpm in microcentrifuge
        pellet contains immune complex
4) wash immune complex (optional)
    4.1) gently aspirate supernatant using P1000 micropipette and tip
    4.2) resuspend pellet in 1 mL of 1x NP-40 lysis buffer (wash #1)
    4.3) centrifuge for 10 sec at 14,000 rpm in microcentrifuge
    4.4) repeat for a total of 2 washes
    4.5) centrifuge for 10 sec at 14,000 rpm in microcentrifuge
    4.6) gently remove supernatant using P1000 micropipette and tip
5) for resolving by SDS-PAGE, reduce immune complex
    5.1) add 10 μL of 2x laemmli sample buffer to pellet in tube containing immune complex
    5.2) continue immediately with SDS-PAGE protocol

Acrylamide Gel Preparation

Equipment:

- glass base for gel assembly
- gel apparatus
  assemble in sequential order (listed from back to front)
  - gel backing
  - alumina plate
    select correct size
  - spacers, 2 each
    select correct thickness
  - glass plate
    select correct size
  - clamps, 4 each
  - comb
    select correct thickness
- dI water squirt bottle
- vortex
- refrigerator

Materials:

- 70% ethanol squirt bottle
- Acrylamide Gel Buffer, 10 mL
  - for plug and running gel
  - see 'Buffers & Solutions'
  - select required % acrylamide gel needed for resolution of protein(s) of interest based on molecular weight
  - prepare fresh
- APS, 10%
  - polymerizing agent
  - see 'Buffers & Solutions'
  - prepare fresh
- TEMED
  - polymerizing agent
  - see 'Buffers & Solutions'
- Stacking Gel, 5 mL
  see 'Buffers & Solutions'
  - prepare fresh
- blotting paper
- microcentrifuge tubes
- Whatman filter paper
- 1x Running Buffer
  see 'Buffers & Solutions'
- plastic wrap

Premium Saran

Protocol:

1) clean components of gel apparatus with 70% ethanol solution
2) assemble gel apparatus (see 'Equipment' above for details)
3) prepare Acrylamide Gel Buffer and Stacking Gel Buffer (see 'Buffers & Solutions')
4) prepare gel plug
   proceed immediately after adding polymerizing agents
   - 4.1) transfer 1 mL of Acrylamide Gel Buffer to microcentrifuge tube
     - 4.1.1) add 10 μL of 10% APS
     - 4.1.2) add 5 μL of TEMED
   - 4.2) vortex
   - 4.3) pipet 1 mL between alumina and glass plates
     add 50% volume to each side of apparatus
   - 4.4) allow ~ 5 mins. to polymerize
5) cast running gel
   - 5.1) add to remaining 9 mL of Acrylamide Gel Buffer:
     to polymerize gel
     - 5.1.1) 45 μL of 10% APS
     - 5.1.2) 9 μL of TEMED
   - 5.2) vortex
   - 5.3) pipet 6-7 mL between alumina and glass plates
     add 50% volume to each side of apparatus
   - 5.4) add dI H_2O above stacking gel to top of alumina plate
     applies pressure to form horizontal line across gel
   - 5.5) allow ~ 30 mins. to polymerize
6) wick away dI H_2O above gel using folded Whatman filter paper
7) cast stacking gel
   - 7.1) add to 5 mL of Stacking Gel
     to polymerize gel
     - 7.1.1) 25 μL 10% APS
     - 7.1.2) 10 μL TEMED
   - 7.2) pipet to top of alumina plate
   - 7.3) add gel comb
     select comb with appropriate thickness to ensure tight fit for proper well formation
   - 7.4) allow ≤ 30 mins. to polymerize completely
8) add 1x Running Buffer to apparatus between gel backing and alumina plate
9) for short term storage, wrap gel apparatus in plastic wrap
   to retain moisture
   - 9.1) keep in refrigerator for 2-3 days

SDS-PAGE

sodium dodecyl sulfate – polyacrylamide gel electrophoresis

Equipment:

- gas line
- bunsen burner and stand
- gel base
- power supply for gel apparatus
  limits: 500 V, 40 mA, 10 W
  run at 12-18 mA
- vortex

Materials:

- beaker containing tap water and boiling chips
- clamps for microcentrifuge tubes
- lysate samples
  - keep at 4°C
- IP samples
  proceed immediately following IP protocol
- protein ladder
  dual colored for identification of protein of interest
- acrylamide gel
  see supporting protocol for details
  - prepared fresh or stored in refrigerator
- 1x Running Buffer

Supporting Protocols:

- Lysis
- Immunoprecipitation
- Acrylamide Gel Preparation

Protocol:

1) denature lysate and/or IP samples
   1.1) boil water in beaker containing boiling chips
      1.1.1) pre-heat for 10 min. in microwave oven
      1.1.2) bring to complete boil using bunsen burner
   1.2) apply clamps to microcentrifuge tubes
      prevents sample loss due to caps popping
   1.3) boil lysate and IP samples for 5 mins.
   1.4) allow tubes to cool before removing caps
   1.5) centrifuge all tubes for 30 sec at 14,000 rpm
      for IP samples:
      - sepharose beads contained in pellet

- IP antibody, target and associated proteins contained in supernatant

2) assemble gel apparatus and base
    2.1) insert completely assembled gel apparatus to base
        adjust position of lower clamps to facilitate
    2.2) add 1x Running Buffer to apparatus between gel backing and alumina plate
    2.3) add 1x Running Buffer to gel base
    2.4) remove comb when completely submerged in buffer
    2.5) add protein ladder and samples to designated wells
    2.6) connect wires from power supply to gel apparatus
    2.7) program settings for power supply
        limits: 500 V, 40 mA, 10 W
3) run gel
    3.1) run gel at 12-18 mA
    3.2) monitor gel for leaks
        if necessary, add 1x Running Buffer
    3.3) after achieving desired separation, turn off power to gel apparatus
    3.4) detach gel apparatus from base
    3.5) remove backing from gel apparatus
    3.6) discard Running Buffer in sink
    3.7) proceed immediately with trans-blotting protocol

Trans-blotting

Equipment:

- trans-blotter apparatus
  - base (negative electrode)
  - top (positive electrode)
  - wires for connection to power supply
- power supply for trans-blotter apparatus
  volts: 14
  limit: 0.4 mA
  timer: 1.0 hr
- glass baking dish
- blotting tray

Materials:

- acrylamide gel apparatus
  proceed immediately following electrophoresis
- PVDF membrane
- methanol, molecular biology grade
- Western Transfer Buffer
- blotting paper

Supporting Protocols:

- SDS-PAGE

Protocol:

1) activate PVDF membrane
   1.1) soak PVDF membrane in methanol (in blotting tray)
   1.2) discard methanol
   1.3) wash with dI H_2O
2) soak 2 pieces of blotting paper and PVDF membrane in Western Transfer Buffer in glass baking dish
3) assemble trans-blotter apparatus in order listed below (from bottom to top)
   - keep all layers saturated in WTB
   - roll each layer with glass rod to remove air bubbles
   3.1) add blotting paper to base of trans-blotter (negative electrode)
   3.2) add acrylamide gel
      3.2.1) flip upside down
      take extreme care removing gel from apparatus to prevent tearing
   3.3) add PVDF membrane
   3.4) add blotting paper

3.5) attach / tighten top of trans-blotter (positive electrode)

4) connect wires from power supply to trans-blotter apparatus

5) program settings for power supply

volts: 14

limit: 0.4 mA

timer: 1.0 hr – will stop automatically

6) remove PVDF membrane

confirm transfer of colored ladder to PVDF membrane

7) store membrane in drawer between two pieces of dry blotting paper until immunoblotting

no longer need to keep membrane wet after transfer

Immunoblotting (IB)

Equipment:

- developer (in dark room)
- rocker platform
- lab timer

Materials:

- PVDF membrane containing transferred proteins
- ballpoint pen
- ultrafine point Sharpie marker
- 1° antibody
  epitope-specific
- 2° antibody, HRP-conjugated
  specific for species and isotype of 1° antibody
- Blocking Buffer
  see 'Buffers & Solutions'
- Blotting Solution
  see 'Buffers & Solutions'
- blotting tray
- ECL (enhanced chemiluminescent) substrate
  Thermo Scientific, Cat. # 34080
- film folder
- film, 8 x 10"
  - protect from light
- forceps
- methanol, molecular biology grade
- plastic wrap
  Premium Saran
- razor blade
- scissors
- TBST
  see 'Buffers & Solutions'
- Thimerasol

Supporting Protocol:

- Trans-blotting

Protocol:

- plan order of serial blotting (e.g., phosphotyrosine blotting followed by loading control)
- once hydrated, keep membrane wet throughout protocol
- equilibrate buffers to room temperature prior to use

1) prepare membrane
    make annotations using ballpoint pen
    1.1) mark location of protein ladder bands
    1.2) write initials and date on edge of membrane
    1.3) wet membrane with methanol (in blotting tray)
    1.4) wash 3x with dI H_2O
2) blocking
    2.1) add 15 mL of Blocking Buffer
    2.2) incubate for ~1 hour with gentle rocking
    2.3) discard Blocking Buffer
3) blotting with 1° antibody, epitope-specific
    3.1) dilute antibody in Blotting Solution
    3.2) add antibody solution to membrane
        3.2.1) retain 15mL Falcon tube for storage
    3.3) incubate for 1 hour with gentle rocking
    3.4) transfer antibody solution to original tube for re-blotting (if needed)
4) wash, repeat 3x
    4.1) add TBST
    4.2) incubate for 5 min. with gentle rocking
    4.3) discard TBST
5) blotting with 2° antibody, HRP-conjugated
    5.1) dilute antibody in Blotting Solution
    5.2) add antibody solution to membrane
    5.3) incubate for 30 minutes with gentle rocking
6) wash, repeat 3x
    6.1) add TBST
    6.2) incubate for 10 min. with gentle rocking
    6.3) discard TBST
7) react with ECL substrate
    7.1) add 3mL of solution 1
    7.2) add 3mL of solution 2
    7.3) mix briefly, allow sufficient contact with membrane
    7.4) transfer membrane to plastic wrap (use forceps)
    7.5) wrap membrane; trim excess plastic using razor blade
8) develop film in dark room
    8.1) bring membrane, film, scissors, timer and film folder
    8.2) turn on developer and allow to warm-up prior to use
    8.3) cut film in half along length
    8.4) clip upper-left corner of film
        for identification of orientation relative to membrane
    8.5) place film directly above membrane and close lid of folder
    8.6) expose film for various time intervals
        e.g., 10 seconds, 1, 5, 10 minutes, etc.

8.7) insert film in developer
8.8) label film with initials, date, blotting antibody, and exposure time
use ultrafine point Sharpie marker
8.9) scan and catalog for analysis and archival

9) serial blotting
9.1) wash membrane 3x for 10 min. with TBST
to remove luminescent product/signal
9.2) for strong signal, wash overnight on rocker
9.3) expose film for various time intervals to verify removal of signal
if necessary, strip membrane
9.4) repeat blotting with different 1° antibody and continue with protocol

Detection of Itk Activation by Immunoblotting

Equipment:

- humidified, 5% CO_2, 37°C incubator
- tube rotator
- bucket with ice
- lab timer

Materials:

- Jurkat cells (20x10^6 cells per tube / condition)
  - serum starved overnight
    see supporting protocol
  - stimulated via TCR (and non-stim. control)
    see supporting protocol
  - stimulated with PMA/IM (and non-stim. control)
    see supporting protocol
- primary lymphocytes (60-80x10^6 cells per tube / condition)
  - freshly isolated
  - rested inside incubator for ≤ 4 hours prior to stimulation
    keep in 50 mL polypropylene tube (rather than polystyrene) to prevent loss of cell yield due to adherence
  - stimulated via TCR (and non-stim. control)
    see supporting protocol
  - stimulated with PMA/IM (and non-stim. control)
    see supporting protocol
- normal rabbit IgG
  pre-clear antibody
- protein G sepharose (PGS)
- rabbit anti-Itk
  immunoprecipitation antibody
- 4G10
  anti-phosphotyrosine immunoblotting antibody
- 2F12
  anti-Itk immunoblotting antibody
- HRP Goat Anti-Mouse
  detection antibody
- ECL substrate

Supporting Protocols:

- T Cell Stimulation / Itk Activation > Jurkat α-TCR Antibody Stimulation
- T Cell Stimulation / Itk Activation > Primary Lymphocyte α-TCR Antibody Stimulation
- Jurkat / Primary Lymphocyte Lysis
- Immunoprecipitation

- SDS-PAGE
- Immunoblotting

Protocol:

1) lyse cells immediately following stimulation
2) pre-clear lysate
   see supporting protocol
   2.1) add 5 μg of normal rabbit IgG per tube
   2.2) incubate on tube rotator for 2 hrs. at 4°C
   2.3) add 30 μL of protein G sepharose
   2.4) incubate on tube rotator for 1 hr at 4°C
3) immunoprecipitate
   see supporting protocol
   3.1) add 5 μg of rabbit anti-Itk to each tube
   3.2) incubate overnight (~16 hrs.) on tube rotator at 4°C
   3.3) add 30 μL of protein G sepharose
   3.4) incubate for 1 hr. on tube rotator at 4°C
4) resolve immune complex by SDS-PAGE
   see supporting protocol
5) transfer to PVDF membrane
   see supporting protocol
6) block membrane for 1 hour at room temp on rocker
7) immunoblot for phosphotyrosine
   7.1) incubate with 4G10 at 1:1,000 for 1 hr at room temp.
   7.2) wash 3x for 5 min each
   7.3) incubate with HRP Goat Anti-Mouse at 1:5,000 for 30 min. at room temp.
   7.4) wash 3x for 10 min each
8) wash membrane 3x for 30 min each; expose film for ~1 min to confirm removal of signal
   wash overnight if necessary
9) immunoblot for Itk (loading control)
   9.1) incubate with 2F12 at 1:1,000 for 1 hr at room temp.
   9.2) wash 3x for 5 min each
   9.3) incubate with HRP Goat Anti-Mouse at 1:5,000 for 30 min. at room temp.
   9.4) wash 3x for 10 min each

References:

(2)

5.2.8. Microscopy / FACS

Surface Staining for FACS Analysis

Equipment:

- microcentrifuge
- bucket with ice
- refrigerator
- refrigerated centrifuge
- vortex
- waste container
    - containing 10% bleach when full
- lab timer
- flow cytometer and computer with BD FACS Diva installed
    e.g., Canto or Aria

Materials:

- single cell suspension
    keep on ice
    - cell line
    - primary tissue
        spleen, lymph nodes, lung or thymus
        - lyse RBCs
            except from lung tissue
- FACS tubes
- microcentrifuge tubes
- 2% paraformaldehyde / PBS
    - keep on ice
- FACS buffer
    - keep on ice
- anti-CD16 / CD32
    Fc blocking antibody
- fluorescently-labeled antibodies (including isotype control antibodies)
    - quick spin vial to recover maximum volume
    - protect from light
- Kimwipes
- CST beads
- dI water

Supporting Protocol:

- Cell Culture > Cell Counting

Protocol:

1) prepare spreadsheet listing each tube / sample and staining antibodies required
    including non-stained and single-color control samples

1.1) for each fluorescently-labeled antibody, check flow cytometer for available laser for excitation and detection filter
1.2) if performing multi-color staining, check fluorescent spectra for compatibility

2) prepare cell suspension
10^6 cells per FACS tube / staining condition
2.1) count cells
see supporting protocol
2.2) centrifuge for 5 min. at 1,500 rpm
2.3) discard supernatant
2.4) resuspend in FACS buffer at 20x10^6 cells/mL
2.5) for primary cells, incubate with 0.5-1 μg of anti-CD16 / CD32 for 5-10 min. on ice prior to staining
to block Fc receptors
2.6) aliquot 50 μL (10^6 cells) to designated FACS tubes

3) prepare staining antibody solution
0.03-2 μg of antibody per 10^6 cells
3.1) dilute to 0.6 - 40 μg/mL in FACS buffer
titrate antibody to determine optimal concentration
3.1.1) prepare common batch for identical staining conditions
3.2) aliquot 50 μL (0.03-2 μg) to designated FACS tubes

4) vortex to mix

5) incubate for 20-45 min. in dark on ice or at 4°C in refrigerator

6) wash cells
to remove unbound antibody
6.1) add 2 mL of FACS buffer to each tube
6.2) vortex (wash)
6.3) centrifuge for 5 min. at 1,000-1,500 (4°C)
6.4) decant supernatant to waste container
6.4.1) wick away excess supernatant using Kimwipe
6.5) repeat for a total of 2-3 washes

7) optional: fix cells
7.1) add 100 μL of ice-cold 2% paraformaldehyde/PBS to each tube
7.2) vortex to resuspend
7.3) incubate for 10 min. in dark on ice or at 4°C in refrigerator

8) wash cells
to remove paraformaldehyde
8.1) add 2 mL of FACS buffer to each tube
8.2) vortex (wash)
8.3) centrifuge for 5 min. at 1,000-1,500 (4°C)
8.4) decant supernatant to waste container

8.4.1) wick away excess supernatant using Kimwipe
8.5) repeat for a total of 2-3 washes
9) add 300-500 μL of FACS buffer to each tube
10) vortex to resuspend
11) store on ice or at 4°C in refrigerator until FACS analysis
- for live cells, acquire FACS data immediately after staining
- for fixed cells, acquire FACS data within 2-3 days
12) acquire FACS data
12.1) perform CST calibration prior to running samples
12.1.1) add dI water to FACS tube
12.1.2) add 2 drops of CST beads
12.1.3) vortex
12.1.4) run on instrument
12.2) start stream
check for proper instrument performance
12.3) setup experiment / tubes
12.4) set up parameters / voltages
12.5) vortex each tube prior to loading
12.6) identify cell population on FSC, SSC dot plot
12.7) adjust flow rate as needed
12.8) identify fluorescent populations on respective dot plots
adjust voltages if needed
12.9) collect ≥ 10,000 events per sample

Viability Assay with 7-AAD

viability exclusion dye

Equipment:

- bucket with ice
- refrigerated centrifuge
- vortex
- waste container
    - containing 10% bleach when full
- lab timer
- flow cytometer
    for analysis

Materials:

- cells
    - stained for surface markers
- FACS tubes
- FACS buffer
    - keep on ice
- 7-AAD, 100x
    - *BioLegend, Cat. # 420404*
    - *detect in PerCP channel:*
        - *ex: 488 nm*
        - *em: 682/33 nm*
    - *can be used in conjunction with FITC and/or PE*
    - stored at 4°C in dark

Supporting Protocol:

- Surface Staining for FACS Analysis

Protocol:

perform in FACS tubes

1) wash cells
    1.1) add 1 mL FACS buffer to each tube
    1.2) vortex / wash
    1.3) centrifuge for 5 min. at 1,000 / 1,500 rpm (4°C)
    1.4) discard supernatant
2) resuspend ~10^6 cells in 100 μL of ice-cold FACS buffer
3) add 1 μL of 100x 7-AAD (1x final)
4) incubate on ice for 10 min. in dark
5) wash cells
    5.1) add 1 mL FACS buffer to each tube
    5.2) vortex / wash

5.3) centrifuge for 5 min. at 1,000 / 1,500 rpm (4°C)
5.4) discard supernatant
6) resuspend cells in 300 μL of ice-cold FACS buffer
7) proceed immediately with FACS analysis
do not fix with paraformaldehyde

Coating Microscopy Slides / Cover Glass with Poly-L-Lysine

for attachment of cells to glass slides or cover glass

Equipment:

- waste container
  - containing 10% bleach when full

Materials:

- 0.01% (w/v) poly-L-lysine (pLL) / H_2O
  see 'Buffers & Solutions'
- microscope slides, 25 x 75 x 1 mm
  Fisher, Cat. # 12-544-7
- for cover glass method:
  - cover glass, 18 x 18 x 1 mm
    Fisher, Cat. # 12-548-A
  - tissue culture dishes, 35 x 10 mm
    BD, Cat. # 353001
- ultrapure water

Protocol:

prepare excess number of slides prior to experiment

1) for coating slides:
   1.1) add 50 μL of 0.01% pLL / H_2O per slide
   1.2) spread evenly in center area using pipet tip
2) for coating cover glass:
   2.1) add 1 cover glass per culture dish
   2.2) add 1 mL of 0.01% pLL / H_2O to each dish
   make sure cover glass is completely covered
   2.3) discard pLL
3) incubate at room temp. for ≤ 30 min.
4) wash 3x with ultrapure water
5) allow to dry completely (≤ 1 hr.)

Growing / Transfecting HEK293 on Cover Glass

Equipment:

- humidified, 37°C, 5% CO_2 incubator
- biosafety cabinet (TC hood)
- waste container
  for decontamination of discarded culture
  - containing 10% bleach when full
- lab timer

Materials:

- cover glass (inside culture dishes)
  see supporting protocol for details
  - coated with poly-L-lysine
- microscopy slides, 25 x 75 x 1 mm (1 per cover glass)
  Fisher, Cat. # 12-544-7
- straight-edge razor
  for removing cover glass from culture dishes
- optional: 35 mm MatTek culture dishes
  contain embedded cover glass
- 293 Wash Medium
- 293 Transfection Medium
- 293 Growth Medium
- 293 Starvation Medium
- microcentrifuge tube (1 per transfection)
- plasmid DNA
- PBS
- 2% paraformaldehyde / PBS
  - keep on ice
- mounting medium
  Invitrogen, Cat. # P7481
  - pre-warm to 37°C before use
- nail polish
- slide folder

Supporting Protocol:

- Coating Microscopy Slides / Cover Glass with Poly-L-Lysine

Protocol:

1) seed dish
   1.1) add 2 mL of 293 Growth Medium to each dish
   1.2) add ~250,000 cells
   1.3) incubate until cover glass is 50-80% confluent
2) prepare DNA-Lipofectamine complexes

prepare separately for each dish

2.1) add 100 μL of 293 Wash Medium to microcentrifuge tube
2.2) add 1 μg of DNA to tube
2.3) add 10 μL of Lipofectamine 2000 to tube
2.4) incubate for 45 min. at room temp.

3) transfect cells
3.1) add 800 μL of 293 Wash Medium to tube containing DNA-Lipofectamine complexes
dilute just prior to adding to cells
3.2) add diluted DNA-Lipofectamine complexes to cells grown in dish
3.3) return dish to incubator for 1 hr.

4) replace medium
4.1) discard medium following transfection
to remove excess DNA-Lipofectamine complexes
4.2) add 2 mL of fresh 293 Growth Medium
4.3) return dish to incubator for 24 hrs.

5) optional: serum starve
5.1) discard medium
5.2) add 2 mL of fresh 293 Starvation Medium
5.3) return to incubator for 3 hrs.

6) wash with PBS
6.1) discard medium
6.2) add 2 mL of PBS
6.3) discard PBS

7) fix
7.1) add 100 μL of 2% paraformaldehyde / PBS
7.2) wait 15 min.

8) wash with PBS as before

9) remove cover glass from bottom of dish

10) apply mounting medium / seal slides
10.1) add 10 μL of mounting medium directly above cells
10.2) invert cover glass with attached cells onto slide
10.3) seal coverslip at each corner with nail polish
do not seal along edges yet
10.4) allow mounting medium to cure overnight at room temp. in dark
10.5) seal edges of cover glass using nail polish

11) store slides at 4°C in slide folder

Antibody Adsorption to Microspheres

for localization / conjugation assays

Equipment:

- tube rotator
- deli fridge
- bench-top microcentrifuge
- hemacytometer

Materials:

- 6 μm diameter polystyrene (PS) microspheres
  - *Polysciences, Cat. # 07312*
  - *2.59% (w/v) stock (25.9 mg/mL)*
  - *provided in water without preservatives*
  - *1 year shelf life*
  - *use aseptic technique*
- microcentrifuge tubes, sterile
- antibody for adsorption
  stock solution in buffer without carrier protein (e.g., BSA)
  - OKT3
  - OKT9
  - UPC10
- FITC-conjugated secondary antibody
  for staining to determine efficiency of antibody adsorption by FACS analysis
- Microsphere Blocking Buffer
  0.1% BSA/PBS (1 mg/mL)
- Microsphere Storage Buffer
  0.05% BSA/PBS (0.5 mg/mL)
- FACS Buffer
  - keep on ice
- FACS tubes

Supporting Protocols:

- General > Measurement of Antibody Concentration by Absorbance
- Cell Culture > Cell Counting

Protocol:

microspheres adsorb protein non-specifically, therefore it is important to add antibody from a stock solution that does not include carrier protein

formula for calculation of microsphere surface saturation is as follows: $S = [6 / (\rho * D)] * C$
where S = surface saturation (mg protein / g of microspheres;
ρ = density of microspheres (e.g., 1.05 g/cm^3)

D = diameter of microspheres in microns (e.g., 6 μm)
C = capacity of microsphere surface for given protein (e.g., 2.5 mg/m^2 for IgG, 3 mg/m^2 for BSA)

example calculation for coating 6 μm microspheres with IgG:
*S = [6 / (1.05 * 6)] * 2.5 = 2.4 mg*

- therefore, 2.4 mg of antibody needed to coat 1 g of microspheres
- multiply by recommended 10x excess for providing driving force for adsorption results in 24 mg for 1 g of microspheres (i.e., 120 μg of antibody for 5 mg of microspheres)

1) determine concentration of antibody stock solution
    see supporting protocol
2) prepare antibody / microsphere mixture
    500 μL total volume in microcentrifuge tube
    2.1) add 120 μg of antibody
        0.24 mg/mL final concentration
    2.2) add calculated volume of PBS
    2.3) add 5 mg of microspheres
        - add microspheres to diluted antibody for even distribution of adsorption
        - 1% (10 mg/mL) final concentration of microspheres
    2.4) mix
3) tumble on tube rotator for 24 hours at 4°C
    to adsorb antibody to microspheres
4) block microspheres
    to saturate exposed hydrophobic surfaces of the microspheres
    4.1) centrifuge at 1,200 g for 15 min.
    4.2) carefully aspirate supernatant using pipet tip
    4.3) resuspend antibody-coated microspheres in 1 mL of Blocking Buffer
    4.4) tumble on tube rotator overnight at 4°C
5) reconstitute in Storage Buffer
    5.1) centrifuge at 1,200 g for 15 min.
    5.2) carefully aspirate supernatant using pipet tip
    5.3) resuspend in 1 mL of Storage Buffer
6) determine final # microspheres / mL using hemacytometer
    see 'Cell Counting' protocol for details
7) verify efficient antibody adsorption for each microsphere preparation by FACS analysis prior to use in cell-based assay

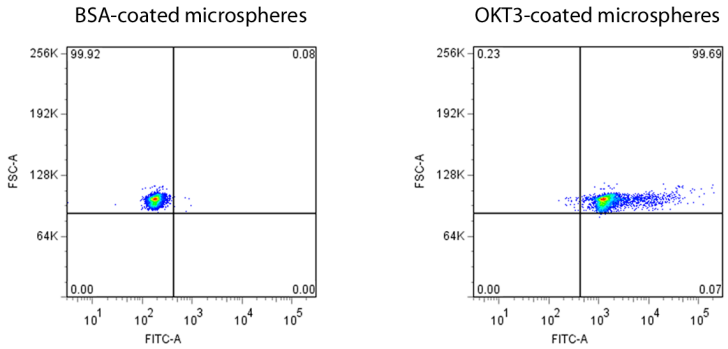


Figure 41: FACS analysis of microspheres coated with antibody.
OKT3-coated microspheres were stained with FITC-conjugated goat anti-mouse antibody and analyzed by FACS to determine efficiency of OKT3 adsorption as compared to control microspheres coated with BSA.

7.1) for each sample, add 1 mL of FACS buffer to microcentrifuge tube
7.2) aliquot 10^6 antibody-coated microspheres to tube
7.3) add fluorophore-conjugated secondary antibody at 5 μg/mL final
7.4) tumble for 30 min. at 4°C
7.5) wash 2x with FACS buffer
    7.5.1) centrifuge at 1,200 g for 15 min.
    7.5.2) carefully aspirate supernatant using pipet tip
    7.5.3) resuspend in 1 mL of FACS buffer (wash)
    7.5.4) repeat
7.6) centrifuge at 1,200 g for 15 min.
7.7) carefully aspirate supernatant using pipet tip
7.8) resuspend in 1 mL of FACS Buffer
7.9) transfer microspheres to designated FACS tubes
7.10) acquire FACS data
7.11) analyze FACS data
    7.11.1) prepare dot plots with negative gate determined by BSA-coated control microspheres
    7.11.2) display overlapping histogram to comparatively assess efficiency of antibody adsorption
8) store at 4°C for ≤ 2 weeks

References:

(94, 143)

Jurkat–Microsphere/OKT3 Conjugation

for localization studies only (reflective property of microspheres interferes with detection of FRET)

Equipment:

- refrigerated centrifuge
- bucket with ice
- 37°C water bath

Materials:

- Jurkat cells cultured to ≤ 10^6 cells/mL
  - serum starved overnight
    see supporting protocol
- OKT3- and UPC10/OKT9-coated microspheres
  see supporting protocol for details
- microscope slides, 25x75x1 mm
  Fisher, Cat. # 12-544-7
  - coated with poly-L-lysine in advance
    see supporting protocol
- microcentrifuge tubes
- RPMI
  - keep on ice

Supporting Protocols:

- Cell Culture > Jurkat Serum Starvation
- Antibody Adsorption to Microspheres
- Coating Microscopy Slides / Cover Glass with Poly-L-Lysine

Protocol:

for each experimental condition, combine according to ratio below:
10^6 Jurkat cells : 2x10^6 microspheres

1) serum starve Jurkat cells overnight
   see supporting protocol for details
2) adsorb antibody to microspheres
   see supporting protocol for details
3) coat microscopy slides with poly-L-lysine
   see supporting protocol for details
4) prepare OKT3- or UPC10/OKT9-coated microspheres
   see supporting protocol for details
   4.1) adjust concentration to 2x in RPMI
   e.g., 8x10^6 microspheres/mL
   4.2) equilibrate on ice
5) prepare Jurkat cells

5.1) adjust concentration to 2x in RPMI
e.g., 4x10^6 cells/mL
5.2) equilibrate on ice

6) mix microspheres and cells
prepare in microcentrifuge tube
6.1) add microspheres (e.g., 2x10^6 microspheres / 250μL)
6.2) add cells (e.g., 10^6 cells / 250μL)
6.3) mix gently
6.4) keep on ice

7) promote contact of microspheres and cells
7.1) centrifuge for 5 min. at 100 g (500 rpm) at 4°C
7.2) carefully remove supernatant using pipet tip
- do not disturb pellet
- leave ~100μL volume
7.3) incubate for 30 min. on ice
allow conjugate formation to occur

8) stimulate
proceed quickly between steps
8.1) transfer microcentrifuge tube(s) to 37°C water bath
8.2) incubate for various time-points
e.g., 1, 3, 5, 10, 30 minutes
8.3) return tube(s) to ice
8.4) proceed immediately with 'Cell Attachment and Fixation on Slides / Cover Glass'

References:

(94, 143)

Surface Staining with Cy5 NHS Ester

- for surface labeling of Raji cells (antigen presenting cell line for Jurkat cells along with super-antigen) for FRET and/or localization experiment(s)
- surface labeling can be used to generate a region of interest (ROI) at the contact site between an APC and a T cell expressing CFP / YFP
- do not label APCs with other fluorescent molecules that may contaminate FRET channel

Equipment:

- biosafety cabinet / TC hood
- -20°C freezer
- refrigerated centrifuge
- bucket with ice

Materials:

- Raji cells
  APCs for Jurkat in the presence of super-antigen
  - cultured to ≤ 10^6 cells/mL
- Cy5 Mono-Reactive Dye Pack
  - GE Healthcare / Amersham Biosciences, Cat. # PA25001
  - kit contains 5 dye packs
  - each pack contains ~30 nmols and is intended for one labeling reaction (i.e., for labeling 1 mg of protein)
  - stored at 4°C
  - protected from light
- anhydrous DMSO
  Sigma, Cat. # 276855; 100 mL
  - protect from moisture
- 50 mL centrifuge tube
- PBS, cell culture grade
  - prepare aliquot
  - keep on ice
- Raji Growth Medium
  cRPMI + 10% FBS
  - prepare aliquot
  - keep on ice

Supporting Protocol:

- In Vivo / Ex Vivo: Mononuclear Cell Isolation Using Density Gradient

Protocol:

for staining 1-5x10^6 cells

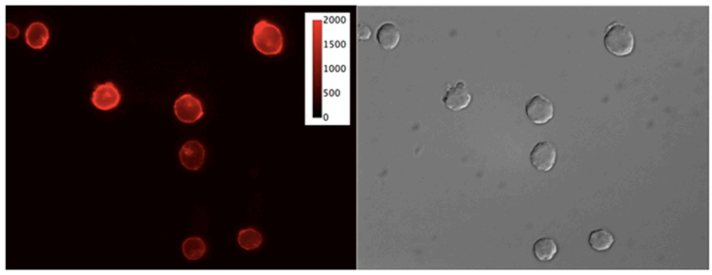


Figure 42: Jurkat cells surface stained with Cy5 NHS ester.

1) reconstitute Cy5 NHS ester
    1.1) dissolve 1 dye pack (see notes above) in 30 µL of anhydrous DMSO
        succinimidyl ester will gradually hydrolyze in the presence of water
    1.2) store unused reconstituted dye in dark at -20°C for 1 month
        if protected from moisture
2) optional: isolate viable cells by separation with histopaque
    - see supporting protocol
    - dead cells become stained very bright intracellularly and can interfere with image analysis
3) transfer cells for staining to 50 mL centrifuge tube
    wider surface area at bottom is better for more uniform staining
4) centrifuge for 5 min. at 1,000 rpm (4°C)
5) discard supernatant
6) wash cells with PBS
    6.1) resuspend / wash pellet in 10 mL of PBS
    6.2) centrifuge for 5 min. at 1,000 rpm (4°C)
    6.3) discard supernatant
7) resuspend cells prior to staining in 500 µL of ice-cold PBS
8) add 5 µL of Cy5 dye as prepared above
    can increase to 10 µL if dye is old
    8.1) protect from light
9) mix immediately
10) incubate in dark for 10 min. on ice
    10.1) mix frequently during incubation
11) wash cells 2x with PBS
12) wash cells 1x with Raji Growth Medium
    to quench un-reacted dye
13) proceed with imaging experiment
    - can use cells immediately if it is determined that Cy5 does not interfere with T cell activation
    - alternatively, return cells to incubator overnight to allow re-expression of surface / MHC proteins needed for T cell activation

- however, avoid longer incubations that may result in bright internalized granules containing dye that may interfere with image analysis

References:

(144)

Jurkat-Raji/SEE Conjugation

for localization and/or FRET assay(s)

Equipment:

- refrigerated centrifuge
- humidified, 5% CO_2 37°C incubator
- bucket with ice
- 37°C water bath

Materials:

- Jurkat cells
  - *10^6 cells per reaction*
  - nucleofected with fluorescent-protein reporter construct
    - *see supporting protocol*
  - serum starved overnight (optional)
- Raji cells
  - *10^6 cells per reaction*
  - surface stained with Cy5 NHS Ester
    - *see supporting protocol*
- staphylococcal enterotoxin E (SEE)
  - *super-antigen for binding to Raji MHC*
  - *Toxin Technologies, Cat. # ET404*
- microscope slides, 25x75x1 mm
  - *Fisher, Cat. # 12-544-7*
  - coated with poly-L-lysine in advance
    - *see supporting protocol*
- microcentrifuge tubes
- RPMI
  - keep on ice
- 4% paraformaldehyde
  - keep on ice

Supporting Protocols:

- Surface Staining with Cy5 NHS Ester
- In Vivo / Ex Vivo > Mononuclear Cell Isolation Using Density Gradient
- Cell Culture > Jurkat Amaxa Nucleofection
- Cell Culture > Jurkat Serum Starvation
- Coating Slides with Poly-L-Lysine

Protocol:

for each experimental condition, combine according to ratio below:
10^6 Jurkat cells : 10^6 Raji cells

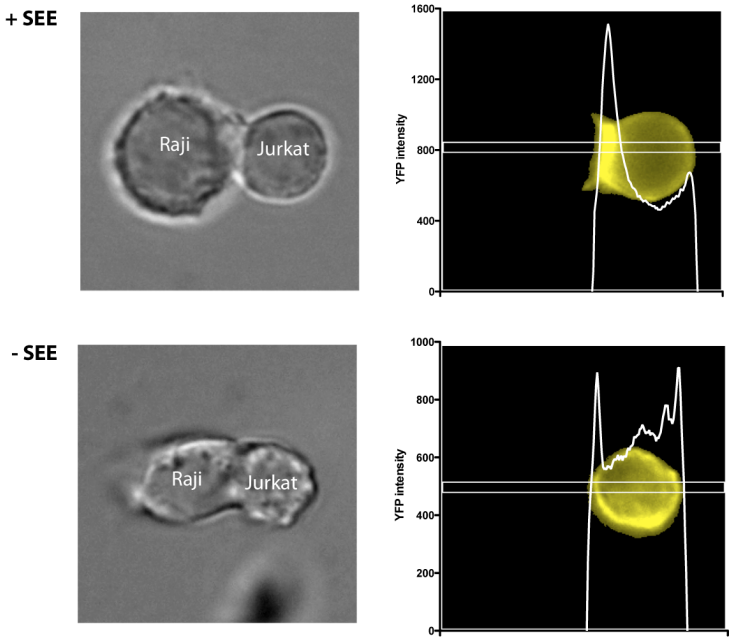


Figure 43: Jurkat–Raji/SEE conjugates.

Jurkat–Raji conjugates formed in the presence or absence of SEE, respectively. Fluorescence micrographs to the right of each DIC image display Itk-CY localization and SEE-dependent polarization at the immunological synapse.

1) pulse surface-labeled Raji cells with SEE
    1.1) optional: isolate viable cells by separation with histopaque
        - see supporting protocol
        - dead cells become stained very bright intracellularly and can interfere with image analysis
    1.2) centrifuge for 5 min. at 1,000 rpm (4°C)
    1.3) resuspend cells at $2x10^6$ cells/mL in RPMI
    1.4) for each reaction, transfer 10^6 cells / 0.5 mL per microcentrifuge tube
    1.5) add SEE at 2 μg/mL final
        or without SEE for non-stimulation control
    1.6) place inside incubator for 1 hr.
        uncap tubes to allow gas exchange
2) prepare nucleofected Jurkat cells
    2.1) optional: isolate viable cells by separation with histopaque
        - see supporting protocol

- dead cells become stained very bright intracellularly and can interfere with image analysis

2.2) centrifuge for 5 min. at 1,000 rpm (4°C)
2.3) resuspend cells at $2x10^6$ cells/mL in RPMI
2.4) keep on ice until Raji cells are ready for conjugation

3) Jurkat-Raji conjugation
3.1) for each reaction, transfer 10^6 / 0.5 mL Jurkat cells to tube containing 10^6 / 0.5 mL SEE-pulsed Raji cells
3.2) pipet up and down to mix
3.3) centrifuge for 5 min. at 200 g / 1,000 rpm (4°C)
3.4) aspirate 900 µL of supernatant
leaving behind 100 µL and pellet
3.5) carefully aspirate supernatant using pipet tip
- do not disturb pellet
- leave ~100 µL volume
3.6) incubate for 30 min. on ice
to allow conjugate formation to occur

4) stimulation
proceed quickly between steps
4.1) transfer microcentrifuge tube(s) to 37°C water bath
4.2) incubate for 10 min.
4.3) return tube(s) to ice
4.4) proceed immediately with 'Cell Attachment and Fixation on Slides'

References:

(145-149)

Cell Attachment and Fixation on Slides / Cover Glass

Equipment:

- bucket with ice
  keep cells at 4°C during attachment if attaching to cover glass (inside culture dishes)
- 37°C water bath
  for reducing viscosity of mounting medium prior to use
- lab timer

Materials:

- cells for attachment
  - keep on ice
- microscopy slides, coated with poly-L-lysine
  see supporting protocol for details
  - cover glass, 18 x 18 x 1 mm
    Fisher, Cat. # 12-548-A
- cover glass (inside culture dishes), coated with poly-L-lysine
  - see supporting protocol for details
  - optional: equilibrate culture dishes on ice prior to attachment
  - microscopy slides, 25 x 75 x 1 mm (1 per cover glass)
    Fisher, Cat. # 12-544-7
  - straight-edge razor
    for removing cover glass from culture dishes
- 2% (w/v) paraformaldehyde (PFA) in PBS
  - keep on ice
- 1x PBS
  - keep on ice
- Kimwipes
- mounting medium
  Invitrogen, Cat. # P7481
  - pre-warm at 37°C before use
- nail polish
- slide folder

Supporting Protocol:

- Coating Microscopy Slides / Cover Glass with Poly-L-Lysine

Protocol:

1) attach cells to pLL-coated slides / cover glass
   1.1) gently resuspend cells prior to attachment
      1.1.1) for conjugates, use razor blade to remove end of 200 μL pipet tip

to reduce shear forces while adding conjugates to slide

1.2) add ~10^5 cells to each slide / cover glass
add 10 µL of $10x10^6$ cells/mL concentrated suspension
1.3) spread evenly in small, circular area using pipet tip
1.4) allow cells to attach for 5 min.
do not allow to dry completely

2) fix with paraformaldehyde
2.1) gently add 100µL of ice-cold 2% PFA/PBS to perimeter of area containing attached cells
2.2) distribute PFA evenly over cells using pipet tip
avoid disturbing attached cells
2.3) incubate for 10 min.
2.4) wash 3x with 1mL of ice-cold PBS
- removes non-attached cells
- do not pipet directly on cells
2.5) allow to dry for ≤ 30 min.
2.6) wick away excess PBS using Kimwipe

3) continue with further staining (if required)

4) apply mounting medium / seal slides
4.1) add 10 µL of mounting medium directly above cells
4.2) gently place cover glass on top of cells / invert cover glass with attached cells onto slide
4.3) seal coverslip at each corner with nail polish
do not seal along edges yet
4.4) allow mounting medium to cure overnight at room temp. in dark
4.5) seal edges of cover glass using nail polish

5) store slides at 4°C in slide folder

Actin Polymerization Assay

Equipment:

- 37°C water bath
- refrigerated centrifuge
- bucket with ice

Materials:

- Jurkat - OKT3-coated microsphere conjugates
  - *including BSA / OKT9-coated microsphere conjugates for negative control*
  - *see supporting protocol for details*
  - attached to slides
    see supporting protocol
  - fixed with paraformaldehyde
    see supporting protocol
- PBS
  - keep on ice
- Permeabilization Buffer
  - *0.1% (v/v) Triton X-100 / PBS*
  - *see 'Buffers & Solutions'*
- Blocking Buffer
  - *1% BSA / PBS*
  - *see 'Buffers & Solutions'*
  - keep on ice
- Texas Red Phalloidin Staining Buffer
  - *5 U/mL Texas Red Phalloidin in Blocking Buffer*
  - *Texas Red is optimally excited at 591 nm and has an emission peak at 608 nm*
  - *see 'Buffers & Solutions'*
- mounting medium, pre-warm to 37°C before use
  Invitrogen, Cat. # P7481

Supporting Protocols:

- Coating Microscopy Slides / Cover Glass with Poly-L-Lysine
- Jurkat-Microsphere/OKT3 Conjugation/Stimulation
- Cell Attachment and Fixation on Slides / Cover Glass

Protocol:

continue following fixation of conjugates on slides

1) permeabilize cells
   1.1) gently add 200 µL of ice-cold permeabilization buffer to area surrounding cells

do not pipet directly on attached conjugates

1.2) distribute evenly over cells using pipet tip

take care to avoid disturbing attached cells

1.3) incubate for 5 min. at room temp.

2) wash 2x with 1 mL of ice-cold PBS

do not pipet directly on attached conjugates

3) block cells prior to staining

3.1) gently add 200 μL of ice-cold blocking solution to area surrounding cells

3.2) distribute evenly over cells using pipet tip

take care to avoid disturbing attached cells

3.3) incubate for 20 min. at room temp.

4) wash 2x with 1 mL of ice-cold PBS

do not pipet directly on attached conjugates

5) stain cells

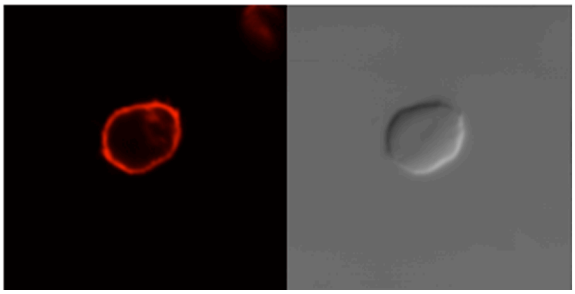


Figure 44: Jurkat cell stained with TR phalloidin.

5.1) gently add 200 μL of ice-cold Texas Red Phalloidin Staining Buffer to area surrounding cells

5.2) distribute evenly over cells using pipet tip

take care to avoid disturbing attached cells

5.3) incubate for 20 min. in dark at room temp.

6) wash 2x with 1 mL of PBS

7) apply mounting medium / seal slide

see supporting protocol

8) store slides at 4°C in slide folder

References:

(91, 94, 115, 143, 145, 146, 150-157)

5.2.9. FRET

Image Acquisition

Equipment:

- eppifluorescence microscope, Zeiss
  upright
  - 63x objective, water immersion
    Zeiss
  - automatic rotating turret containing filter cubes
    - CFP filter cube (DD channel)
      donor excitation / donor emission
      ex: 436 nm +/- 10
      dichroic: 455 nm
      em: 480 nm +/- 20
    - YFP filter cube (AA channel)
      acceptor excitation / acceptor emission
      ex: 500 nm +/- 10
      dichroic: 515 nm
      em: 535 nm +/- 15
    - FRET filter cube (DA channel)
      donor excitation / acceptor emission
      ex: 436 nm +/- 10
      dichroic: 455 nm
      em: 535 nm +/- 15
    - DIC filter cube
    - TRITC filter cube
      ex: 545 nm +/- 15
      dichroic: 565 nm
      em: 610 nm +/- 38
  - slider containing neutral density filters
    for reducing light intensity
- Apotome
  for acquiring deconvolved images
- camera, 14-bit grayscale
- computer
  - Axiovision 4.2 software installed
- external hard drive
  for storage of images
- dI water bottle and transfer pipet
  for objective
- slide folder
- lab timer

Materials:

- transfectants expressing donor and/or acceptor fluorescent protein(s) including the following:

fixed and mounted on microscopy slides
- CFP-only (single color control)
- YFP-only (single color control)
- CFP-YFP (pYC)
  FRET positive control (CFP and YFP co-expressed on same molecule separated by short linker
- CFP + YFP
  FRET negative control (CFP and YFP co-expressed on separate molecules)
- FRET biosensor with protein of interest
  - stimulated / non-stimulated

- CD / DVD
  for saving images
- lab notebook
  for recording log of images acquired at microscope

Supporting Protocols:
- Cell Culture > Jurkat Amaxa Nucleofection
- Microscopy / FACS > Jurkat-Raji/SEE Conjugation/Stimulation
  - Surface Staining with Cy5 NHS Ester
- Microscopy / FACS > Cell Attachment and Fixation on Slides / Cover Glass

Protocol:
1) set up microscope
   1.1) turn on power for fluorescent bulb
      1.1.1) adjust bulb strength to 100%
   1.2) turn on power for computer
   1.3) turn on power for microscope
   1.4) connect camera to microscope using plug
   1.5) open Axiovision software
      1.5.1) select 'Multidimensional Acquisition > Load > CFP-YFP FRET > OK'
      1.5.2) create folder on hard drive and name with current date
         for storage of images
2) scan slide for fluorescent cells
   2.1) slide neutral density into light path
   2.2) select 40x objective
   2.3) place slide on stage
   2.4) turn on 'Hal' for transmitted light
   2.5) focus on cells in DIC channel
   2.6) switch to YFP channel to search for fluorescent cells

- YFP is less sensitive to photobleaching as compared to CFP

- therefore, scan slide for fluorescent cells using YFP channel when possible

- reduce YFP photobleaching by always using neutral density filter and turning off reflector when not in use

3) acquire YFP reference image

capture image using fixed exposure time for comparing brightness / expression level from cell to cell

3.1) switch to 63x objective

3.2) adjust focus as needed

3.3) capture YFP image using fixed exposure time

determined exposure time from 'representative' image in terms of brightness / expression level

3.4) record image number and identification in notebook

4) acquire sensitized emission images

- includes CFP, YFP and FRET images used to calculate E-FRET

- acquire control images from cells expressing CFP-only, YFP-only and pYC

4.1) adjust exposure times proportionally for CFP, YFP and FRET images

- ratio of exposure times for CFP : YFP : FRET images must remain constant in order to calculate / compare E-FRET from cell to cell

- for good image quality, need ≥ 1,000 and < 10,000 average pixel counts for all images (range 0 - 16,384 pixel counts for 14-bit camera)

- CFP requires ~2x longer exposure time compared to YFP when expressed at 1:1 stoichiometry

4.2) run FRET acquisition program to capture series of images as follows: CFP, YFP, FRET, DIC, Cy5 (for imaging APC / Raji cell)

4.3) record image number and identification in notebook

5) acquire Apotome image

- used for images of conjugates to define ROI at contact site

- acquires images above and below focus plane to mechanically deconvolve eppifluorescence image

5.1) slide Apotome into light path

5.2) run Apotome acquisition program

5.3) record image number and identification in notebook

6) acquire pre / post acceptor photobleaching images

- for determination of G parameter from cells expressing pYC

- use separate slide designated for photobleaching to avoid unintended photobleaching of cells outside of field

- acquire control images from cells expressing CFP-only and YFP-only

6.1) run FRET acquisition program prior to photobleaching YFP
6.2) turn on YFP reflector to photo-bleach YFP by ≥ 80%
- determine time required in advance using cells expressing YFP-only (e.g., 10 min.)
- use timer to remain consistent from cell to cell
6.3) run FRET acquisition program after photobleaching YFP
6.4) record image number and identification in notebook
7) save all images as 16-bit '.tif' files
de-select check boxes in window
8) burn images to DVD

References:

(158)

Initial Image Processing

image alignment and background subtraction

Equipment:

- computer
  - Image J software installed
    TurboReg plugin installed

Materials:

- raw fluorescence images
  16-bit (1388 x 1040 pixels) grayscale images (.tif file extension) in the following channels:
  - CFP
  - YFP
  - FRET
  - Cy5
    for Raji cells

Supporting Protocol:

- FRET Image Acquisition

Protocol:

1) open 16-bit raw fluorescence image from within Image J
   File > Open
2) align correlated raw fluorescence images
   except YFP_{after} images depending on extent of photobleaching
   2.1) convert images for alignment into stack
   Image > Stack > Convert Images to Stack
   2.2) run TurboReg plugin to align images
   2.3) convert stack back into individual images
3) convert images to 32-bit
4) adjust brightness / contrast
5) subtract background
   proceed independently for each raw image
   5.1) create ROI in cell-free area of background
   5.2) record mean pixel count
   5.3) subtract mean background value from entire raw image
6) save processed images
7) continue with further analysis

References:

(158)

Binary Mask Generation

for removing background and/or defining synapse ROI

Equipment:

- computer
  - Image J software installed

Materials:

- fluorescence image(s)
- for defining synapse ROI, fluorescence images acquired using Apotome
  - YFP image
    for defining Jurkat cell
  - Cy5 image
    for defining Raji cell

Supporting Protocol:

- Image Acquisition

Protocol:

1) open fluorescence image from within Image J
   File > Open
2) duplicate image
3) adjust brightness / contrast as needed to visualize fluorescent cell of interest
   Image > Adjust > Brightness/Contrast
4) adjust threshold to select cell of interest in red
   Image > Adjust > Threshold
5) select 'Apply > OK'
   - image is converted to 8-bit
   - cell is given value of 0
   - background is given value of 255
6) select 'Edit > Invert'
   - cell is given value of 255
   - background is given value of 0
7) select 'Image > Math > Subtract' and enter 254
   - cell is given value of 1
   - background is given value of 0
8) adjust brightness / contrast as needed so that cell of interest appears white with a black background
9) save 8-bit binary image as 'mask'
10) for defining synapse ROI, multiply YFP and Cy5 mask images
    contact site is given value of 1

Image Analysis for Acceptor Photobleaching Method

FRET efficiency measured by donor recovery after acceptor photobleaching

Equipment:

- computer
  - Image J software installed

Materials:

- raw images of cell expressing the following fluorescently-tagged proteins:

  16-bit (1388 x 1040 pixels) grayscale images (.tif file extension) in the following channels:
  CFP, YFP and DIC

  - acquired before and after acceptor photobleaching
    - including images acquired after photobleaching the same cell at different time points
  - aligned
  - background subtracted

  - CFP
  - YFP
  - CFP-YFP
  - CFP + YFP
  - biosensor with protein of interest
    - stimulated / non-stimulated

Supporting Protocols:

- Image Acquisition
- Initial Image Processing
- Binary Mask Generation

Protocol:

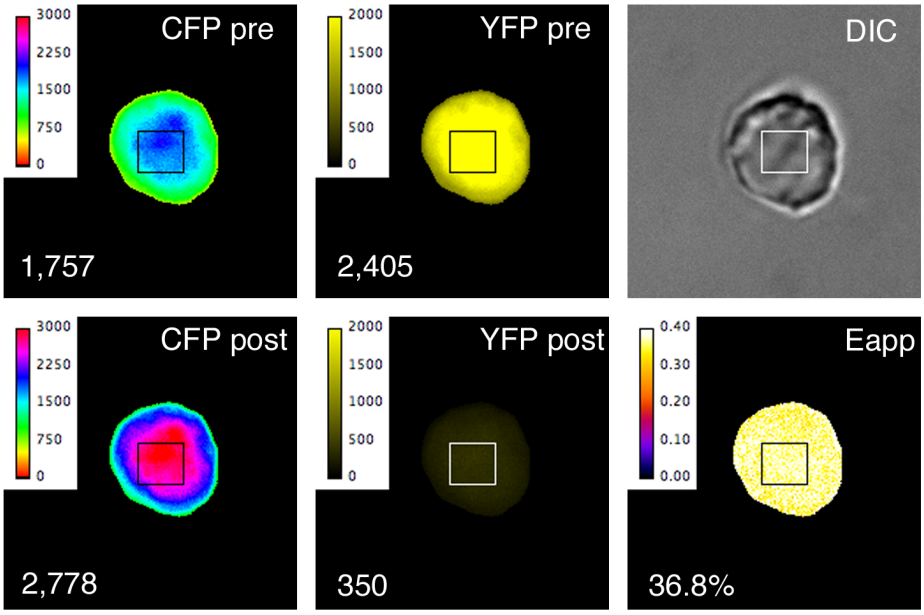


Figure 45: FRET efficiency of positive control (CFP-YFP) as determined by acceptor photobleaching method.

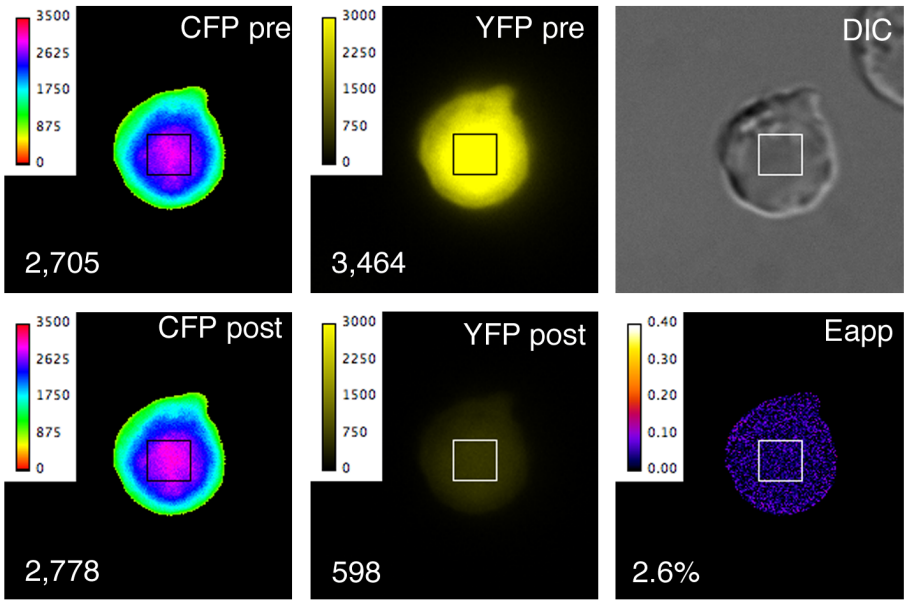


Figure 46: FRET efficiency of negative control (CFP + YFP) as determined by acceptor photobleaching method.

1) determine correction factor for CFP photobleaching effect (if any)
   - *proceed for cells expressing CFP-only*
   - *corrects for cross-excitation of CFP during YFP photobleaching*
   - *value depends on YFP photobleaching time interval*

   correction factor = I_{DD} before / I_{DD} after

   1.1) create ROI within cell
   1.2) record mean pixel count for CFP_{before} and CFP_{after} images
   1.3) normalize by dividing all values by CFP_{before} value for each time interval
   1.4) plot % photobleaching efficiency versus time interval
   1.5) repeat for several cells / images
   1.6) determine correction factor required for given time interval using equation above

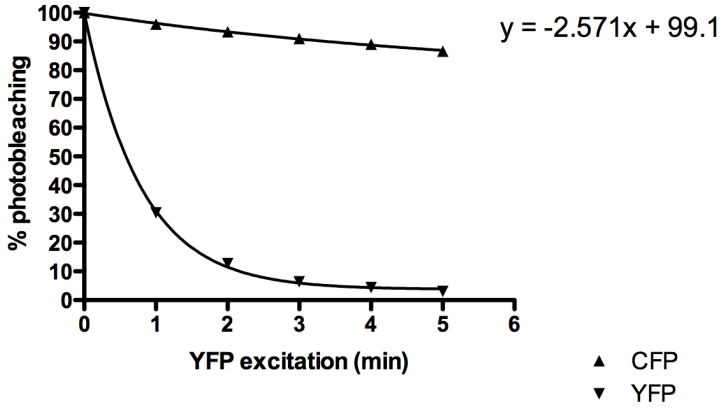


Figure 47: Progressive CFP 'bystander' photobleaching versus exposure time to YFP excitation light.

2) apply correction factor for CFP photobleaching effect (if required)
   2.1) multiply entire raw CFP_{after} image by correction factor
3) calculate YFP photobleaching efficiency
   YFP photobleaching efficiency (%): 1 - (I_{AA} after / I_{AA} before)
   3.1) select YFP_{before} image
   3.2) create ROI within cell
   3.3) save ROI
   3.4) record mean pixel count
   3.5) repeat from above for YFP_{after} image
   3.6) substitute mean ROI values into equation described above
4) calculate FRET efficiency
   E_{app} (%) = 1 - (CFP_{before} / CFP_{after})
   4.1) select CFP_{before} image

4.2) create saved ROI within cell
4.3) record mean pixel count
4.4) repeat from above for CFP_{after} image
4.5) substitute mean ROI values into equation described above

5) generate FRET efficiency image
for display (i.e., not calculation) purposes
5.1) create 32-bit ratio image by dividing CFP_{before} image by CFP_{after} image
5.1.1) select 'Process > Image Calculator > Image 1 > CFP_{before}'
5.1.2) select 'Operation > Divide'
5.1.3) select 'Image 2 > CFP_{after} > 32-bit result > OK'
5.2) create new 32-bit image
5.2.1) add '1' to entire image
5.3) subtract ratio image from '1' image
5.4) continue with final image processing

References:

(158)

Image Analysis for E-FRET Method

FRET efficiency calculated from sensitized emission (FRET) image

Equipment:

- computer
  - Image J software installed
    - TurboReg plugin installed
    - E-FRET macro installed

Materials:

- raw images of cells expressing the following fluorescently-tagged proteins:

  16-bit (1388 x 1040 pixels) grayscale images (.tif file extension) in the following channels:
  CFP, YFP, FRET and DIC

  - aligned
  - background subtracted
  - CFP
  - YFP
  - CFP-YFP
  - CFP + YFP
  - CFP-YFP

    for calculation of G parameter
    - acquired before and after acceptor photobleaching
    - including images acquired after photobleaching the same cell at different time points
  - biosensor with protein of interest
    - stimulated / non-stimulated

Supporting Protocols:

- Image Acquisition
- Initial Image Processing
- Image Analysis for Acceptor Photobleaching Method

Protocol:

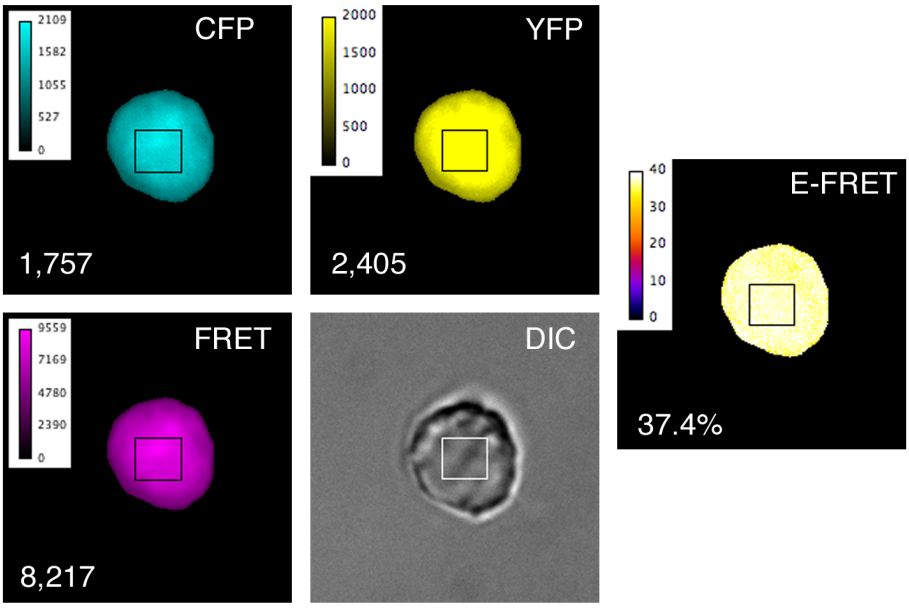


Figure 48: FRET efficiency of representative Jurkat cell expressing CFP-YFP as determined by E-FRET method.

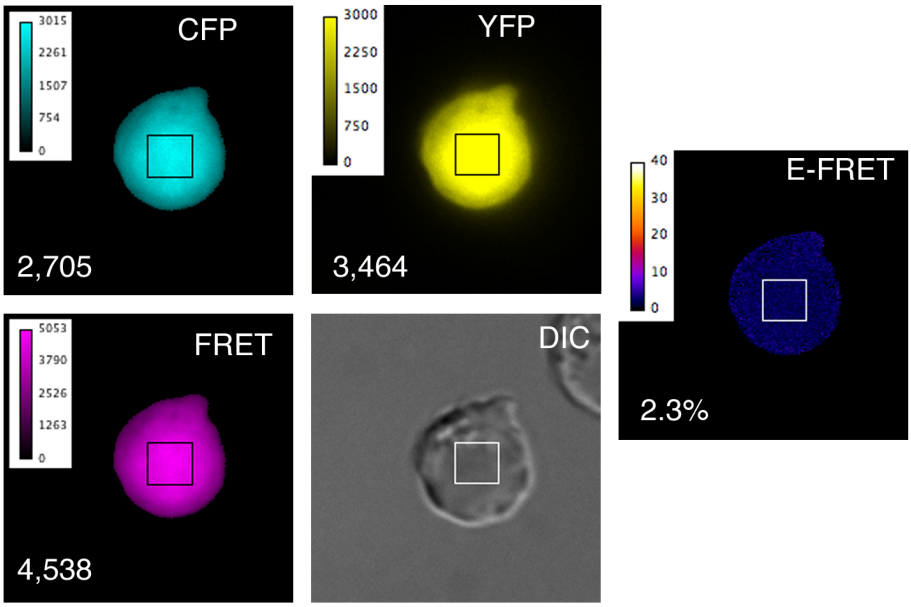


Figure 49: FRET efficiency of representative Jurkat cell expressing CFP + YFP as determined by E-FRET method.

1) calculate spectral bleed-through constants
   - *requires CFP, YFP and FRET images*
   - *for cells expressing YFP / acceptor (A)-only*
      - *bleed-through to FRET (DA) channel: $a = I_{DA(A)} / I_{AA(A)}$*
      - *bleed-through to donor (DD) channel: $b = I_{DD(A)} / I_{AA(A)}$*
   - *for cells expressing CFP / donor (D)-only*
      - *bleed-through to acceptor channel (AA) channel: $c = I_{AA(D)} / I_{DD(D)}$*
      - *bleed-through to FRET (DA) channel: $d = I_{DA(D)} / I_{DD(D)}$*

1.1) create ROI within cell
1.2) record mean pixel count in each channel
1.3) substitute mean ROI values into relevant equations described above
1.4) repeat for several cells / images and determine average value

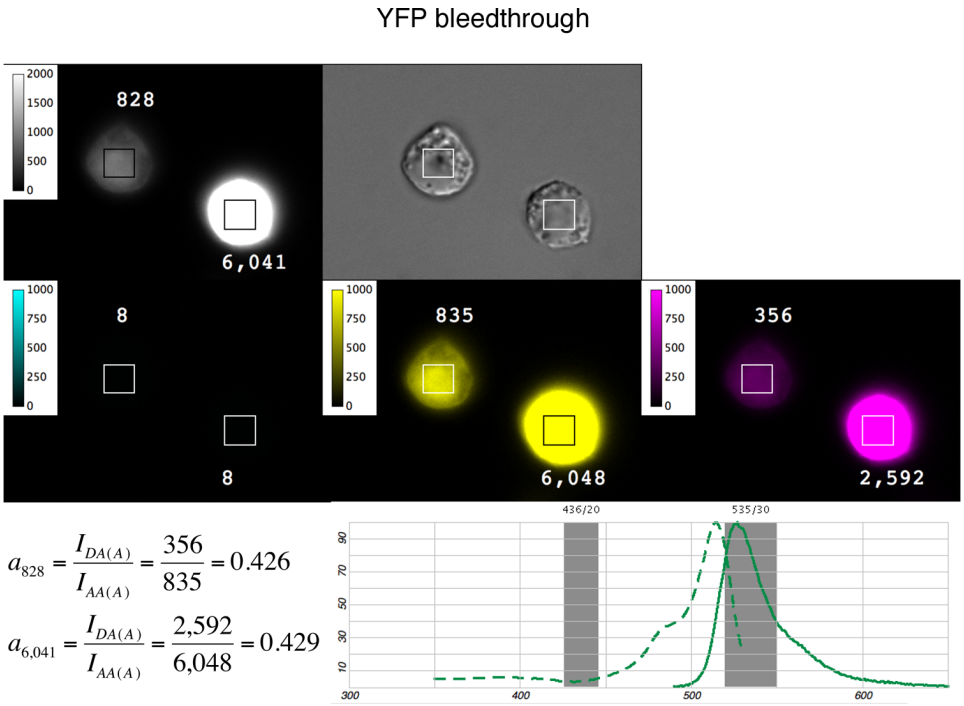


Figure 50: YFP bleed-through.
Jurkat cells expressing (acceptor) imaged in CFP, YFP and FRET channels; bleed-through to FRET channel calculated from selected ROIs.

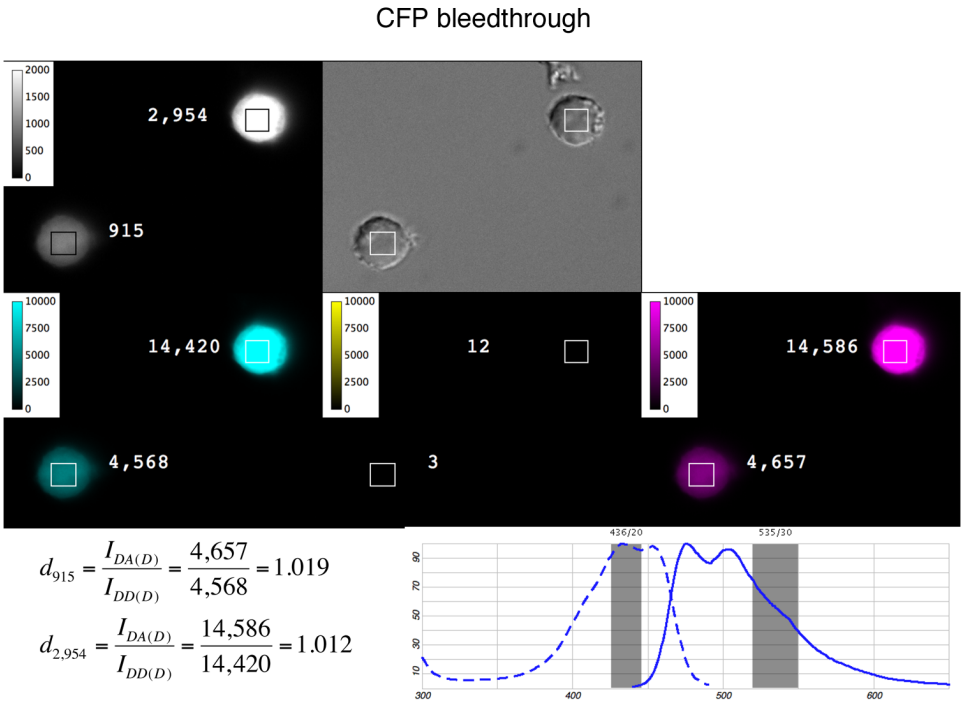


Figure 51: CFP bleed-through.

Jurkat cells expressing CFP (donor) imaged in CFP, YFP and FRET channels; bleed-through to FRET channel calculated from selected ROIs.

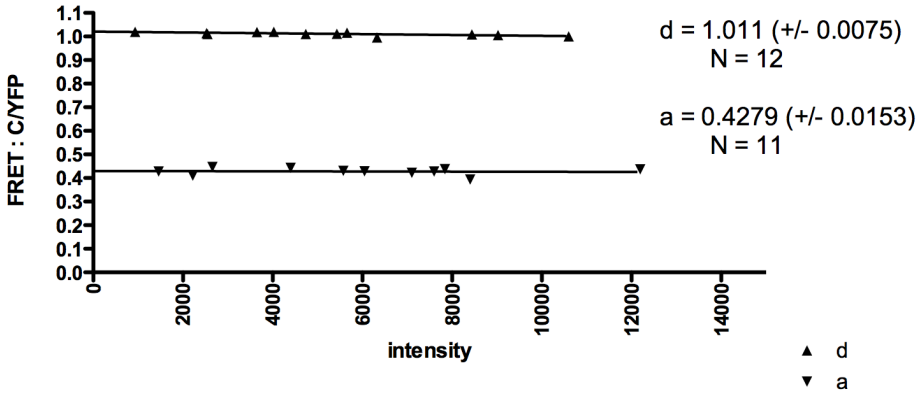
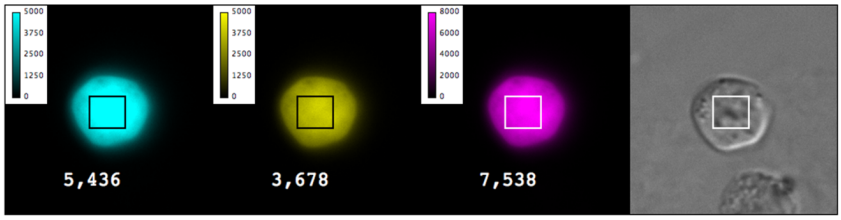


Figure 52: Linearity of bleed-through constants 'a' and 'd' as a function of expression level.

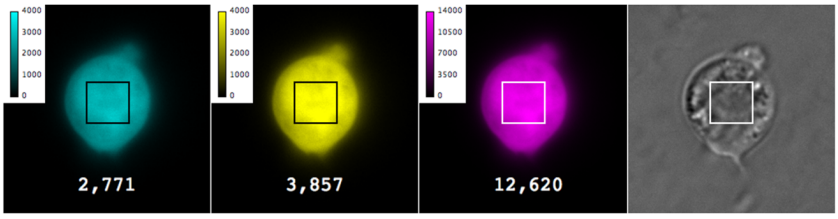
2) calculate bleed-through-corrected FRET (F_c)

F_c (corrected FRET) = I_{DA} - aI_{AA} - d*I_{DD}*

(assumes that b = c = 0)



CFP + YFP $F_c = 7{,}538 - (.4279)(3{,}678) - (1.011)(5{,}436) = 468$



CFP-YFP $F_c = 12{,}620 - (.4279)(3{,}857) - (1.011)(2{,}771) = 8{,}169$

Figure 53: Calculation of Fc from representative fluorescence images. Jurkat cells expressing CFP + YFP or CFP-YFP and imaged in CFP, YFP and FRET channels; Fc calculated from selected ROIs.

2.1) select CFP image
2.2) create ROI within cell
2.3) save ROI
2.4) record mean pixel count
2.5) repeat from above for YFP and FRET images
2.6) substitute mean ROI values into equation described above

3) calculate G parameter from ROI within cell

- requires set of images (CFP, YFP and FRET) acquired before and after YFP photobleaching (see supporting protocol)
- proceed for cells expressing CFP-YFP (constitutive FRET construct)
- correlates FRET efficiency as measured by acceptor photobleaching and sensitized emission methods, respectively

G = (F_c before - F_c after) / (I_{DD} after - I_{DD} before)

3.1) calculate Fc as described above for 'before photobleaching' set of images
3.2) calculate Fc as described above for 'after photobleaching' set of images

3.2.1) apply correction factor to CFP_{after} image to correct for photobleaching effect (if any)
see supporting protocol

3.3) substitute mean ROI values into equation described above

3.4) repeat for several cells / images and determine average value

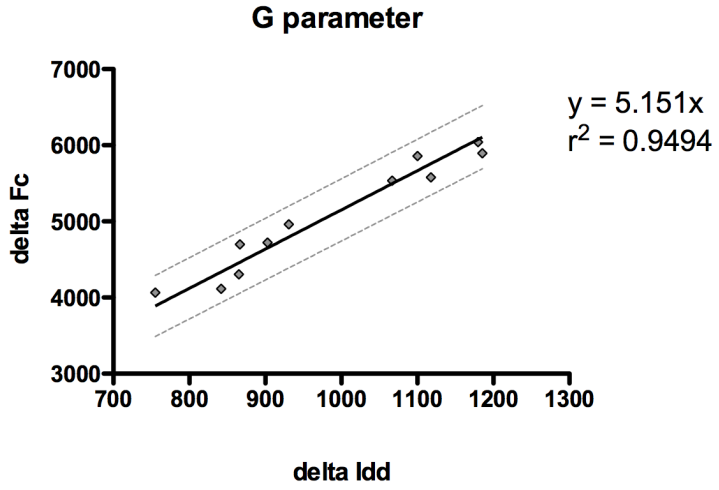


Figure 54: Calculation of G parameter from Jurkat cells expressing CFP-YFP.

4) define ROI at Jurkat-Raji contact site
- for calculation of E-FRET of stimulated cells
- use images acquired using Apotome (mechanically deconvolved image) for precise definition of contact site

4.1) generate binary mask for Jurkat cell expressing biosensor from YFP image
see supporting protocol

4.2) generate binary mask for Cy-5 surface-labeled Raji cell from Cy5 image
see supporting protocol

4.3) multiply YFP mask image by Cy5 mask image
- contact site is given value of 1
- background is given value of 0

4.4) save resulting image as 'ROI mask'

4.5) use wand tool to select ROI at contact site

4.6) save ROI

5) calculate E-FRET from selected ROI

$R = F_c / I_{DD}$

$E_{app} = R / (R + G)$

5.1) calculate Fc as described above

5.2) substitute mean ROI values into equation above to determine 'R'
5.3) substitute R into equation above to determine E_{app}

6) generate E-FRET image
for display (i.e., not calculation) purposes
6.1) create 32-bit F_c image
6.1.1) multiply YFP image by 'a' parameter
6.1.2) multiply CFP image by 'd' parameter
6.1.3) subtract image generated from product of YFP*a from FRET image
6.1.4) subtract image generated from product of CFP*d from above image
6.2) create F_c/I_{DD} ratio image
6.3) duplicate above image
6.3.1) add G value to entire image
6.4) divide F_c/I_{DD} ratio image by above image

7) create and multiply images by binary mask
for display purposes
7.1) duplicate raw image (CFP, YFP, or FRET)
7.2) name image as 'bin'
7.3) adjust threshold and select cell in red
pixel count in cell is '255'
7.4) continue with final image processing

References:

(158)

Final Image Processing

for displaying processed images

Equipment:

- computer
  - Image J software installed

Materials:

- fluorescence image(s) for mask generation
- image(s) for display
  - fluorescence image(s)
  - calculated / derived image(s)

Supporting Protocol:

- Binary Mask Generation

Protocol:

1) generate binary mask
   for removing background
2) apply binary mask to selected image(s)
   2.1) open image(s)
   2.2) multiply by binary mask
      2.2.1) select 'Process > Image Calculator > Image 1 > mask'
      2.2.2) select 'Operation > Multiply'
      2.2.3) select 'Image 2 > CFP/YFP/FRET/ratio > 32-bit result > OK'
   2.3) save resulting 32-bit image
   2.4) repeat for additional images
3) apply lookup table (LUT)
   3.1) select 'Image > Lookup Tables >'
      e.g., Fire
   3.2) adjust brightness for cell of interest
      define min and max
   3.3) draw saved ROI to display on image
4) crop image(s) for display
   e.g., 250 x 250 pixels
5) annotate cropped images
   5.1) add calibration bar
      5.1.1) select 'Analyze > Tools > Calibration Bar > OK'
         converts to RGB image
   5.2) add image description
   5.3) add pixel count for ROI
6) save final RGB image

5.2.10. Cytokine Detection

Primary Lymphocyte α-TCR Antibody Stimulation

30-hour stimulation of naive splenocytes to assess cytokine secretion

Equipment:

- bucket with ice
- refrigerated centrifuge
- tissue culture hood
- humidified, 37°C, 5% CO_2 incubator
- -20 / -80°C freezer

Materials:

- splenocyte suspension
  - *do not lyse RBCs*
  - freshly isolated
  - purified using CD4+ negative isolation kit (optional)
- 96-well plate, round-bottom
  - *Sarstedt, Cat. # 83.1837*
- T Cell Medium (TCM)
- antibodies
  - anti-CD3ε, clone 145-2C11
    - *BD, Cat. # 553058*
    - *0.5 mg/mL stock*
  - anti-mouse CD28, clone 37.51
    - *BD, Cat. # 553295*
    - *0.5 mg/mL stock*
  - goat anti-armenian hamster IgG
    - *Jackson ImmunoResearch, Cat. # 127-005-099*
    - *1.8 mg/mL stock*
- 1,000x Brefeldin A (BFA)
  - *BioLegend, Cat. # 420601*
  - dilute 1:10 in TCM to prepare 100x working concentration
- GolgiStop / Monensin
  - *BD, Cat. # 554724*

Supporting Protocol:

- Peptide > In Vitro Peptide Transduction

Protocol:

1) optional: pre-treatment with peptide
2) prepare cells
   - 2.1) centrifuge require number of cells for 5 min. at 1,500 rpm (4°C)
   - 2.2) discard supernatant
   - 2.3) resuspend at $5x10^6$ cell/mL in TCM

2.4) add 10^6 lymphocytes (200 μL) per well of round-bottom plate

3) 1° TCR stimulation
- 3.1) add anti-TCR and anti-CD28 antibodies
  do not add to wells designated for non-stimulation control
  - 3.1.1) add 2C11 at 5 μg/mL final
    2 μL of 0.5 mg/mL stock
  - 3.1.2) add anti-CD28 at 5 μg/mL final
    2 μL of 0.5 mg/mL stock
- 3.2) gently resuspend each well
- 3.3) keep in refrigerator for 30 min.
  for binding
- 3.4) add cross-linking antibody
  do not add to wells designed for non-stimulation control
  - 3.4.1) add goat anti-armenian hamster IgG at 5 μg/mL final
  - 3.4.2) gently resuspend each well
- 3.5) keep in refrigerator for 15 min.
  for binding

4) place in TC incubator for 24 hours

5) 2° TCR stimulation
- 5.1) add anti-TCR and anti-CD28 antibodies
  do not add to wells designed for non-stimulation control
  - 5.1.1) add 2C11 at 5 μg/mL final
    2 μL of 0.5 mg/mL stock
  - 5.1.2) add anti-CD28 at 5 μg/mL final
    2 μL of 0.5 mg/mL stock
- 5.2) gently resuspend each well
- 5.3) gently tap plate to mix
  do not resuspend
- 5.4) keep in TC hood for 15 min. (room temp.)
  for binding
- 5.5) add cross-linking antibody
  do not add to wells designed for non-stimulation control
  - 5.5.1) add goat anti-armenian hamster IgG at 5 μg/mL final
  - 5.5.2) gently resuspend each well
- 5.6) keep in TC hood for 15 min. (room temp.)
  for binding

6) for cytokine analysis by ICCS:
- 6.1) add Brefeldin A or GolgiStop at 1x final
  including non-stimulation control well(s)
- 6.2) place in TC incubator for 6 hours
- 6.3) proceed with ICCS protocol

7) for cytokine analysis by ELISA

7.1) place in TC incubator for 6 hours
7.2) harvest supernatants
    7.2.1) gently resuspend each well
    7.2.2) transfer cell suspensions to labeled microcentrifuge tubes
    7.2.3) centrifuge for 10 min. at 1,500 rpm (4°C)
    7.2.4) gently aspirate supernatants
    7.2.5) transfer to new microcentrifuge tubes
7.3) store at -20°C / -80°C until cytokine analysis by ELISA

References:

(2)

Primary Lymphocyte PMA/IM Stimulation

30-hour stimulation of naive splenocytes to assess cytokine secretion

Equipment:

- bucket with ice
- refrigerated centrifuge
- tissue culture hood
- humidified, 37°C, 5% CO_2 incubator
- -20 / -80°C freezer

Materials:

- primary lymphocytes
  - *do not lyse RBCs*
  - freshly isolated
  - purified using CD4+ negative isolation kit (optional)
- T Cell Medium (TCM)
- 96-well plate, round-bottom
  - *Sarstedt, Cat. # 83.1837*
- 100x PMA/IM
- 1,000x Brefeldin A (BFA)
  - *BioLegend, Cat. # 420601*
  - dilute 1:10 in TCM to prepare 100x working concentration
- GolgiStop / Monensin
  - *BD, Cat. # 554724*

Protocol:

1) prepare cells
   - 1.1) centrifuge require number of cells for 5 min. at 1,500 rpm (4°C)
   - 1.2) discard supernatant
   - 1.3) resuspend at $5x10^6$ cell/mL in TCM
   - 1.4) add 10^6 lymphocytes (200 μL) per well of round-bottom plate
2) PMA/IM stimulation
   - 2.1) add PMA/IM at 1x final
     - *do not add to wells designated for non-stimulation control*
   - 2.2) gently resuspend each well
3) for cytokine analysis by ICCS:
   - 3.1) add Brefeldin A or GolgiStop at 1x final
     - *including non-stimulation control well(s)*
   - 3.2) place in TC incubator for 6 hours
   - 3.3) proceed with ICCS protocol
4) for cytokine analysis by ELISA
   - 4.1) place in TC incubator for 6 hours

4.2) harvest supernatants
- 4.2.1) gently resuspend each well
- 4.2.2) transfer cell suspensions to labeled microcentrifuge tubes
- 4.2.3) centrifuge for 10 min. at 1,500 rpm (4°C)
- 4.2.4) gently aspirate supernatants
- 4.2.5) transfer to new microcentrifuge tubes
- 4.2.6) store at -20°C / -80°C until cytokine analysis by ELISA

References:

(2, 159)

Reconstitution of Recombinant Cytokines

for use in skewing / differentiation

Equipment:

- -20°C / -80°C freezer

Materials:

- murine rIL-2
  - *PeproTech, Cat. # 212-12*
  - *100 µg/mL / H_2O [stock]*
  - *0.5 µg/mL / TCM [100x working]*
  - *5 ng/mL / TCM [1x final]*
- murine rIL-4
  - *PeproTech, Cat. # 214-14*
  - *100 µg/mL / H_2O [stock]*
  - *1 µg/mL / TCM [100x working]*
  - *10 ng/mL / TCM [1x final]*
- murine rIL-12
  - *PeproTech, Cat. # 210-12*
  - *200 µg/mL / PBS [stock]*
  - *0.1 µg/mL / TCM [100x working]*
  - *1 ng/mL / TCM [1x final]*

Protocol:

1) centrifuge stock vial prior to opening
2) reconstitute lyophilized stock in H_2O or PBS as indicated
3) dilute to working concentration in TCM
4) store at -20°C or -80°C

References:

(100)

2C11 Adsorption to 96-well Plates

Equipment:

- humidified, 37°C, 5% CO_2 incubator

Materials:

- clone 2C11
  anti-CD3ε
  BD, Cat. # 553058
  0.5 mg/mL
- PBS, tissue culture grade
- microcentrifuge tube
- paper towels
- round-bottom 96-well plates
  Sarstedt, Catalog # 83.1837

Protocol:

1) dilute 2C11 to 1 μg/mL [final] in PBS
   e.g., add 2 μL of 0.5 mg/mL [stock] to 1 mL PBS
2) add 30 μL of 2C11 to each well
3) cover plate and incubate plate for ~2 hrs. in 37°C incubator
4) optional: keep in refrigerator overnight
5) wash wells 3x with PBS
   5.1) add 200 μL of PBS to each well (wash #1)
   5.2) invert plate on stack of paper towels to aspirate
   5.3) repeat for a total of 3 washes
6) remove residual PBS from wells using sterile pipet tips
7) proceed with plate-bound stimulation

In Vitro Splenocyte Differentiation

5 day in vitro priming followed by re-stimulation

Equipment:

- bucket with ice
- humidified, 37°C, 5% CO_2 incubator
- refrigerated centrifuge
- hemacytometer
- -80°C freezer

Materials:

- splenocyte suspension
  - prepared fresh
  - keep on ice
- T Cell Medium (TCM)
- round-bottom 96-well plates (2: 1 each for 1° and 2° stimulation)
  Sarstedt, Cat. # 83.1837
  - coated with 2C11 antibody
    see supporting protocol
- PBS, cell culture grade
- antibodies
  - anti-CD3ε (2C11)
    BD, Cat. # 553058
    0.5 mg/mL
  - anti-mouse CD28 (37.51)
    BD, Cat. # 553295
    0.5 mg/mL
  - rat anti-mouse IL-12
    BD, Cat. # 554475
    1 mg/mL
  - rat anti-mouse IL-4
    BD, Cat. # 554433
    0.5 mg/mL
- recombinant cytokines
  see supporting protocol
  - murine rIL-2, 100x
    500 ng/mL / TCM
  - murine rIL-4, 100x
    1 µg/mL / TCM
  - murine rIL-12, 100x
    100 ng/mL / TCM
- microcentrifuge tubes
- 1,000x Brefeldin A (BFA)
  BioLegend, Cat. # 420601
  - dilute 1:10 in TCM to prepare 100x working concentration

- GolgiStop / Monensin
  BD, Cat. # 554724

Supporting Protocols:

- Plate-Bound 2C11
- Splenocyte Preparation
- Recombinant Cytokine Reconstitution
- Cell Culture > Cell Counting

Protocol:

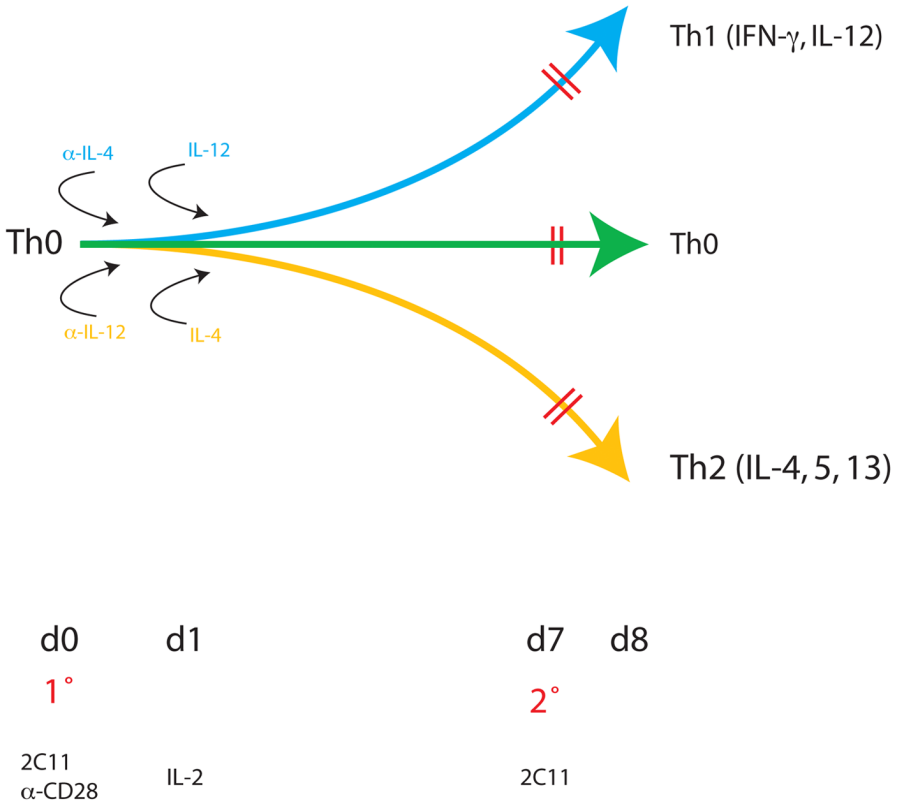


Figure 55: *In vitro* skewing model.

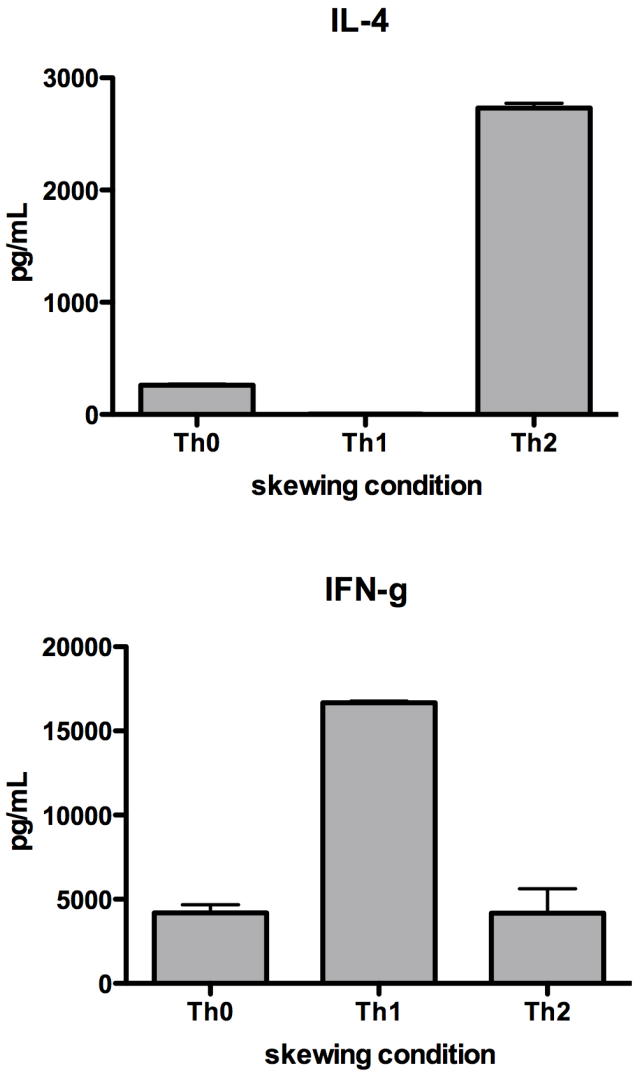


Figure 56: Measurement of cytokine secretion after Th0, Th1 or Th2 skewing *in vitro*, respectively.

1) 1° stimulation

day 0

1.1) adsorb 2C11 at 1 μg/mL final to designated wells of 96-well plate for 2 hrs and wash with PBS

see supporting protocol

1.2) adjust splenocyte concentration to 10^6 cells/mL in TCM

1.3) add $2x10^6$ cells (200 μL) per well containing plate-bound 2C11

1.4) add soluble anti-CD28 at 1 μg/mL final

1.4.1) dilute 0.5 mg/mL stock to 0.1 mg/mL in PBS

1.4.2) add 2 μL per well

2) add neutralizing antibody

day 0

2.1) for Th 1 conditions: add Th2 neutralizing antibody

2.1.1) add anti-IL-4 at 5 μg/mL final

2.2) for Th 2 conditions: add Th1 neutralizing antibody

2.2.1) add anti-IL-12 at 20 μg/mL final

4 μL of 1 mg/mL stock per well

2.2.2) mix each well using micropipettor and tip

2.2.3) return plate to incubator for 24 hours

3) addition of recombinant cytokine

day 1

3.1) Th 0 / 1 / 2 conditions

3.1.1) add mrIL-2 at 5 ng/mL final

2 μL of 100x stock per well

3.2) Th 1 conditions

3.2.1) add mrIL-12 at 1 ng/mL final

3.3) Th 2 conditions

3.3.1) add mrIL-4 at 10 ng/mL final

3.4) gently tap edges of plate to mix

3.5) return plate to incubator for 4 days

for proliferation and differentiation

4) replace 50% medium as needed

days 3/4

4.1) carefully remove / discard 100 μL per well

do not disturb cells

4.2) add 100 μL fresh TCM

4.3) return plate to incubator

5) harvest differentiated splenocytes

day 5

keep cells and medium at 4°C throughout

5.1) gently resuspend each well

5.2) transfer to sterile, pre-labeled microcentrifuge tubes

5.3) wash 3x

removes cytokines from medium prior to restimulation

5.3.1) wash with 1mL of ice-cold TCM

5.3.2) centrifuge for 5 min at 1500 rpm (4°C)

5.3.3) aspirate / discard supernatant

5.4) resuspend in estimated volume of TCM for counting using hemacytometer

5.5) adjust concentration to 2.5 $x10^5$ cells/mL in TCM

6) 2° stimulation

day 5

6.1) adsorb 2C11 at 1 μg/mL final to designated wells of 96-well plate for 2 hrs and wash with PBS

see supporting protocol

6.2) add $5x10^4$ cells (200 μL) per well containing plate-bound 2C11

6.3) gently tap edges of plate to mix

6.4) return to incubator for 24 hours

for cytokine production / secretion

7) assay

day 6

7.1) for cytokine analysis by ICCS:

7.1.1) add Brefeldin A or GolgiStop at 1x final

including non-stimulation control well(s)

7.1.2) place in TC incubator for final 6 hours

7.1.3) proceed with ICCS protocol

7.2) for ELISA

7.2.1) gently resuspend each well

7.2.2) transfer cell suspensions to labeled microcentrifuge tubes

7.2.3) centrifuge for 10 min at 1500 rpm (4°C)

7.2.4) gently aspirate supernatants

7.2.5) transfer to new microcentrifuge tubes

7.2.6) store supernatants at -80°C until cytokine analysis by ELISA

References:

(100, 101, 160)

ELISA

enzyme-linked immunosorbent assay

Equipment:

- microplate spectrophotometer
- computer with Softmax Pro installed
- multi-channel pipettor

Materials:

- samples
  - stored at -20°C / -80°C
- buffers
  equilibrate to room temp. prior to use
  - PBS / Coating Buffer
  - Blocking Buffer
  - Reaction Buffer
  - Wash Buffer
  - Stop Solution
- 96-well ELISA plate(s)
- plate cover(s)
- parafilm
- pipet tips, racked
- pipet basins
- 15 mL centrifuge tubes
- microcentrifuge tubes
- paper towels
- recombinant standard
  - stored at -80°C
  - dilute in Reaction Buffer
- streptavidin (HRP-conjugated)
  - dilute in Reaction Buffer
- TMB substrate
- IL-4
  sensitivity: 4 pg/mL
  - capture: clone 11B11
    eBioscience, Cat. # 14-7041; 250x
    2 µg/mL [final] in Coating Buffer
  - standard: 1 µg/mL mrIL-4
    eBioscience, Cat. # 14-8041-62
    500 pg/mL - 7.8 pg/mL, diluted serially in Reaction Buffer 1:2
  - detection: biotin clone BVD6-24G2
    eBioscience, Cat. # 13-7042; 250x
    2 µg/mL [final] in Reaction Buffer
- IL-5

sensitivity: 4 pg/mL

- capture: clone TRFK5
  eBioscience, Cat. # 13-7051; 250x
  2 µg/mL [final] in Coating Buffer
- standard: 1 µg/mL mrIL-5
  eBioscience, Cat. # 14-8051-62
  5,000 pg/mL - 6.9 pg/mL, diluted serially in Reaction Buffer 1:3
- detection: biotin clone TRFK4
  eBioscience, Cat. # 13-7051; 250x
  BioLegend, Cat. # 504402; 0.5 mg/mL (250x)
  2 µg/mL [final] in Reaction Buffer

- IL-13

sensitivity: 4 pg/mL

- capture: clone eBio13A
  eBioscience, Cat. # 14-7133; 250x
  2 µg/mL [final] in Coating Buffer
- standard: 1 µg/mL mrIL-13
  eBioscience, Cat. # 14-8131-62
  10,000 pg/mL - 13.7 pg/mL, diluted serially in Reaction Buffer 1:3
- detection: biotin clone eBio1316H
  eBioscience, Cat. # 13-7135; 250x
  2 µg/mL [final] in Reaction Buffer

- IFN-γ
  - capture: clone R4-6A2
    BD, Cat. # 551216; 250x
    eBioscience, Cat. # 14-7312-85; 0.5 mg/mL (250x)
    4 µg/mL [final] in Coating Buffer
  - standard: mrIFN-γ
    eBioscience, Cat. # 14-8311-63
    10,000 pg/mL - 13.7 pg/mL, diluted serially in Reaction Buffer 1:3
  - detection: biotin clone XMG1.2
    BD, Cat. # 554410; 250x
    eBioscience, Cat. # 13-7311-85; 0.5 mg/mL (250x)
    2 µg/mL [final] in Reaction Buffer

Protocol:

1) coat plate with capture antibody
    1.1) dilute in coating buffer / PBS according to recommendations
        prepare in 15 mL tube
    1.2) transfer to pipet basin
    1.3) add 50 µL per well
    1.4) tap plate to cover each well evenly
    1.5) cover plate
    1.6) seal plate with parafilm
    1.7) incubate overnight at 4°C
2) block plate
    2.1) remove plate from refrigerator
    2.2) decant plate in sink
    2.3) wash 3x with 300 µL wash buffer per well
    2.4) tap plate on top of paper towels to remove excess liquid
    2.5) add 150 µL of blocking buffer to each well
        3x volume of coating solution
    2.6) incubate for 1.5 - 2 hrs. at room temp.
3) add standards / samples
    3.1) equilibrate samples / standards to room temp.
    3.2) prepare 7 standard concentrations by performing serial dilutions in reaction buffer according to recommendations
    3.3) decant plate in sink
    3.4) wash 3x with 300 µL wash buffer per well
    3.5) tap plate on top of paper towels to remove excess liquid
    3.6) add 50 µL of standards / samples to designated wells
    3.7) incubate for 1.5 - 2 hrs. at room temp.
4) add biotin-conjugated detection antibody
    4.1) dilute biotin-conjugated detection antibody in reaction buffer according to recommendations
        prepare in 15 mL Falcon tube
    4.2) decant plate in sink
    4.3) wash 3-5x with 300 µL wash buffer per well
    4.4) tap plate on top of paper towels to remove excess liquid
    4.5) add 50 µL of detection antibody solution per well
    4.6) incubate for 1.5 - 2 hrs. at room temp.
5) add streptavidin-HRP
    5.1) dilute streptavidn-HRP to 1x in reaction buffer
        prepare in 15 mL Falcon tube
    5.2) decant plate in sink
    5.3) wash 3-5x with 300 µL wash buffer per well
    5.4) tap plate on top of paper towels to remove excess liquid
    5.5) add 50 µL of streptavidin-HRP per well
    5.6) incubate for 3/4 to 1 hr. at room temp.

6) add substrate
    6.1) equilibrate substrate solution to room temp. prior to use
    6.2) decant plate in sink
    6.3) wash 5-7x with 300 μL wash buffer per well
    6.4) tap plate on top of paper towels to remove excess liquid
    6.5) add 50 μL of 1x substrate solution to each well
    6.6) observe development of blue product in wells containing standards / samples
    6.7) incubate for ≤ 30 min. at room temp.
7) add stop solution
    7.1) add 25 μL of stop solution to each well
        observe color change from blue to yellow product
    7.2) measure absorbance at 450 nm and 650 nm
        7.2.1) subtract absorbance at 650 nm from each well
            to normalize for optical differences in plastic from well to well

ELISPOT

ELISA Spot Assay
- cytokine captured at location of production by cell on filter plate
- allows for quantification of # of cytokine producing cells

Equipment:

- multi-channel pipettor
- humidified, 37°C, 5% CO_2 incubator
- biosafety cabinet (tissue culture hood)
- Zeiss ELISpot reader, automated
    - with KS ELISpot software installed

Materials:

- MultiScreen HTS 96-well plates
    Millipore, Cat. # MSIPS4510
- PBS
    for dilution of capture antibody
- Wash Buffer
    0.05% Tween-20 / PBS
- ELISPOT Blocking Buffer
    1% BSA / PBS + 0.05% Tween-20, pH 7.4
- primary lymphocytes
    see supporting protocol
    - prepare fresh
    - lyse RBCs
    - keep on ice
- IL-4
    - capture: clone 11B11
        eBioscience, Cat. # 14-7041; 125x
        4 µg/mL [final] in PBS
    - detection: biotin clone BVD6-24G2
        eBioscience, Cat. # 13-7042; 1,000x
        0.5 µg/mL [final] in ELISPOT Blocking Buffer
- IL-5
    - capture: clone TRFK5
        eBioscience, Cat. # 14-7052; 125x
        4 µg/mL [final] in PBS
    - detection: biotin clone TRFK4
        eBioscience, Cat. # 13-7051; 500x
        1 µg/mL [final] in ELISPOT Blocking Buffer
- IL-10
    - capture:
        4 µg/mL [final] in PBS
    - detection:
        2 µg/mL [final] in ELISPOT Blocking Buffer

- IL-13
    - capture: clone eBio13A
        eBioscience, Cat. # 14-7133; 125x
        4 µg/mL [final] in PBS
    - detection: biotin clone eBio1316H
        eBioscience, Cat. # 13-7135; 500x
        1 µg/mL [final] in ELISPOT Blocking Buffer
- IFN-γ
    - capture: clone R4-6A2
        BD, Cat. # 551216; 250x
        4 µg/mL [final] in PBS
    - detection: biotin clone XMG1.2
        BD, Cat. # 554410; 250x
        2 µg/mL [final] in ELISPOT Blocking Buffer
- HRP-streptavidin, 250x stock
    BD, Cat. # 51-9002812
- parafilm
- pipet tips, racked
- filter pipet tips
- pipet basins, sterile
    for dispensing capture antibody
- pipet basins, non-sterile
    for spot development

Supporting Protocols:

- In Vivo / Ex Vivo > Splenocyte Preparation
    - Tissue Dissociation
- OVA System > OVA Restimulation (*in vitro*)

Protocol:

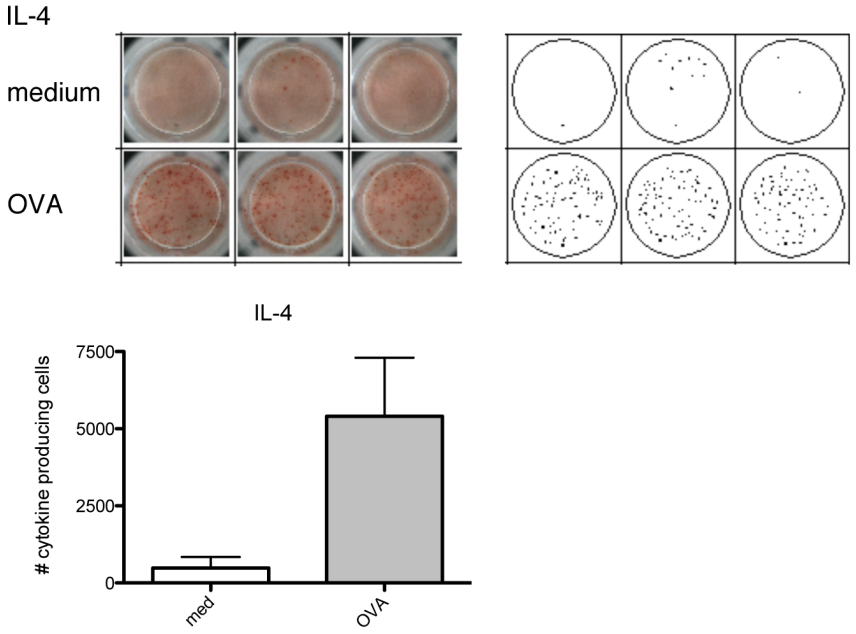


Figure 57: Detection of IL-4 producing cells by as measured by ELISpot assay.

On day 0, a C57BL/6 mouse was sensitized with OVA/alum. After 7 days, splenocytes were restimulated on an ELISPOT plate coated with anti-IL-4 capture antibody. Spots were developed according to protocol below after being cultured *in vitro* for 4 days.

1) coat ELISPOT plate with capture antibody
   work in tissue culture hood
   1.1) dilute capture antibody in PBS to 2-4 μg/mL final in PBS
      see recommendations above
   1.2) add 50 μL to designated wells of ELISPOT plate
   1.3) firmly tap edges of plate to cover bottom of each well completely
   1.4) cover plate and seal with parafilm
   1.5) place in refrigerator overnight
2) block ELISPOT plate
   work in tissue culture hood
   2.1) decant capture antibody solution from ELISPOT plate
      carefully remove residual volume using pipet tips
   2.2) add 150 μL ELISPOT Blocking Buffer to each well
   2.3) cover plate and place inside 37°C tissue culture incubator for 2 hrs.
3) seed ELISPOT plate
   see 'OVA Restimulation (in vitro)' supporting protocol

3.1) cover plate and place inside 37°C tissue culture incubator for 2-4 days

cytokine-specific kinetics

4) develop ELISPOT plate

work at lab bench

4.1) incubation with detection antibody
4.2) decant cell suspension from each well
4.3) carefully remove residual volume using pipet tips
4.4) add 300 μL of wash buffer to designated wells (wash #1)
4.5) decant wash buffer
4.6) repeat for a total of 3 washes
4.7) dilute biotin-conjugated detection antibody to 0.5 - 2 μg/mL final in ELISPOT blocking buffer

see recommendations above

4.8) incubate for 2 hrs. at room temp. in dark

5) incubation with streptavidin-HRP

5.1) decant detection antibody solution from each well

carefully remove residual volume using pipet tips

5.2) add 300 μL of wash buffer to designated wells (wash #1)
5.3) decant wash buffer
5.4) repeat for a total of 3 washes
5.5) dilute streptavidin-HRP stock 1:250 in ELISPOT blocking buffer
5.6) add 50 μL of streptavidin-HRP solution to designated wells
5.7) incubate for 45 min. at room temp. in dark

6) substrate addition

6.1) decant streptavidin-HRP solution from each well

carefully remove residual volume using pipet tips

6.2) add 300 μL of wash buffer to designated wells (wash #1)
6.3) decant wash buffer
6.4) repeat for a total of 5 washes
6.5) add 100 μL ELISPOT substrate solution to designated wells
6.6) incubate for 5 - 30 min.

monitor plate for development of spots

6.7) decant substrate solution
6.8) remove plastic backing from plate
6.9) wash plate with running tap water
6.10) allow plate to dry overnight

ICCS, Part I

surface staining (e.g., CD4) followed by fixation

Equipment:

- vortex
- refrigerated centrifuge
- bucket with ice
- refrigerator

Materials:

- lymphocytes
  - stimulated / non-stimulated
  - incubated with Brefeldin A / GolgiStop at 1x for 6 hours
- FACS Buffer
  - keep on ice
- FACS tubes (1 per staining condition)
- blocking agent
  - *select to match species of staining antibody*
  - *prepare for multiple tubes (100 µL each) at 1% (w/v) final in FACS Buffer*
  - *keep on ice*
  - normal rat serum
    Jackson ImmunoResearch, Cat. # 012-000-001
    60 mg/mL
- staining antibodies
  - *prepare for multiple tubes (200 µL each) at 2.5 µg/mL final in FACS Buffer*
  - *keep on ice*
  - Alexa Fluor 647 rat anti-mouse CD4
    BioLegend, Cat. # 100530
    0.5 mg/mL stock
    ex: 633 / 635 nm
    em: 668 nm
  - Pacific Blue rat anti-mouse CD4
    BioLegend, Cat. # 100531
    0.5 mg/mL stock
    ex: 405 nm
    em: 455 nm
  - PE anti-TCRβ, clone H57-597
    BioLegend, Cat. # 109208
  - APC anti-TCRβ, clone H57-597
    BioLegend, Cat. # 109211
- PBS
  - keep on ice

- 2% paraformaldehyde / PBS
    - keep on ice

Supporting Protocols:

- Cell Culture > Cell Counting
- Primary Lymphocyte Stimulation
- In Vitro Splenocyte Differentiation

Protocol:

keep cells on ice throughout to reduce secretion of intracellular cytokines

1) perform cell count
    see supporting protocol
2) aliquot cells to designated FACS tubes
    2.1) add $4x10^6$ cells to each tube
    2.2) add 2 mL of ice-cold FACS Buffer
    2.3) vortex / wash
    2.4) centrifuge for 5 min. at 1,500 rpm (4°C)
    2.5) discard supernatant
3) optional: block
    this step is important if cells were stimulated using a secondary / cross-linking antibody that may capture the antibody used for surface staining and thus create a high background signal
    3.1) add 100 μL of ice-cold 1% (w/v) normal rat serum in FACS Buffer to each tube
    3.2) vortex / resuspend
    3.3) block on ice for 20 min.
4) stain cells
    4.1) add 2 mL of ice-cold FACS Buffer to each tube
    4.2) vortex to wash
    4.3) centrifuge for 5 min. at 1,500 rpm (4°C)
    4.4) discard supernatant
    4.5) add 200 μL of ice-cold 2.5 μg/mL staining antibody in FACS Buffer to each tube
    4.6) vortex / resuspend
    4.7) stain on ice for 20 min. in dark
5) fix cells
    5.1) add 2 mL of ice-cold PBS to each tube
    5.2) vortex / wash
    5.3) centrifuge for 5 min. at 1,500 rpm (4°C)
    5.4) discard supernatant
    5.5) add 500 μL of 2% paraformaldehyde / PBS to each tube
    5.6) vortex / resuspend
    5.7) fix on ice for 10 min. in dark

6) wash cells
    6.1) add 2 mL of ice-cold FACS Buffer to each tube
        to quench paraformaldehyde
    6.2) centrifuge for 5 min. at 1,500 rpm (4°C)
    6.3) discard supernatant
    6.4) add 500 μL ice-cold FACS Buffer to each tube
    6.5) vortex / resuspend
    6.6) store at 4°C in dark
        proceed with Intracellular Cytokine Staining (ICCS) protocol within 24 hours

References:

(159)

ICCS, Part II

permeabilization and intracellular cytokine staining

Equipment:

- vortex
- refrigerated centrifuge
- bucket with ice
- refrigerator

Materials:

- lymphocytes ($4x10^6$ per FACS tube)
  - stimulated (and non-stimulated control)
  - incubated with Brefeldin A / GolgiStop at 1x for 6 hours
  - stained for expression of surface protein(s)
  - fixed
- FACS Buffer
  - keep on ice
- FACS tubes (1 per staining condition)
- Permeabilization Buffer
  see Buffers & Solutions
- Blocking Buffer
  see Buffers & Solutions
- staining antibodies
  - prepare for multiple tubes (50 µL each) at 2.5 µg/mL final in Blocking Buffer
  - keep on ice
  - AlexaFluor 647 rat isotype control
    BioLegend, Cat. # 400526
    ex: 633 / 635 nm
    em: 668 nm
  - AlexaFluor 647 rat anti-mouse CD4
    ex: 633 / 635 nm
    em: 668 nm
  - AlexaFluor 488 rat isotype control
    BioLegend, Cat. # 400417
    ex: 488 nm
    em: 519 nm
  - AlexaFluor 488 rat anti-mouse IFN-γ
    BioLegend, Cat. # 505813
    ex: 488 nm
    em: 519 nm

Supporting Protocol:

- ICCS, Part I

Protocol:

- saponin must be present in all staining and washing buffers as it is a reversible permeabilization agent
- stain with isotype-control antibody to determine background and for defining negative gate for FACS analysis

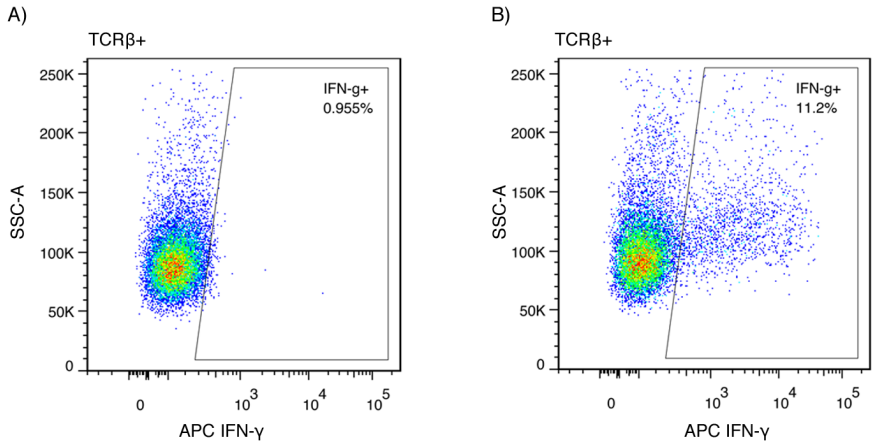


Figure 58: Measurement of IFN-γ expression by ICCS method.
BL/6 splenocytes left untreated (A) or stimulated with PMA/IM for 6 hrs. (B) were surface stained for TCRβ and IFNγ according to ICCS protocol described above.

1) permeabilize cells
    1.1) add 2 mL of ice-cold FACS Buffer to each tube
    1.2) centrifuge for 5 min. at 1,500 rpm (4°C)
    1.3) discard supernatant
    1.4) add 1 mL of Permeabilization Buffer to each tube
    1.5) vortex / resuspend
2) block
    2.1) transfer $2x10^6$ cells to new FACS tube for intracellular staining
    2.2) centrifuge for 5 min. at 1,500 rpm (4°C)
    2.3) aspirate supernatant
    2.4) add 50 μL of Blocking Buffer to each tube
    2.5) vortex / resuspend
    2.6) incubate for 30 min. on ice in dark
3) stain cells
    3.1) add 1 mL of Blocking Buffer to each tube
    3.2) centrifuge for 5 min. at 1,500 rpm (4°C)
    3.3) aspirate / supernatant
    3.4) add 50 μL of ice-cold 2.5 μg/mL staining antibody in Blocking Buffer to each tube

3.5) incubate for 30 min. on ice in dark

4) wash (2x)
   - 4.1) add 1 mL of Permeabilization Buffer to each tube
   - 4.2) vortex / wash
   - 4.3) centrifuge for 5 min. at 1,500 rpm (4°C)
   - 4.4) discard supernatant
   - 4.5) repeat
   - 4.6) add 300-500 μL of FACS Buffer to each tube
   - 4.7) vortex / resuspend

5) store at 4°C in dark

complete FACS analysis within 24 hours

References:

(159)

5.2.11. Lung Inflammation / Asthma

Serum Immunoglobulin (Ig) Detection by ELISA

total and/or OVA-specific for IgG, IgE

Equipment:

- microplate spectrophotometer
- computer with Softmax Pro installed
- multi-channel pipettor

Materials:

- C57BL/6J mice (6-12 weeks old)
  - sensitized with OVA/alum
    see supporting protocol
  - challenged
    see supporting protocol
  - serum collected
    see supporting protocol
  - serum samples stored at -20°C / -80°C
    - for IgG1, 1:1,000-10,000 starting, diluted serially 1:4 in PBS
    - for other isotypes, 1:100 starting, diluted serially 1:4 in PBS
- buffers
  equilibrate to room temp. prior to use
  - PBS
  - Blocking Buffer
  - Reaction Buffer
  - Wash Buffer
  - Stop Solution
- 96-well ELISA plate(s)
- plate cover(s)
- parafilm
- pipet tips, racked
- pipet basins
- 15 mL centrifuge tubes
- microcentrifuge tubes
- paper towels
- capture
  - goat anti-mouse IgG
    - for IgG1, IgG2a, IgG2c, IgG3
    - SouthernBiotech, Cat. # 1010-01; 1 mg/mL (1,000x)
    - 1 µg/mL final in PBS
  - anti-mouse IgG2b
    - SouthernBiotech, Cat. # 553396; 0.5 mg/mL (250x)
    - 2 µg/mL final in PBS
  - goat anti-mouse IgE
    - SouthernBiotech, Cat. # 1110-01; 1 mg/mL (200x)

- *5 µg/mL final in PBS*
- OVA/PBS
  - *prepare at 0.5 mg/mL in PBS; 50x*
  - *10 µg/mL final in PBS*

- standard
  - mouse IgG1, clone 15H6
    - *SouthernBiotech, Cat. # 0102-01; 1 mg/mL*
    - *10 ng/mL starting, diluted serially 1:2 in PBS*
  - mouse IgG2a, clone HOPC-1
    - *SouthernBiotech, Cat. # 0103-01; 1 mg/mL*
    - *10 ng/mL starting, diluted serially 1:2 in PBS*
  - mouse IgG2b, clone A-1
    - *SouthernBiotech, Cat. # 0104-01; 1 mg/mL*
    - *1,000 ng/mL starting, diluted serially 1:2 in PBS*
  - mouse IgG2c
    - *Bethyl Laboratories - ALEXIS, Cat. # RS110-112; 0.55 mg/mL*
    - *10 ng/mL starting, diluted serially 1:2 in PBS*
  - mouse IgG3
    - *BD, Cat. # 553486; 0.5 mg/mL*
    - *5 ng/mL starting, diluted serially 1:2 in PBS*
  - mouse IgE, clone 15.3
    - *SouthernBiotech, Cat. # 0114-01; 0.25 mg/mL*
    - *200 ng/mL starting, diluted serially 1:2 in PBS*
- detection
  - biotin goat anti-mouse IgA
    - *SouthernBiotech, Cat. # 1040-08; 0.5 mg/mL (5,000x)*
    - *0.1 µg/mL final in Reaction Buffer*
  - biotin goat anti-mouse IgM
    - *SouthernBiotech, Cat. # 1020-08; 0.5 mg/mL (5,000x)*
    - *0.1 µg/mL final in Reaction Buffer*
  - biotin goat anti-mouse IgG
    - *SouthernBiotech, Cat. # 1034-08; 0.5 mg/mL (5,000x)*
    - *0.1 µg/mL final in Reaction Buffer*
  - biotin goat anti-mouse IgG1
    - *SouthernBiotech, Cat. # 1070-08; 0.5 mg/mL (5,000x)*
    - *0.1 µg/mL final in Reaction Buffer*
  - biotin goat anti-mouse IgG2a
    - *SouthernBiotech, Cat. # 1080-08; 0.5 mg/mL (5,000x)*
    - *0.1 µg/mL final in Reaction Buffer*
  - biotin anti-mouse IgG2b, clone R12-3
    - *Pharmingen, Cat. # 553393*
    - *2 µg/mL final in Reaction Buffer*
  - biotin goat anti-mouse IgG2c
    - *SouthernBiotech, Cat. # 1079-08; 0.5 mg/mL (5,000x)*
    - *0.1 µg/mL final in Reaction Buffer*

- biotin goat anti-mouse IgG3
  - *SouthernBiotech, Cat. # 1100-08; 0.5 mg/mL (5,000x)*
  - *0.1 µg/mL in Reaction Buffer*
- biotin rat anti-mouse IgE, clone 23G3
  - *SouthernBiotech, Cat. # 1130-08; 0.5 mg/mL (5,000x)*
  - *0.1 µg/mL in Reaction Buffer*
- streptavidin (HRP-conjugated)
  dilute to 1x in Reaction Buffer
- TMB substrate

Supporting Protocols:

- In Vivo / Ex Vivo > Retroorbital Blood Collection
- Cytokine Detection > ELISA

Protocol:

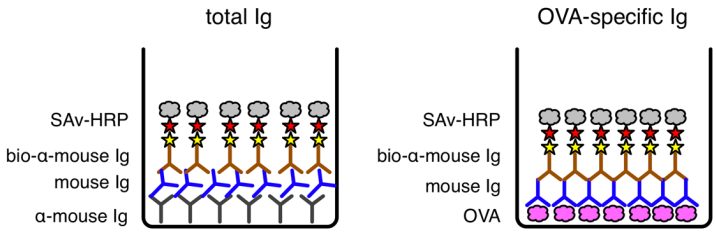


Figure 59: Detection of mouse immunoglobulin by ELISA.

1) coat plate with anti-mouse Ig isotype antibody / OVA
   - *for detection of total Ig, coat both sample and standard wells with anti-mouse isotype Ig*
   - *for detection of OVA-specific Ig, coat sample wells with OVA and standard wells with anti-mouse isotype Ig*

   1.1) dilute anti-mouse Ig isotype antibody to final concentration in PBS
   - *see above for recommended titer*
   - *prepare in 15 mL tube*

   1.2) dilute OVA to 10 µg/mL final in PBS
   prepare in 15 mL tube

   1.3) transfer to pipet basin
   1.4) add 50 µL per well
   1.5) tap plate to cover each well evenly
   1.6) cover plate
   1.7) seal plate with parafilm
   1.8) incubate overnight at 4°C

2) block plate

   2.1) remove plate from refrigerator
   2.2) decant plate in sink

2.3) wash 3x with 300 μL wash buffer per well
2.4) tap plate on top of paper towels to remove excess liquid
2.5) add 150 μL of blocking buffer to each well
3x volume of coating solution
2.6) incubate for 1.5 - 2 hrs. at room temp.
3) prepare dilutions of standard
purified mouse Ig isotype
3.1) prepare starting concentration in PBS
see above for recommended titer
3.2) prepare 7 standard titers by diluting serially 1:2 in PBS
4) prepare dilutions of samples
4.1) equilibrate serum samples to room temp.
4.2) prepare starting concentration in PBS
see above for recommended titer
4.3) prepare 7 sample titers by diluting serially 1:4 in PBS
5) add standards / samples to plate
5.1) decant plate in sink
5.2) wash 3x with 300 μL wash buffer per well
5.3) tap plate on top of paper towels to remove excess liquid
5.4) add 50 μL of standards / samples to designated wells
5.5) incubate for 1.5 - 2 hrs. at room temp.
6) add detection antibody
biotin anti-mouse Ig isotype antibody
6.1) dilute biotin anti-mouse Ig isotype antibody to final concentration in reaction buffer
- see above for recommended titer
- prepare in 15 mL Falcon tube
6.2) decant plate in sink
6.3) wash 3-5x with 300 μL wash buffer per well
6.4) tap plate on top of paper towels to remove excess liquid
6.5) add 50 μL of detection antibody solution per well
6.6) incubate for 1.5 - 2 hrs. at room temp.
7) continue with ELISA protocol as described for cytokine detection

Bronchoalveolar Lavage

perform on assay day

Equipment:

- bucket with ice
- light source for dissection
- dissection board
- cannula
  - polyethylene tubing
    - *Intramedic, Cat. # 427411*
    - *I.D. 0.58 mm; O.D. 0.965 mm; length 30.5 m*
  - 23G needle
- sharp surgical scissors (1)
- forceps (2)
- refrigerated centrifuge
- vortex
- -20°C / -80°C freezer
  for storage of BALF supernatant

Materials:

- C57BL/6J mice (6-12 weeks old)
  - sensitized with OVA/alum
    see supporting protocol
  - challenged with OVA
    see supporting protocol
- PBS
  - keep on ice
- microcentrifuge tubes (2 per mouse)
  - *for collection of BALF from lavage #1 (1 per mouse)*
  - *for storage of BALF supernatant from lavage #1 (1 per mouse)*
  - label with mouse ID
- FACS tubes with caps (1 per mouse)
  - *for collection of BALF from lavage #s 2-5*
  - *5 mL round-bottom (12 x 75 mm)*
  - *BD, Cat. # 352054*
  - label with mouse ID
- surgical suture
  - *size 4-0 silk*
  - *CA Surgical Instruments & Supplies, Cat. # SP116*
  - cut piece for each mouse
- 1 mL syringe
  BD, Cat. # 309602
- ACK Lysis Buffer
- FACS Buffer

- keep on ice
- TCM
  cRPMI + 10% FBS
- keep on ice

Supporting Protocols:

- OVA System > OVA/Alum Sensitization
- OVA System > OVA Challenge
- In Vivo / Ex Vivo > Mouse Euthanasia Using Isoflurane
- In Vivo / Ex Vivo > Mouse Dissection
- Cell Culture > Cell Counting

Protocol:

- BALF collected from lavage #1 contains the highest titer of cytokines and the supernatant is retained for cytokine analysis by ELISA

- BALF collected from lavage #s 2-5 are pooled to increase the cell number for preparation of cytospin slides and/or FACS analysis

1) sacrifice mouse
   - *proceed serially for each mouse*
   - *see supporting protocol*
2) perform chest cavity dissection
   - *see supporting protocol*
   - *do not use pins to open rib cage*
3) perform tracheotomy
   - 3.1) using forceps, separate muscle at midline covering trachea
   - 3.2) using forceps, remove membrane above trachea at cartilage ring
     avoid rupturing blood vessels on either side of trachea
   - 3.3) using forceps, puncture membrane below trachea
   - 3.4) thread surgical suture below trachea using forceps
   - 3.5) make single, small incision through trachea at cartilage ring
   - 3.6) insert cannula through opening of trachea and extend ~1 mm
   - 3.7) fasten cannula in place by tying knot with surgical suture
4) lavage #1
   keep on ice following collection
   - 4.1) fill syringe with 1 mL of cold PBS
   - 4.2) attach to cannula
   - 4.3) depress plunger to dispense PBS into lungs
     confirm proper delivery by observing expansion of lungs
   - 4.4) steadily withdrawal plunger to aspirate BALF
   - 4.5) dispense BALF from lavage #1 to designated microcentrifuge tube

4.6) detach syringe from cannula

5) lavage #s 2-5

keep on ice following collection

5.1) repeat 4x as for lavage #1

5.2) dispense BALF from lavage #s 2-5 into same FACS tube

6) repeat from above for each mouse

7) centrifuge microcentrifuge and FACS tubes containing BALF for 5 min. at 1,500 rpm (4°C)

proceed in parallel for all BALF samples

8) for each mouse, transfer supernatant from microcentrifuge tube (containing BALF from lavage #1) to separate microcentrifuge tube

retain BALF cells in pellet

8.1) store BALF supernatant samples at -20 / -80°C

for cytokine measurement by ELISA

9) discard supernatants from FACS tubes

retain BALF cells in pellet

10) lyse RBCs

see supporting protocol

10.1) add 200 μL of ACK lysis buffer to each tube

10.2) for each mouse, transfer cells from microcentrifuge tube to corresponding FACS tube

10.3) incubate for 5 min. at room temp.

10.4) add 2 mL of ice-cold FACS buffer to each tube

10.5) centrifuge for 5 min. at 1,500 rpm (4°C)

10.6) discard supernatant

11) resuspend BALF cells in 300 μL of FACS buffer / TCM

optional: resuspend BALF cells in TCM and store overnight at 4°C

12) perform cell count

see supporting protocol

13) keep cells on ice

14) proceed to prepare cytospin slides and/or FACS staining

Cytospin Preparation

- perform on assay day
- modified Wright-Giemsa stain

Equipment:

- centrifuge with FACS tube adaptors
- Shandon Cytoclips
    - *Thermo Scientific, Cat. # 59910052*
    - *for assembling slide / filter card / funnel*
- Shandon Cytospin 2 centrifuge
- dI water supply
- staining dishes
- slide rack for staining
- slide folder
- refrigerator

Materials:

- BALF cell suspension
    - collected fresh (≤ 24 hours)
    - keep on ice
- FACS buffer
- microcentrifuge tubes (1 per mouse / sample)
- glass slides (1 per mouse / sample)
    - *25 x 75 x 1 mm*
    - *Fisher, Cat. # 12-544-7*
- single cytology funnels with attached cards and caps (1 per mouse / sample)
    Biomedical Polymers, Inc., Cat. # BMP-CYTO-S50 / VWR, Cat. # 80094-254
- Hema 3 stain set
    - *Protocol, Cat. # 122-911 / Fisher, Cat. # 22-122-911*
    - *fixative solution, Solution I, Solution II*
- coverslips, No. 1 (1 per mouse / sample)
    - *18 x 18 mm*
    - *Fisher, Cat. # 12-548-A*
- Permount mounting medium
    Fisher, Cat. # SP15-100

Supporting Protocol:

- Bronchoalveolar Lavage

Protocol:

1) prepare in advance for each mouse / sample:
    1.1) label microcentrifuge tubes
        for aliquot of 10^5 BAL cells for slide attachment
    1.2) label glass slides
        - write on frosted area of slide with pencil
        - include date and mouse ID
    1.3) set aside coverslips
    1.4) set aside cytology funnels (with attached cards and caps)
2) aliquot 10^5 cells to designated microcentrifuge tube for each mouse / sample
3) adjust to 400 μL final volume per tube with FACS buffer
4) assemble slide / filter card / funnel using Shandon Cytoclip
    listed in order from back to front
5) attach 10^5 cells / 400 μL to each slide using Cytospin 2 centrifuge
    5.1) add cell suspension to funnel
    5.2) attach cap
    5.3) centrifuge for 5 min at 600 rpm (low acceleration)
        display reads '05' for 5 minutes and '60' for 600 rpm
    5.4) remove slides from adaptors
6) allow cells to air dry for 5-10 mins.
7) place slides in rack for staining
8) stain cells using Hema 3 stain set
    proceed in order below
    8.1) pour solutions into respective staining dishes
    8.2) dip slides 5x into fixative solution
    8.3) dip slides 5x into Solution I
    8.4) dip slides 5x into Solution II
    8.5) rinse slides in running dI water
9) remove slides from staining rack
10) allow to air dry for 5-10 mins.
11) add 10 μL of Permount mounting medium over cells
12) apply coverslip
13) allow to mounting medium to cure
14) store slides in folder in refrigerator
    protect from light

Lung Tissue Fixation

- perform on assay day
- extract separate lung lobes for fixation and preparation of cell suspension, respectively

Equipment:

- light source for dissection
- forceps (1)
- scissors (1)
- beaker with 70% ethanol
  for sterilizing surgical instruments
- bucket with ice

Materials:

- C57BL/6J mice (6-12 weeks old)
  - sensitized with OVA/alum
  - challenged with OVA
- 10 cm diameter petri dishes (1 per group)
  Corning, Cat. # 430167
  - fill with PBS
- PBS, sterile
- scintillation vials, 20 mL (1 per mouse / sample)
  Fisher, Cat. # 03-337-5
  - label on cap with mouse ID and assay date
  - add 15 mL of 10% zinc formalin to each vial
- 10% zinc formalin
  Protocol, Cat. # 313-095 (Fisher, Cat. # 23-313-095)

Supporting Protocols:

- In Vivo / Ex Vivo > Mouse Euthanasia Using Isoflurane
- In Vivo / Ex Vivo > Mouse Dissection
- Bronchoalveolar Lavage (optional)

Protocol:

proceed serially for each mouse

1) sacrifice mouse
   see supporting protocol
2) perform chest cavity dissection
   see supporting protocol
3) perform lavage (optional)
   see supporting protocol
4) use forceps and scissors to harvest lung lobe(s)
5) place in petri dish containing PBS

6) gently rinse tissue
7) transfer single lung lobe to labeled vial containing formalin
8) seal with screw cap
9) store in dark at room temp.

Lung Cell Preparation

- perform on assay day
- extract separate lung lobes for fixation and preparation of cell suspension, respectively

Equipment:

- light source for dissection
- bucket with ice
- forceps (2)
    - sterilize in beaker with 70% ethanol
- scissors (1)
    - sterilize in beaker with 70% ethanol
- beaker with 70% ethanol
    for sterilizing surgical instruments
- 37°C tissue culture incubator
- lab timer
- refrigerated centrifuge
- vortex

Materials:

- C57BL/6J mice (6-12 weeks old)
    - sensitized with OVA/alum
    - challenged with OVA
- 10 cm diameter petri dishes (1 per group)
    Corning, Cat. # 430167
- PBS, sterile
- FACS tubes (1 per mouse / sample)
    - label with mouse ID
- TCM
    cRPMI + 10% FBS
- 3.5 cm diameter petri dishes (1 per mouse)
    for mincing lung tissue
- collagenase, 100 mg/mL (30 μL per mouse)
- DNAse I, 10 mg/mL (10 μL per mouse)
- FACS Buffer
    - keep on ice
- TCM
    cRPMI + 10% FBS
    - keep on ice

Supporting Protocols:

- In Vivo / Ex Vivo > Mouse Euthanasia Using Isoflurane
- In Vivo / Ex Vivo > Mouse Dissection
- Bronchoalveolar Lavage (optional)

- In Vivo / Ex Vivo > Tissue Dissociation
- Cell Culture > Cell Counting

Protocol:

do not perform RBC lysis for lung cells

1) sacrifice mouse
   see supporting protocol
2) perform chest cavity dissection
   see supporting protocol
3) perform lavage (optional)
   see supporting protocol
4) harvest lung tissue
   4.1) use forceps and scissors to harvest lung lobe(s)
   4.2) place in 10 cm diameter petri dish containing PBS
   4.3) gently rinse tissue
   4.4) transfer single lung lobe to labeled FACS tube containing TCM
   4.5) keep at 4°C
5) mince lung tissue
   proceed serially for each tissue sample
   5.1) decant lung tissue and TCM to 3.5 cm diameter petri dish
      retain FACS tube
   5.2) using pair of forceps, mince tissue into small pieces (1-2 mm)
   5.3) return minced tissue and TCM to retained FACS tube
   5.4) tap tube to collect minced tissue at bottom
   5.5) keep at 4°C
6) digest with collagenase and DNAse
   proceed in parallel for all tissue samples
   6.1) add 30 μL of 100 mg/mL collagenase stock to each tube
      3 mg/mL final
   6.2) add 10 μL of 10 mg/mL DNAse I stock to each tube
      100 μg/mL final
   6.3) vortex tube to mix
   6.4) place in 37°C tissue culture incubator for 1 hour
      to enzymatically digest tissue
7) dissociate tissue into cell suspension
   see supporting protocol
8) add 4 mL of FACS Buffer / TCM
9) vortex / resuspend cells
10) count cells
   see supporting protocol
11) adjust to desired cell concentration

DLN Preparation

mediastinal draining lymph nodes

Equipment:

- light source for dissection
- biosafety cabinet (TC hood)
- forceps (2)
  sterilize in beaker with 70% ethanol
  - fine point (1)
  - standard (1)
- beaker with 70% ethanol
  for sterilizing surgical instruments
- bucket with ice

Materials:

- C57BL/6J mice (6-12 weeks old)
  - sensitized with OVA/alum
  - challenged with OVA
- Kimwipes
- TCM
  cRPMI + 10% FBS
- 15 mL conical tubes (1 per group)

Supporting Protocols:

- In Vivo / Ex Vivo > Mouse Euthanasia Using Isoflurane
- In Vivo / Ex Vivo > Mouse Dissection
- Bronchoalveolar Lavage (optional)
- In Vivo / Ex Vivo > Tissue Dissociation
- In Vivo / Ex Vivo > RBC Lysis
- Cell Culture > Cell Counting

Protocol:

1) add 10 mL of ice-cold TCM to 15 mL tube for each group
   prepare inside TC hood
   1.1) keep on ice
2) sacrifice mouse
   see supporting protocol
3) perform chest cavity dissection
   see supporting protocol
   3.1) separate rib cage using pins
4) perform lavage (optional)
   see supporting protocol
5) harvest DLN

5.1) fold Kimwipe into quarters and aspirate blood from chest cavity
5.2) using fine point forceps, harvest mediastinal lymph nodes
- small, spherical and shiny in appearance
- 2 each on left and right sides
- 1 at midline
5.3) transfer to tube with TCM
pool with additional mice from same treatment group to increase cell number

6) dissociate DLN to prepare cell suspension
see supporting protocol
7) lyse RBCs
see supporting protocol
8) resuspend in 0.5 mL of TCM
9) count cells
see supporting protocol
10) optional: store overnight at 4°C

References:

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FACS Staining for Eosinophils and Macrophages

using BALF and/or lung cells

Equipment:

- flow cytometer for analysis
  e.g., BD Canto or Aria
- refrigerated centrifuge
- bucket with ice

Materials:

- BALF cell suspension
  see supporting protocol
- lung cell suspension
  see supporting protocol
- FACS buffer
  - keep on ice
- FACS staining tubes
- antibodies
  confirm sufficient stock prior to staining cells
  - rat anti-mouse CD16/CD32 (Mouse BD Fc Block)
    BD, Cat. # 553141; 0.5 mg/mL
  - PE rat anti-Siglec-F
    BD, Cat. # 552126; 0.2 mg/mL
    ex: 488 nm laser
    em: 585/42 filter
  - PerCP-Cy5.5 rat anti-mouse CD45
    BD, Cat. # 550994; 0.2 mg/mL
    ex: 488 nm laser
    em: 670 LP filter
  - FITC hamster anti-mouse Cd11c
    BD, Cat. # 557400; 0.5 mg/mL
    ex: 488 nm laser
    em: 530/30 filter
- 2% paraformaldehyde / PBS, keep on ice
- USB drive

Supporting Protocols:

- Bronchoalveolar Lavage
  - BALF Processing
- Harvesting Lung Tissue
  - Lung Cell Preparation

Protocol:

for staining ≤ 10^6 BALF or lung cells

1) create spreadsheet listing all samples and staining conditions including the following:
   - *unstained sample*
   - *single-color control samples (prepare from OVA/alum sensitized and challenged mouse / mice)*
   - *experimental samples for each mouse / group*
2) for each mouse / sample, transfer 100 μL of BALF or lung cells to new FACS tube
3) add 0.5 μg (1 μL) Fc blocking antibody to each sample
4) incubate for 5 min.
5) add 0.5 μg of eosinophil markers to each sample
   5.1) add 2.5 μL of anti-Siglec-F antibody
   5.2) add 2.5 μL of anti-CD45 antibody
   5.3) add 1 μL of anti-CD11c antibody
6) incubate for 45 min. in refrigerator (in dark at 4°C)
7) add 2 mL FACS buffer per tube (wash)
8) centrifuge for 5 min. at 1,500 rpm (4°C)
9) discard supernatant
   invert tube
10) resuspend in 100 μL of 2% PFA/PBS per sample
11) incubate for 10 min. in refrigerator (in dark at 4°C)
12) add 2 mL FACS buffer per tube to wash
13) centrifuge for 5 min. at 1,500 rpm (4°C)
14) discard supernatant
   invert tube
15) resuspend cells in 300 μL of FACS buffer
16) store samples in refrigerator until analysis (in dark at 4°C)
17) acquire FACS data within 72 hours
   example voltages for FACS acquisition:

FSC:	*175*
SSC:	*450*
FITC:	*450*
PE:	*450*
PerCP-Cy5.5:	*400*

   17.1) select 'medium' or 'high' flow rate
   17.2) for BALF cells, collect ≥ 10,000 $CD45^+$ events
   17.3) for lung cells, collect ≥ 20,000 $CD45^+$ events
   17.4) export FCS files
   17.5) save to USB drive
18) analyze FACS data

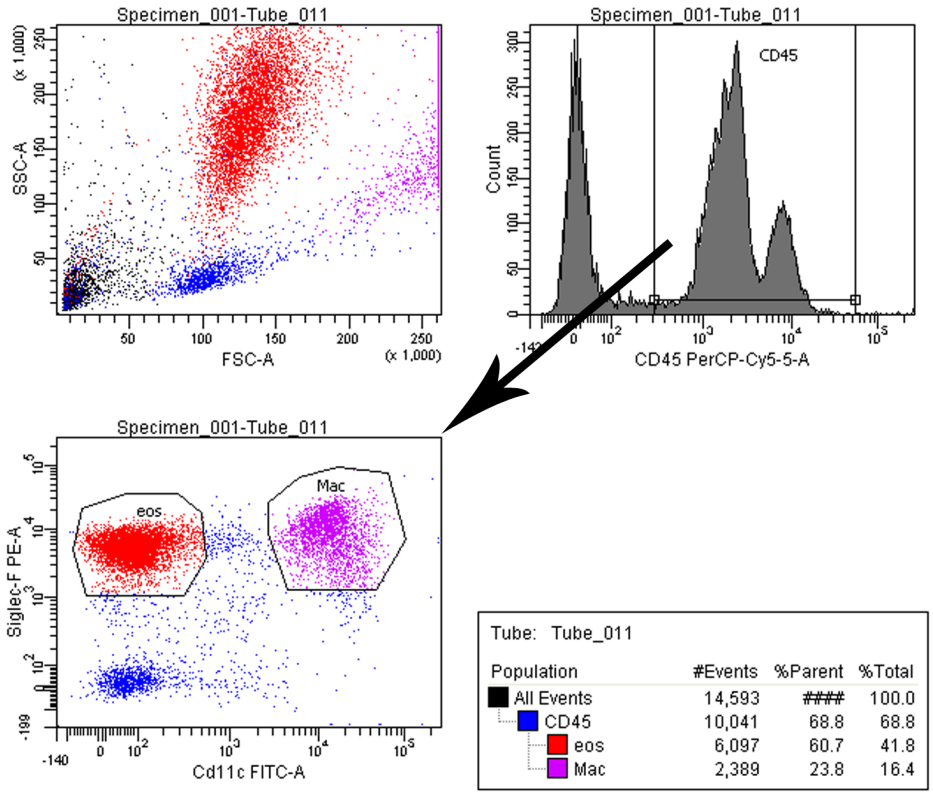

Figure 60: Identification and quantification of eosinophils and macrophages by FACS.

18.1) determine compensation parameters from single-color control samples
- *e.g., FITC - PE = 3.0%*
- *e.g., PE - FITC = 14.0%*
- *perform separately for BAL cells and lung cells*

18.2) create dot plot with SSC on y-axis and FSC on x-axis

18.3) create CD45 PerCP-Cy5.5 histogram
- 18.3.1) select biexponential display for x-axis
- 18.3.2) draw gate on $CD45^+$ events
  - 18.3.2.1) label as 'CD45'

18.4) create dot plot with Siglec-F PE on y-axis and Cd11c FITC on x-axis
- 18.4.1) select biexponential display for x- and y-axes
- 18.4.2) select 'Show Population' > 'CD45 PerCP-Cy5.5' *to display $CD45^+$ events*
- 18.4.3) draw Siglec-F^+, Cd11c^- gate
  - 18.4.3.1) label as 'eos'
  - 18.4.3.2) record eosinophil (eos) percentage

18.4.3.2.1) label on graph as '% (Siglec-F$^+$ Cd11c$^-$) / CD45$^+$'

18.4.4) draw Siglec-F$^+$, Cd11c$^+$ gate

18.4.4.1) label as 'Mac'

18.4.4.2) record macrophage (Mac) percentage

18.4.4.2.1) label on graph as '% (Siglec-F$^+$ Cd11c$^+$) / CD45$^+$'

References:

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FACS Staining for T Cells

using BALF cells

Equipment:

- flow cytometer for analysis
  e.g., BD Canto or Aria
- refrigerated centrifuge
- bucket with ice

Materials:

- BALF cell suspension
- FACS buffer
  - keep on ice
- FACS staining tubes
- antibodies
  - PerCP-Cy5.5 rat anti-mouse CD45
    BD, Cat. # 550994; 0.2 mg/mL
    ex: 488 nm laser
    em: 670 LP filter
  - PE-Cy7 hamster anti-mouse CD3ε, clone 145-2C11
    BD, Cat. # 552774; 0.2 mg/mL
    ex: 488 nm laser
    em: 780/60 nm laser
- USB drive

Supporting Protocols:

- Bronchoalveolar Lavage
  - BALF Processing

Protocol:

for staining ≤ 10^6 BALF cells

1) prepare in advance:
   1.1) create spreadsheet listing all samples and staining conditions including the following:
   - unstained sample
   - single-color control samples (prepare from OVA/alum sensitized and challenged mouse / mice)
   - experimental samples for each mouse / group
   1.2) confirm sufficient stock of antibodies
2) for each mouse, transfer 100 μL of BALF cell suspension to new FACS tube
3) add 0.5 μg (1 μL) Fc blocking antibody to each sample
4) incubate for 5 min.

5) add 0.5 μg of eosinophil markers to each sample
    5.1) add 2.5 μL of anti-CD45 antibody
    5.2) add 2.5 μL of anti-CD3ε antibody
6) incubate for 45 min. in refrigerator (in dark at 4°C)
7) add 2 mL FACS buffer per tube to wash
8) centrifuge for 5 min. at 1,500 rpm (4°C)
9) discard supernatant
    invert tube
10) resuspend in 100 μL of 2% PFA/PBS per sample
11) incubate for 10 min. in refrigerator (in dark at 4°C)
12) add 2 mL FACS buffer per tube to wash
13) centrifuge for 5 min. at 1,500 rpm (4°C)
14) discard supernatant
    invert tube
15) resuspend cells in 300 μL of FACS buffer
16) store samples in refrigerator until analysis (in dark at 4°C)
17) acquire FACS data within 72 hours
    example voltages for FACS acquisition:
    FSC: 175
    SSC: 450
    PerCP-Cy5.5: 500
    PE-Cy7: 500
    17.1) select 'medium' or 'high' flow rate
    17.2) collect ≥ 20,000 $CD45^+$ events
    17.3) export FCS files
    17.4) save to USB drive
18) analyze FACS data

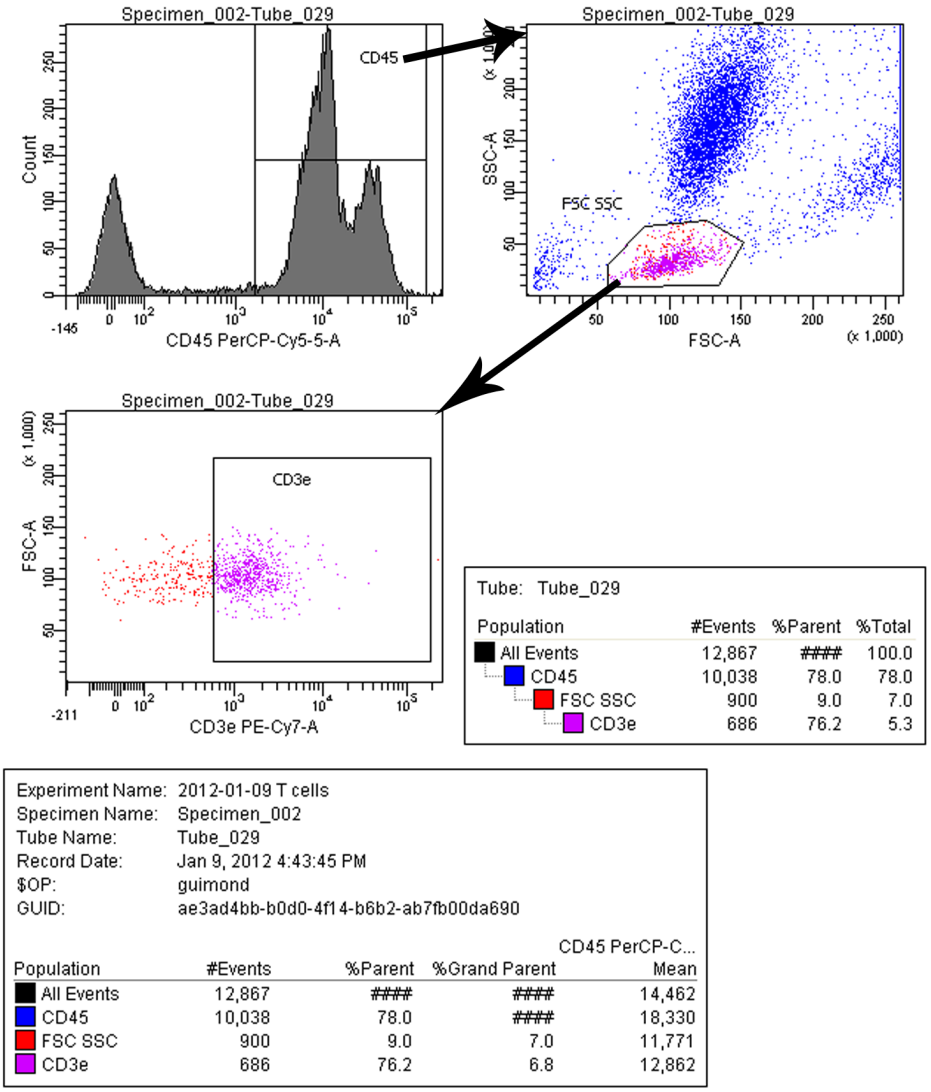

Figure 61: Identification and quantification of BAL T cells by FACS.

18.1) determine compensation parameters from single-color control samples

e.g., PE-Cy7 - PerCP-Cy5.5 = 12.0%

e.g., PerCP-Cy5.5 - PE-Cy7 = 6.0%

18.2) create CD45 PerCP-Cy5.5 histogram

18.2.1) select biexponential display for x axis

18.2.2) draw gate on CD45+ events

18.2.2.1) label as 'CD45'

18.3) create dot plot with SSC on y-axis and FSC on x-axis

18.3.1) select 'Show Population' > 'CD45 PerCP-Cy5.5' *to display CD45+ events*

18.3.2) draw gate on lymphocytes by scatter profile

18.3.2.1) label as 'FSC SSC'
18.4) create dot plot with PE-Cy7 on x-axis and FSC on y-axis
18.4.1) select biexponential display for x axis
18.4.2) select 'Show Population' > 'FSC SSC'
to display $CD45^+$ SSC^+ FSC^+ events
18.4.3) draw CD3e PE-$Cy7^+$ gate based on CD45 PerCP-Cy5.5 single-color control sample
'negative' gate
18.4.3.1) label as 'CD3e'
18.4.4) create statistics view and select to display % grandparent
18.4.4.1) record percentage of $CD3\varepsilon^+$ events as a percentage of $CD45^+$ events
18.4.4.1.1) label on graph as '% $CD3\varepsilon^+$ / $CD45^+$'

References:

(133)

Histological Examination

mucus severity score and inflammation score

Equipment:

- microtome
  at histology lab
- slide folder
- light microscope with 40x objective

Materials:

- lung tissue specimens
  - stored in formalin
- for mucus analysis:
  - PAS (Schiff's reagent)
    stain for mucus
  - hematoxylin
    nuclear counter-stain
- for inflammation analysis:
  - hematoxylin
    nuclear stain
  - eosin
    stains cytoplasm

Supporting Protocols:

- Lung Tissue Fixation

Protocol:

- tissue embedding, sectioning and staining performed at pathology lab
- stain separate slides cut from same tissue for mucus and inflammation

1) embed lung tissue in paraffin
   save tissue block for re-cutting and further staining if needed
2) make 5 μm sections around middle of tissue
3) stain for mucus:
   3.1) stain for 5 min. with Schiff's reagent
   3.2) rinse in cold water
   3.3) stain with hematoxylin
   counter-stain
   3.4) skip acid
4) stain for inflammation:
   4.1) stain with hematoxylin
   4.2) rinse in cold water
   4.3) stain with eosin
5) assign unique, random code number to each slide

6) evaluate slides
    6.1) view slides using 40x objective of light microscope
    6.2) qualitatively assess staining quality in reference to positive and negative control slides
    6.3) focus on cross-section of individual bronchioles
    6.4) for mucus analysis, assign score to each bronchiole according to the percentage of the total circumference staining bright pink / fuchsia as follows:

score	*description*
0	*0% +*
1	*≤ 10% +*
2	*≤ 25% +*
3	*≤ 50% +*
4	*≤ 75% +*
5	*100%*

    6.5) for inflammation analysis, assign score to each bronchiole according to the degree of inflammatory infiltrate tangential to the circumference as follows:

score	*description*
0	*no cellular infiltrate*
1	*1 cell layer deep*
2	*2-4 cell layers deep*
3	*5-7 cell layers deep*
4	*> 7 cell layers deep*

7) create spreadsheet listing scores for each mouse
8) decode slides to reveal mouse / group identity
9) calculate average score for each mouse
10) graph average scores +/- SEM for each group

References:

(69, 162)

Airway Hyperresponsiveness (AHR)

Equipment:

- Scireq FlexiVent system
  - *small rodent ventilator*
  - *Part # FV-BU; Serial # 1062328*
  - *for technical assistance, call 877-5-SCIREQ*
- transducer
- nebulizer
- Y tubing
  used to connect components
- monometer
- PC computer
  - FlexiVent software installed
    - *Version 5.3*
    - *Service Pack 2; Build 532*
- dissection board
- scale
  to weigh mice
- endotracheal tube (cannula), 18 gauge
  - *BD Medical, Cat. # 408208*
  - *with beveled end to facilitate cannulation*
  - *keep clean and free of obstructions – replace as needed*

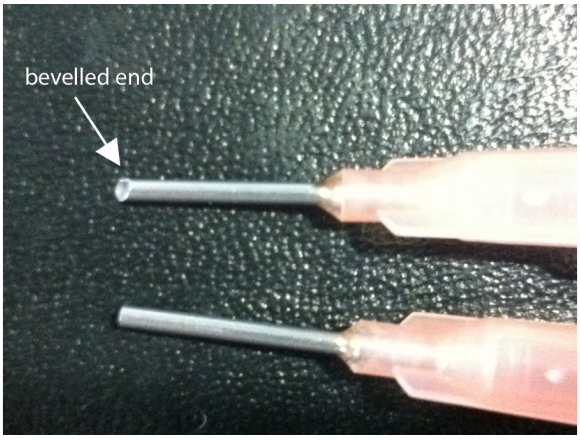

Figure 62: Image of blunt vs. bevelled cannula for tracheotomy.

Materials:

- C57BL/6 mice (6-12 weeks old)
  including negative control and experimental groups
  - sensitized with OVA/alum
    see supporting protocol
  - challenged with OVA
    see supporting protocol
- pins for dissection board
- ketamine / xylazine cocktail
  - prepare fresh
- 27G 1/2 " needle (1)
  for ketamine / xylazine administration -- reuse for multiple mice
- methacholine doses (100 μL / dose / mouse)
  0, 3, 6, 12 & 24 mg/mL
  - prepare dilutions of stock solution in PBS
- 1 mL syringes (6)
  - one for each dose of methacholine
    reuse for multiple mice
  - one for ketamine / xylazine injection
    reuse for multiple mice
- paper towels
- curved forceps (1)
- straight forceps (1)
- surgical scissors
- surgical suture
- 70% ethanol spray bottle
- USB drive
- timer

Supporting Protocols:

- OVA/Alum Sensitization (i.p.)
- OVA Challenge (i.n. or aerosol)

Protocol:

1) connect components for FlexiVent system according to figures below
begin by administering anesthesia (see below) during system setup

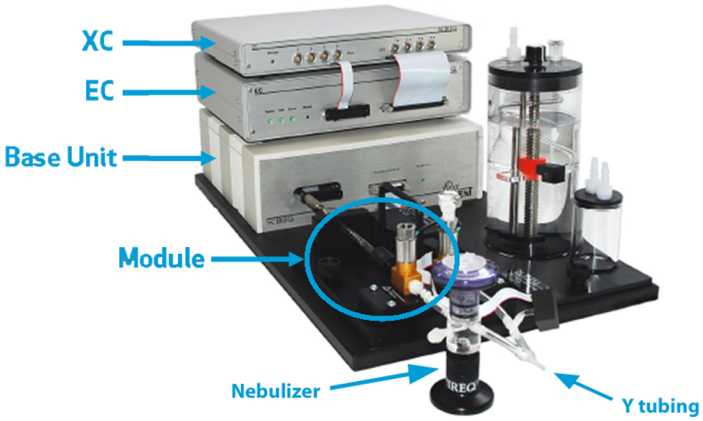

Figure 63: FlexiVent system overview.
Diagram courtesy of Zineb Idrissi and Dr. Annette Roubichaud of SCIREQ.

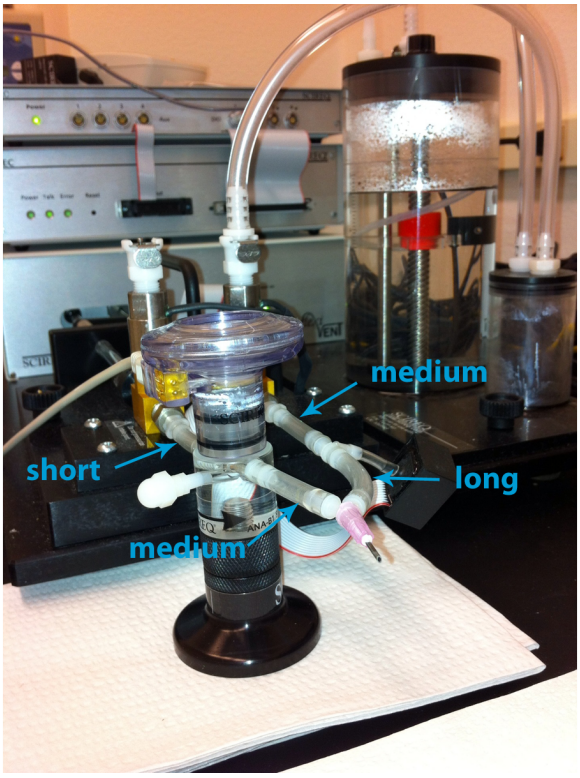

Figure 64: Identification of tubing used for FlexiVent system.
Confirm that the arrow on the Aeroneb adapter is pointing from the module towards the subject.

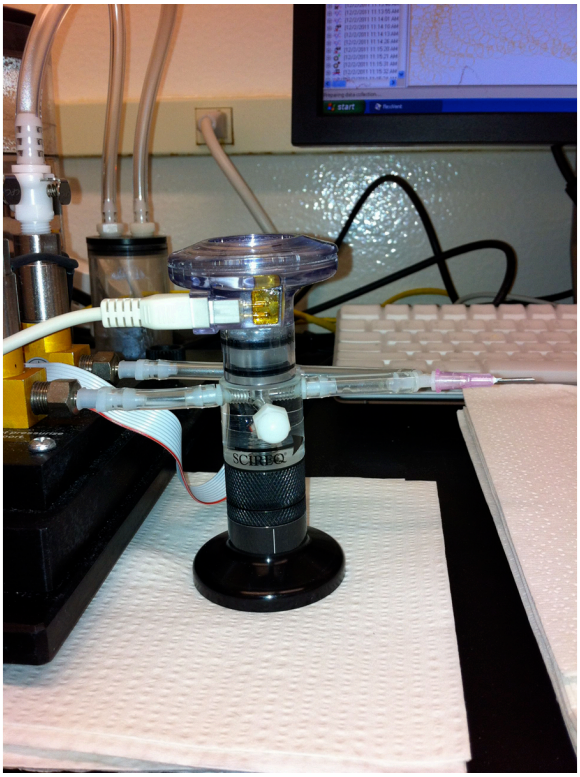

Figure 65: Proper setup for FlexiVent system.

2) start computer
    2.1) turn on power to power strip controlling FlexiVent system and computer
    2.2) turn on power to computer and monitor
    2.3) simultaneously press control, option & delete to get login window
    2.4) log in to computer (control, option & delete)
3) launch FlexiVent software
    3.1) select 'Start a new experiment from template'
    3.2) select 'Wei tem' > 'Open'
    3.3) name and save experiment to 'My flexivent data'
    3.4) name operator
    3.5) select 'OK'
4) create new subject
    4.1) name subject
    4.2) enter weight (e.g., 25 g)
        enter actual mass as determined from scale
    4.3) enter gender (e.g., female)
    4.4) select 'OK'

5) prime nebulizer
    perform once at beginning of each session
    5.1) label and fill each 1 mL syringe with respective dose of methacholine (100 μL per dose per mouse)
    5.2) follow software prompts
    5.3) detach nebulizer from base
    5.4) dispense 100 μL of PBS to nebulizer
    5.5) firmly re-attach nebulizer
6) perform channel calibration
    perform once at beginning of each session
    6.1) select to calibrate cylinder and airway pressure
    6.2) select Calibration Method > two collected points
    6.3) attach manometer and use attached syringe to adjust pressure to 0 and 300 mm H_2O as directed
7) perform dynamic tube calibration
    repeat for each subject / mouse
    7.1) connect endotracheal cannula to end of Y tubing
    7.2) add 100 μL of PBS to nebulizer during calibration to avoid having air leak during closed tube calibration
        leave PBS in reservoir for baseline (0 mg/mL methacholine) reading during data acquisition
    7.3) select all perturbation signals for calibration
    7.4) follow software prompts for:
        7.4.1) closed tube calibration
            block end of cannula with finger when directed
        7.4.2) open tube calibration
    7.5) below TLC section of report; verify that $R_S > 2000$ and that $R_T < 0.5$
        7.5.1) R_S refers to resistance of system and R_T refers to resistance of tubing
        7.5.2) tighten connections and repeat if necessary
8) anesthetize mouse
    preform in advance for subsequent mice
    8.1) proceed individually for each mouse
        allow 30 min. for each mouse
    8.2) inject 200 μL of ketamine / xylazine intraperitoneally
        reduce likelihood of overdose by slowly delivering 50 μL aliquots followed by monitoring until entire volume is dispensed
    8.3) allow ≥ 20 min. for anesthetic to take effect
        mice must be passive (no breathing efforts) when connected to ventilator
    8.4) weigh mouse on scale and record mass in grams

9) perform surgery to reveal trachea
    9.1) use pins to secure mouse to dissection board facing up
    9.2) spray furn above trachea with ethanol prior to surgery
    9.3) remove skin and connective tissue above trachea
    9.4) expose trachea using forceps
10) perform tracheotomy
    take care to avoid rupturing trachea
    10.1) thread surgical suture below trachea
    10.2) create small incision through trachea at cartilage ring
    10.3) hold curved forceps below trachea
    10.4) insert cannula with luer end aligned with the tip of the subject's nose according to diagram below and with beveled end of cannula against bottom of trachea

Figure 66: Proper placement of cannula relative to head of mouse. Diagram courtesy of Zineb Idrissi and Dr. Annette Roubichaud of SCIREQ.

    10.5) secure cannula using surgical suture
11) connect mouse to ventilator
    11.1) assess reflex response by squeezing front and hind paws using forceps
        11.1.1) desired depth of anesthesia is reached when:
            11.1.1.1) respiratory rate is regular and relaxed
            11.1.1.2) withdrawal reflex is absent
            11.1.1.3) there is no response to an external stimulus
    11.2) adjust height of dissection board using folded paper towels to match height of tube on ventilator
    11.3) connect cannula to end of Y tubing
    11.4) tuck end of cannula below skin for proper placement
        to allow unobstructed air flow
    11.5) move head aside to make room for cannula

Figure 67: Proper connection of mouse to FlexiVent system via endotracheal cannula.

12) acquire data
- 12.1) execute ventilation script
  - *e.g., select script 'AN-MI v5.3'*
- 12.2) start ventilation
  - 12.2.1) confirm that mouse is passive (breathing efforts absent) and breathing in rhythm with ventilator
    - 12.2.1.1) if needed, detach cannula from Y tubing, adjust position of cannula inside trachea, reconnect to Y tubing and restart ventilation
    - 12.2.1.2) if needed, inject supplemental ketamine / xylazine i.p. (1/2 dose max) and wait until desired effect
- 12.3) when prompted, dispense 100 μL of PBS in nebulizer
  - *or, use remaining PBS in reservoir from dynamic tube calibration above*
- 12.4) ensure proper baseline reading before proceeding to first methacholine dose
  - *if required, detach from ventilator, adjust / replace cannula and reattach*

12.5) when prompted, select 'OK' to continue with next dose
series of methacholine doses are as follows: 0, 3, 6, 12 and 24 mg/mL
12.6) use folded paper towels to wick away remaining volume from nebulizer
12.7) when prompted, enter methacholine dose
12.8) repeat for each dose of series
12.9) stop ventilation after final dose
12.10) sacrifice mouse by cervical dislocation

13) for additional subjects / mice, select Edit > Subject > Replace
13.1) name new subject
13.2) repeat from 'dynamic tube calibration' above

14) analyze / validate data
14.1) select File > Export > Export parameters > Export All
14.2) save in ASC II format to custom folder using default file name
separator character: tab
14.3) copy files below located at the path 'My Documents > My FlexiVent data' to personal USB drive
- experiment file
- raw data file
- parameters file
14.4) open exported parameters file using spreadsheet application on personal computer
14.5) for each subject / mouse, create a list of the highest resistance value (units of '$cmH_2O.s/mL$') for each dose of methacholine
14.6) for each group, calculate the average resistance value (+/- SEM) for each dose of methacholine
14.7) display cumulative data as a graph of resistance versus methacholine dose
14.7.1) connect each datapoint with a line and display SEM

5.2.12. OVA System

OVA/Alum Sensitization (i.p.)

- perform in vivarium
- for short / 10 day protocol, sensitize on day 0
- for long / 16 day protocol, sensitize on days 0 & 5

Materials:

- C57BL/6J mice (6-12 weeks old)
  3-4 mice per group
- OVA/alum, 2:1 (v/v) for sensitization
  see 'Buffers & Solutions'
- PBS/alum
  for negative control group

Supporting Protocol:

- In Vivo / Ex Vivo > Intraperitoneal Injection

Protocol:

20 µg OVA / 2 mg alum per mouse

1) mix OVA/alum well prior to filling syringe
2) fill syringe for injection(s)
   see supporting protocol
3) deliver 150 µL intraperitoneally to upper-right quadrant
   see supporting protocol

References:

(11, 163)

OVA Challenge (i.n.)

- for short / 10 day protocol, challenge on days 7-9
- for long / 16 day protocol, challenge on days 12-15

Equipment:

bucket with ice

Materials:

- C57BL/6J mice (6-12 weeks old)
  - *OVA/alum sensitized group*
  - *PBS/alum sensitized group (control)*
  - *experimental groups as needed*
- 0.5 mg/mL OVA/PBS
  see 'Buffers and Solutions'
  - keep on ice

Supporting Protocols:

- OVA/Alum sensitization (i.p.)
- In Vivo / Ex Vivo > Intranasal Treatment
  - Anesthesia Machine Setup

Protocol:

for short / 10 day protocol, 10 µg OVA per mouse per challenge day

1) set up anesthesia machine
   see supporting protocol
2) deliver 20 µL bolus of 0.5 mg/mL OVA/PBS by intranasal treatment
   see supporting protocol
3) repeat for 3-4 daily challenges
   allow ~24 hours between consecutive challenges

References:

(11, 163)

OVA Challenge (aerosol)

- for short / 10 day protocol, challenge mice for 20 min. per day with 1% OVA/ PBS on days 7-9
- for long / 16 day protocol, challenge mice for 20 min. per day with 1% OVA/ PBS on days 12-15
- allow ~24 hours between consecutive challenges

Equipment:

- lab timer

Materials:

- C57BL/6J mice (6-12 weeks old)
  - *OVA/Alum sensitized group*
  - *PBS/Alum sensitized group (control)*
  - *experimental groups as needed*
- ~20 mL, 1% (10 mg/mL) OVA/PBS
- fine tip sharpie marker, red
  for labeling tails to distinguish groups

Supporting Protocols:

- OVA/Alum Sensitization (i.p.)
- In Vivo / Ex Vivo > Aerosol Treatment

Protocol:

1) dispense 1% OVA/PBS solution in medicine cup of nebulizer
   see supporting protocol
2) connect nebulizer components
   see supporting protocol
3) label tails to distinguish different groups using sharpie marker
   re-label for each challenge day if needed
4) place mice for challenge inside induction chamber
5) set timer to allow 20 minutes exposure to aerosolized OVA
6) repeat for 3-4 daily challenges
   allow ~24 hours between consecutive challenges

References:

(69)

OVA Restimulation (in vitro)

restimulation of splenocytes, DLN cells or lung cells to assess in vitro cytokine production by ELISA

Equipment:

- biosafety cabinet / TC hood
- refrigerated centrifuge
- humidified, 5% CO_2, 37°C incubator
- -20 / -80°C freezer

Materials:

- C57BL/6 mice (6-12 weeks old)
  - *optimal strain for lung inflammation*
  - sensitized with OVA/Alum
    - *see supporting protocol*
  - challenged with OVA
    - *see supporting protocol*
- BALB/c mice (6-12 weeks old)
  - *optimal strain for systemic Th2 production*
  - sensitized with OVA/Alum
    - *see supporting protocol*
- for ELISA:
  - 96-well culture plate, round-bottom
    - *Sarstedt (Cat. # 83.1837)*
    - *to promote cell-cell contact for antigen presentation*
  - microcentrifuge tubes (2 per condition)
    - *for collecting cell suspension for centrifugation (1)*
    - *for storage of cell culture supernatant (1)*
    - label each tube
- for ELISPOT:
  - 96-well filter plate
    - *Millipore, Cat. # MSIPS4510*
- TCM
  - *cRPMI + 10% FBS*
  - keep on ice
- 10 mg/mL OVA/PBS
  - *100 µL per tube*
  - stored at -80°C

Supporting Protocols:

- In Vivo / Ex Vivo > Splenocyte Preparation
- Lung Inflammation / Asthma > Lung Cell Preparation
- Lung Inflammation / Asthma > DLN Preparation
- In Vivo / Ex Vivo > Tissue Dissociation

- In Vivo / Ex Vivo > RBC Lysis
- Cell Culture > Cell Counting

Protocol:

culture conditions (# of cells per well of 96-well plate):
- 10^6 splenocytes / 200 µL = $5x10^6$ cells/mL
- 10^5 DLN cells / 200 µL = $0.5x10^6$ cells/mL (for IL-5 and IL-13)
- 10^6 DLN cells / 200 µL = $5x10^6$ cells/mL (for IL-4 and IL-5)
- 10^6 lung cells / 200 µL = $5x10^6$ cells/mL
- 50 µg/mL OVA (for DLN or lung cells cultures)
- 100 µg/mL OVA (for splenocyte cultures)

1) prepare cell suspension
    1.1) dissociate tissue to prepare cell suspension
        see supporting protocol
    1.2) lyse RBCs (except for lung cells)
        see supporting protocol
    1.3) count cells
        see supporting protocol
2) add 2x cell suspension to designated wells
    - including for non-stimulation control wells
    - prepare wells in duplicate or triplicate depending on available cell number
    2.1) adjust cell concentration to 2x in TCM
    2.2) add 100 µL to designated wells
3) add 2x OVA solution to designated wells
    add medium for non-stimulation control wells
    3.1) dilute 10 mg/mL OVA/PBS to 2x final concentration in TCM
    3.2) add 100 µL to designated wells
4) incubate for 2-4 days
    cytokine-specific kinetics:
    - 2 days for detection of IFN-γ
    - 4 days for detection of IL-4, IL-5, IL-13
5) harvest supernatants
    5.1) resuspend each well
        combine cell suspension from replicate wells
    5.2) transfer to designated microcentrifuge tubes
    5.3) centrifuge for 5 min. at 1,500 rpm (4°C)
    5.4) transfer supernatant to new microcentrifuge tube
        for storage
6) store tubes in -20 / -80°C freezer until cytokine analysis by ELISA

References:

(164)

5.3. Vendor List

Airgas West
3737 Worsham Avenue
Long Beach, CA 90808
www.airgas.com
800-2-Airgas

American Type Culture Collection
ATCC
10801 University Boulevard
Manassas, VA 20110
www.atcc.org
800-638-6597

AnaSpec
34801 Campus Drive
Fremont, CA 94555
www.anaspec.com
800-452-5530

Baker Company, Inc.
P.O. Drawer E
161 Gatehouse Road
Sanford, Maine 04073
www.bakerco.com
800-992-2537

Bangs Laboratories
9025 Technology Dr.
Fishers, IN 46038-2886
www.bangslabs.com
800-387-0672

BD Biosciences
2350 Qume Drive
San Jose, CA 95131
www.bdbiosciences.com
877-232-8995

BD Medical
1 Becton Drive
Franklin Lakes, NJ 07417
www.bd.com
201-847-6800

Bio-Rad
1000 Alfred Nobel Drive
Hercules, CA 94547
www.bio-rad.com
800-424-6723

BioLegend
9727 Pacific Heights Blvd.
San Diego, CA 92121
www.biolegend.com
877-246-5343

Biomedical Polymers, Inc.
42 Liinus Allain Ave.
Gardner, MA 01440
www.biomedicalpolymers.com
800-253-3684

BioPioneer
3956 Sorrento Valley Blvd. Suite 200
San Diego, CA 92121
858-232-9588

Biotechnical Services, Inc.
BTS
9373 Activity Road, Suite J
San Diego, CA 92126
www.biotechserv.com
800-274-0287

Calbiochem / EMD Millipore
290 Concord Rd.
Billerica, MA 01821
www.emdmillipore.com
800-645-5476

Carl Zeiss AG
Carl-Zeiss-Strasse 22
73447 Oberkochen,
Germany
www.zeiss.com

Cell Signaling Technology
3 Trask Lane
Danvers, MA 01923
www.cellsignal.com
877-616-2355

Dako
6392 Via Real
Carpinteria, CA 93013
www.dako.com
800-235-5763

Denville Scientific, Inc.
3005 Hadley Road
South Plainfield, NJ 07080
www.denvillescientific.com
800-453-0385

eBioscience, Inc.
6042 Cornerstone Court W
San Diego, CA 92121
www.ebioscience.com
858-642-2058

EMD Millipore
290 Concord Road
Billerica, MA 01821
www.emdmillipore.com
978-715-4321

EMD Millipore Chemicals
290 Concord Rd.
Billerica, MA 01821
www.emdmillipore.com/chemicals
866-645-5476

Epitomics
863 Mitten Road, Suite 103
Burlingame, CA 94010-1303
www.epitomics.com
877-772-2622

Fermentas Life Sciences / Thermo Scientific
798 Cromwell Park Drive, Suites R-S
Glen Burnie, MD 21061
www.fermentas.com
800-340-9026

Fisher HealthCare
9999 Veterans Memorial Drive
Houston, TX 77038
www.fishersci.com
800-766-7000

Fisher Scientific, Inc.
81 Wyman Street
Waltham, MA 02454
www.fishersci.com
800-766-7000

Fort Dodge Animal Health / Pfizer
9225 Indian Creek Parkway
Building 32, Suite 400
Overland Park, KS 66210
animalhealth.pfizer.com
973-660-5000

GE Healthcare Life Sciences
800 Centennial Avenue
Piscataway, NJ 08855-1327
www.gelifesciences.com
800-526-3593

GL Biochem, Ltd.
519 Zi Yue Road
Shanghai 200241,
China
www.glschina.com
86-21-61263322

GraphPad Software
2236 Avenida de la Playa
La Jolla, CA 92037
www.graphpad.com
858-454-5577

HyClone / Thermo Fisher Scientific, Inc.
925 West 1800
South Logan, UT 84321
www.thermoscientific.com
435-792-8000

Invitrogen / Life Technologies
1600 Faraday Avenue
Carlsbad, CA 92008
www.invitrogen.com
800-955-6288

Jackson ImmunoResearch
872 West Baltimore Pike
West Grove, PA 19390
www.jacksonimmuno.com
800-367-5296

Jackson Laboratory
600 Main Street
Bar Harbor, Maine 04609
jaxmice.jax.org
800-422-MICE

LabRepCo
101 Witmer Road, Suite 700
Horsham, PA 19044
www.labrepco.com
800-521-0754

Lloyd, Inc.
P.O. Box 130
Shenandoah, IA 51601
www.lloydinc.com
800-831-0004

Lonza
Muenchensteinerstrasse 38
CH-4002 Basel,
Switzerland
www.lonza.com
800-521-0390

MatTek Corporation
200 Homer Avenue
Ashland, MA 01721
www.mattek.com

Mediatech Cellgro / Corning, Inc.
9345 Discovery Blvd.
Manassas, VA 20109
www.cellgro.com
800-235-5476

Miltenyi Biotec
2303 Lindbergh Street
Auburn, CA 95602
www.miltenyibiotec.com

Mo Bio Laboratories
2746 Loker Avenue West
Carlsbad, CA 92010
www.mobio.com
800-606-6246

Molecular Devices, LLC
1311 Orleans Drive
Sunnyvale, CA 94089-1136
www.moleculardevices.com
800-635-5577

MP Biomedicals
29525 Fountain Parkway
Solon, OH 44139
www.mpbio.com
800-854-0530

New England BioLabs
240 County Road
Ipswich, MA 01938
www.neb.com
800-632-5227

Pacific Pathology
9292 Chessapeake Dr., Suite 100
San Diego, CA 92123
United States of America
www.pacificpathology.com
858-576-9630

Pall Life Sciences
25 Harbor Park Drive
Port Washington, NY 11050
800-645-6532

Pierce / Thermo Fisher Scientific, Inc.
3747 N Meridian Rd.
Rockford, IL 61101
www.piercenet.com
800-874-3723

Polysciences, Inc.
400 Valley Road
Warrington, PA 18976-2522
www.polysciences.com
800-523-2575

R&D Systems Inc.
614 McKinley Place NE
Minneapolis, MN 55413
www.rndsystems.com
800-343-7475

Roboz Surgical Instruments Co, Inc.
P.O. Box 10710
Gaithersburg, MD 20898-0710
www.roboz.com
800-424-2984

Roche Applied Science
P.O. Box 50414
9115 Hague Road
Indianapolis, IN 46250-0414
www.roche-applied-science.com

Rockland Immunochemicals, Inc.
P.O. Box 326
Gilbertsville, PA 19525
800-656-7625

SAFC Biosciences
13804 W. 107th Street
Lenexa, KS 66215
www.safcglobal.com
913-469-5580

Santa Cruz Biotechnology
2145 Delaware Avenue
Santa Cruz, CA 95060
www.scbt.com
800-457-3801

Sarstedt AG & Co.
Sarstedtstraße
Postfach 1220
51582 Nümbrecht,
Germany
www.sarstedt.com
800-257-5101

Scireq, Inc.
6600 St-Urbain, Suite 300
Montreal, QC H2S 3G8
Canada
www.scireq.com
877-572-4737

SeqXcel
11526 Sorrento Valley Road, Ste. B2
San Diego, CA 92121
www.seqxcel.com
858-481-7569

Sigma Aldrich
3050 Spruce St.
St. Louis, MO 63103
www.sigmaaldrich.com
800-325-3010

Southern Biotech
P.O. Box 26211
Birmingham, AL 35260
www.southernbiotech.com
800-722-2255

Tree Star, Inc.
340 A Street #101
Ashland, OR 97520
www.flowjo.com
800-366-6045

TW Medical Veterinary Supply / Animal Health International
3610 Lohman Ford Road
Lago Vista, TX 78645-8068
www.twmedical.com
888-787-4483

Upstate / EMD Millipore
300 5th Ave., 6th Floor
Waltham, MA 02451
www.millipore.com
800-645-5476

Ushio America, Inc.
5440 Cerritos Ave
Cypress, CA 90630
www.ushio.com
800-838-7446

VetEquip
P.O. Box 10785
Pleasanton, CA 94588-0785
www.vetequip.com
800-466-6463

VWR
1310 Goshen Parkway
West Chester, PA 19380
www.vwr.com
800-932-5000

Worthington Biochemical Corp.
730 Vassar Ave
Lakewood, NJ 08701
www.worthington-biochem.com
800-445-9603

5.4. Inventory

5.4.1. Antibodies

AF 488 anti-IFNγ, clone XMG1.2
BioLegend; Cat. #: 505813; 100 µg

AF 647 anti-IFNγ, clone XMG1.2
BioLegend; Cat. #: 505814; 100 µg

anti-CD28, syrian hamster IgG2 λ1, clone 37.51
BD; Cat. #: 553295; 0.5 mg

anti-Itk, clone 2F12
Millipore/Upstate; Cat. #: 05-476; 100 µL
for immunoblotting

anti-LAT, clone 2E9
Millipore; Cat. #: 05-561; 100 µg

anti-mouse IFN-γ, clone R4-6A2; 1.0 mg/mL
BD; Cat. #: 551216; 1 mL
ELISA capture, 250x; ELISPOT capture, 250x

anti-mouse IL-12, clone C17.8; 1 mg/mL
eBioscience; Cat. #: 16-7123-85; 500 µg

anti-mouse IL-13, clone eBio13A; 0.5 mg/mL
eBioscience; Cat. #: 14-7133-81; 50 µg
ELISA capture, 250x; ELISPOT capture, 125x

anti-mouse IL-13, clone eBio13A; 0.5 mg/mL
eBioscience; Cat. #: 14-7133-85; 500 µg
ELISA capture, 250x; ELISPOT capture, 125x

anti-mouse IL-4, clone 11B11; 0.5 mg/mL
eBioscience; Cat. #: 14-7041-85; 500 µg
ELISA capture, 250x; ELISPOT capture, 125x

anti-mouse IL-5, clone TRFK5; 0.5 mg/mL
eBioscience; Cat. #: 14-7052-81; 50 µg
ELISA capture, 250x; ELISPOT capture, 125x

anti-mouse IL-5, clone TRFK5; 0.5 mg/mL
eBioscience; Cat. #: 14-7052-85; 500 *μ*g
ELISA capture, 250x; ELISPOT capture, 125x

anti-phosphotyrosine,clone 4G10
Millipore/Upstate; Cat. #: 05-321; 100 *μ*g

anti-TCR CD3ε, armenian hamster IgG1 κ, clone 2C11; 0.5 mg/mL
BD; Cat. #: 553058; 0.5 mg

APC anti-TCRβ, clone H57-597
BioLegend; Cat. #: 109211; 100 *μ*g

biotin anti-mouse IFN-γ, clone XMG1.2; 0.5 mg/mL
BD; Cat. #: 554410; 1 mL
ELISA detection, 250x; ELISPOT detection, 250x

biotin anti-mouse IL-13, clone eBio1316H; 0.5 mg/mL
eBioscience; Cat. #: 13-7135-81; 50 *μ*g
ELISA detection, 250x; ELISPOT detection, 500x

biotin anti-mouse IL-13, clone eBio1316H; 0.5 mg/mL
eBioscience; Cat. #: 13-7135-85; 500 *μ*g
ELISA detection, 250x; ELISPOT detection, 500x

biotin anti-mouse IL-4, clone BVD6-24G2; 0.5 mg/mL
eBioscience; Cat. #: 13-7042-85; 500 *μ*g
ELISA detection, 250x; ELISPOT detection, 1,000x

Biotin anti-mouse IL-5, Clone TRFK4
BioLegend; Cat. #: 504402; 500 *μ*g
ELISA detection; ELISPOT detection

biotin anti-mouse IL-5, clone TRFK4; 0.5 mg/mL
eBioscience; Cat. #: 13-7051-81; 50 *μ*g
ELISA detection, 250x; ELISPOT detection, 500x

biotin anti-mouse IL-5, clone TRFK4; 0.5 mg/mL
eBioscience; Cat. #: 13-7051-85; 500 *μ*g
ELISA detection, 250x; ELISPOT detection, 500x

biotin rat anti-mouse IgE, clone 23G3; 0.5 mg/mL
SouthernBiotech; Cat. #: 1130-08; 0.5 mg
ELISA detection, 5,000x

DyLight 405 goat anti-armenian hamster; 1.0 mg/mL
Rockland; Cat. #: 620-146-440; 100 *µ*g

FITC hamster anti-mouse Cd11c, clone HL3; 0.5 mg/mL
BD; Cat. #: 557400; 0.1 mg

FITC-conjugated goat anti-mouse
Upstate / Millipore; Cat. #: 12-506; 0.5 mg

goat anti-armenian hamster; 1.8 mg/mL
Jackson ImmunoResearch; Cat. #: 127-005-099; 1.5 mg

goat anti-mouse IgE, 1.0 mg/mL
SouthernBiotech; Cat. #: 1110-01; 1.0 mg
ELISA capture, 200x

goat anti-syrian hamster; 1.3 mg/mL
Jackson ImmunoResearch; Cat. #: 107-005-142; 1.0 mg

HRP goat anti-mouse IgG, 0.8 mg/mL
Jackson ImmunoResearch; Cat. #: 115-035-146; 1.5 mL

mouse anti-IFN-γ, clone R4-6A2; 0.5 mg/mL
eBioscience; Cat. #: 14-7312-85; 500 *µ*g
ELISA capture

mouse IgE, clone 15.5; 0.25 mg/mL
SouthernBiotech; Cat. #: 0114-01; 0.25 mg
ELISA standard

mouse IgG2a,κ, clone UPC10
Sigma; Cat. #: M5409; 100 *µ*g
isotype control

PE anti-TCRβ, clone H57-597
BioLegend; Cat. #: 109208; 200 *µ*g

PE rat anti-mouse Siglec-F; 0.2 mg/mL
BD; Cat. #: 552126; 0.1 mg

PE-Cy7 hamster anti-mouse CD3ε, clone 145-2C11; 0.2 mg/mL
BD; Cat. #: 552774; 0.1 mg

PerCP-Cy5.5 rat anti-mouse CD45; 0.2 mg/mL
BD; Cat. #: 550994; 0.1 mg

Purified anti-mouse/human IL-5, Clone TRFK5
BioLegend; Cat. #: 504302; 500 μg
ELISA capture; ELISPOT capture

rabbit anti-human SLP-76 (aa 230-260)
Cell Signaling; Cat. #: 4958S; 100 μL

rabbit polyclonal IgG anti-Itk; 1.0 mg/mL
Millipore/Upstate; Cat. #: 06-546; 250 μg
for IP

rat anti-mouse CD16/CD32 (Fc block), clone 2.4G2; 0.5 mg/mL
BD; Cat. #: 553141; 0.1 mg

5.4.2. Chemicals

2-Mercaptoethanol
Sigma; Cat. #: M7522-100ML; 100 mL

2x YT media
BD; Cat. #: 244020; 500 g

7-AAD
BioLegend; Cat. #: 420404; 500 tests
protect from light

acetyl-β-methylcholine
Sigma; Cat. #: A2251-25G; 25 g

acetyl-β-methylcholine
Sigma; Cat. #: A2251-25G; 25 g

AEC
Sigma; Cat. #: A6926-50TAB; 50 tablets

agar, granulated
Fisher; Cat. #: BP-1423-500; 500 g

anhydrous DMSO
Sigma; Cat. #: 276855; 100 mL
protect from moisture

BSA, fraction V, 98% purity
EMD Chemicals; Cat. #: 2930; 100 g

BSA, fraction V, 98% purity
Fisher; Cat. #: BP1600-100; 100 g

carbon dioxide
Airgas; Cat. #: CD USP50; 50 lb.

collagenase D
Roche Applied Science; Cat. #: 11088858001; 100 mg

collagenase D
Roche Applied Science; Cat. #: 11088874103; 1 g

collagenase, type 4
Worthington Biochemical Corp.; Cat. #: LS004186; 100 mg

DMF
Sigma; Cat. #: D4551-250ML; 250 mL

DNAse (deoxyribonuclease) I
Sigma; Cat. #: DN25-10MG; 10 mg

DNAse (deoxyribonuclease) I
Sigma; Cat. #: DN25-100MG; 100 mg

ethanol, 200 proof, molecular biology grade
Fisher; Cat. #: AC615095000; 500 mL

ethanol, 95%, denatured (general purpose)
Fisher; Cat. #: S73985A; 3.8 L

ethanol, 95%, denatured (general purpose)
EMD; Cat. #: EX0280-3; 4 L

glycine
Fisher; Cat. #: BP381-1; 1 kg
FW: 75.07

hydrogen peroxide, 30% w/v, ACS grade
VWR; Cat. #: BDH3690; 500 mL

Imject alum
Pierce / Thermo Scientific; Cat. #: 77161; 50 mL
adjuvant

Indo-1, AM
Molecular Probes; Cat. #: I-1223; pack
20 x 50 µg

isoflurane
TW Medical Veterinary Supply; Cat. #: 21233484; 100 mL
store in chemical fume hood

isopropanol, ACS grade
Fisher; Cat. #: BP2632-4; 4 L

ketamine HCl (Ketaset III), 100 mg/mL
Fort Dodge Animal Health; Cat. #: NDC 0856-4403-01; 10 mL

LB broth, Lennox
BD; Cat. #: 240230; 500 g

liquid nitrogen, low pressure
Airgas; Cat. #: 160LT22; 160 L

methanol, ACS grade
Sigma; Cat. #: 179337-500ML; 500 mL

methanol, ACS grade
Fisher; Cat. #: A412-1; 1 L

methanol, ACS grade
VWR; Cat. #: BDH1135-1LP; 1 L

mouse recombinant IFN-γ
eBioscience; Cat. #: 14-8311-63; 10 μg
ELISA standard

mouse recombinant IL-13; 0.1 mg/mL
eBioscience; Cat. #: 14-8131-62; 5 μg
ELISA standard; dilute to 1 μg/mL in ELISA blocking buffer; prepare aliquots and store at -80°C

mouse recombinant IL-4; 0.1 mg/mL
eBioscience; Cat. #: 14-8041-62; 5 μg
ELISA standard; dilute to 1 μg/mL in ELISA blocking buffer; prepare aliquots and store at -80°C

mouse recombinant IL-5
eBioscience; Cat. #: 14-8051-62; 5 μg
ELISA standard

ovalbumin, grade V
Sigma; Cat. #: A-5503; 10 g

oxygen, E-tank
Airgas; Cat. #: OX USPE; E-tank

phosphoric acid, ACS grade
Fisher; Cat. #: A242-1; 1 L
CAS #: 7664-38-2

poly-L-lysine, 0.1%
Sigma; Cat. #: P8920-100ML; 100 mL

SEE (staphylococcal enterotoxin E)
Toxin Technologies; Cat. #: ET404; 100 μg

sodium acetate
Fisher; Cat. #: S209-500; 500 g

TMB ELISA substrate solution, 1x
eBioscience; Cat. #: 00-4201-56; 100 mL

Tris base
Fisher; Cat. #: BP152-500; 500 g
FW: 121.14

xylazine (AnaSed), 20 mg/mL
Lloyd Laboratories; Cat. #: 4811; 20 mL

5.4.3. Consumables

1 L vacuum filtration units, 0.22 μm
Nalgene; Cat. #: 567-0020; case of 12

1 mL filter tips, 100/rack
Axygen; Cat. #: TF-1000-R-S; pack of 10

1 mL pipet tips, bulk, non-sterile
VWR; Cat. #: 82028-564; pack of 1,000

1 mL serological pipets, disposable, individually-wrapped, pyrogen-free
Fisher; Cat. #: 13-678-11B; case of 1,000

10 mL serological pipets, disposable, individually-wrapped, pyrogen-free
Fisher; Cat. #: 13-678-27F; case of 500

10 mL serological pipets, disposable, individually-wrapped, pyrogen-free
Fisher; Cat. #: 13-678-11E; case of 200

10 μL pipet tips, bulk, non-sterile
VWR; Cat. #: 82028-528; pack of 1,000

10% zinc formalin
Protocol; Cat. #: 313-095; 1 gallon

100 cell dividers for freezer boxes
Fisher; Cat. #: 03-395-457; pack of 12

115 mL vacuum filtration units; 45 μm, PES
Nalgene; Cat. #: 124-0045; pack of 6

12-well culture plates
Corning Costar; Cat. #: 3513; case of 50

15 mL centrifuge tubes w/ screw caps
BD Falcon; Cat. #: 352097; case of 500

15 mL centrifuge tubes w/ screw caps
Denville; Cat. #: C1018-P; case of 500

150 cm² flasks, w/ membrane cap
Corning; Cat. #: 430825; case of 50

150 mL vacuum filtration units, 0.22 *μ*m, PES
Nalgene; Cat. #: 565-0020; case of 12

18 gauge tubing adaptor
BD Medical; Cat. #: 408208; pack of 25
tracheal cannula

20 *μ*L filter tips, 96/rack
Axygen; Cat. #: TF-20-R-S; pack of 10

200 *μ*L filter tips, 96/rack
Axygen; Cat. #: TF-200-R-S; pack of 10

200 *μ*L gel-loading pipet tips, bulk, non-sterile
Fisher; Cat. #: 02-707-181; pack of 1,000

200 *μ*L pipet tips, bulk, non-sterile
Fisher; Cat. #: 02-681-134; pack of 1,000

24-well culture plates
Corning Costar; Cat. #: 3524; case of 100

25 cm² flasks
Corning; Cat. #: 430168; case of 500

25 mL serological pipets, disposable, individually-wrapped, pyrogen-free
Fisher; Cat. #: 13-678-11; case of 200

25 mL serological pipettes, disposable, individually-wrapped, pyrogen-free
VWR; Cat. #: 89130-900; case of 200

250 mL vacuum filtration units, 0.22 *μ*m, PES
Nalgene; Cat. #: 568-0020; case of 12

250 mL vacuum filtration units, 0.22 *μ*m, PES
Denville Scientific; Cat. #: F5225; case of 12

27G 1/2" needles
BD; Cat. #: 305109; pack of 100

5 mL serological pipets, disposable, individually-wrapped, pyrogen-free
Fisher; Cat. #: 13-678-27E; case of 500

5 mL serological pipets, disposable, individually-wrapped, pyrogen-free
Fisher; Cat. #: 13-678-11D; case of 200

50 mL centrifuge tubes, 25/rack
BD Falcon; Cat. #: 352098; case of 500

50 mL vacuum filter tubes
Millipore; Cat. #: SCGP00525; pack of 25

500 mL vacuum filter units, 0.22 *µ*m, 75 mm, PES
Nalgene; Cat. #: 566-0020; case of 12

500 mL vacuum filter units, 0.22 *µ*m, 75 mm, PES
Denville Scientific; Cat. #: F5227; case of 12

6x gel loading dye
NEB; Cat. #: B7021S; each

70 *µ*m nylon strainers
Fisher; Cat. #: 22-363-548; case of 50

75 cm^2 flasks, w/ membrane cap
Corning; Cat. #: 430641; case of 100

96-well filter plates
Millipore; Cat. #: MSIPS4510; pack of 10
ELISPOT plates

96-well plates, round bottom
Sarstedt; Cat. #: 83.1837; case of 100

alcohol prep pads
Fisher; Cat. #: 06-669-62; case of 4,000

bench paper (absorbent surface liner), 20”x30’ roll
Fisher; Cat. #: 14-127-47; case

BioTrace PVDF membrane, 30 cm x 3 m
Pall Life Science; Cat. #: 66543; each
thickness: 165 µm; pore size: 0.45 µm

blotting pads, 11x18 cm
VWR; Cat. #: 28297-994; pack of 50

broken glass disposal boxes
Fisher; Cat. #: 12-009-7A; pack of 6

broken glass disposal boxes
VWR; Cat. #: 56617-801; pack of 6

C57BL6/J mice, female, 6 weeks
Jackson Laboratory; Cat. #: 000664; each

C57BL6/J mice, female, 8 weeks
Jackson Laboratory; Cat. #: 000664; each

caps for culture tubes, 16 mm diameter
KimKap; Cat. #: 73660 16; case of 1,000

CD4 Negative Isolation Kit
Invitrogen; Cat. #: 114-16D; 4 mL

cryogenic vial closures, blue
Nalgene; Cat. #: 5045-0003; pack of 100

cryogenic vial closures, green
Nalgene; Cat. #: 5045-0004; pack of 100

cryogenic vial closures, red
Nalgene; Cat. #: 5045-0005; pack of 100

cryogenic vial closures, white
Nalgene; Cat. #: 5045-0000; pack of 100

cryogenic vial closures, yellow
Nalgene; Cat. #: 5045-0002; pack of 100

cryogenic vials, 1.2 mL, pack of 25
Nalgene; Cat. #: 5000-0012; case of 500

cryogenic vials, 2.0 mL, pack of 25
Nalgene; Cat. #: 5000-0020; case of 500

culture tubes, 16 x 100 mm, disposable glass
Fisher; Cat. #: 14-961-29; case of 1,000

Cy5 monoreactive kit
GE Healthcare; Cat. #: PA25001; kit
kit contains 5 packs for labeling 1 mg of protein each

DH5α E. coli, sub-cloning efficiency, chemically competent
Invitrogen; Cat. #: 18265-017; 2 mL

DMEM, 500 mL
Mediatech Cellgro; Cat. #: 15-013-CV; pack of 6

DNA ladder, 2-log; 1,000 μg/mL
NEB; Cat. #: N3200L; 500 gel lanes

electroporation cuvettes, 4 mm gap
Molecular BioProducts; Cat. #: 5540; pack of 50

ELISA plates, 96 well
BD; Cat. #: 353279; case of 100

FACS tubes, 5 mL, sterile, round-bottom, 12x75 mm
BD; Cat. #: 352054; pack of 125

film, 8”x10”
Kodak; Cat. #: 8646770; pack of 50 sheets
protect from light

film, 8”x10”
Denville Scientific; Cat. #: E3018; pack of 100 sheets
protect from light

freezer boxes, fiberboard, w/ covers
Fisher; Cat. #: 11-678-24A; pack of 12

glass coverslips, No. 1, 18 x 18 mm
Fisher; Cat. #: 12-548-A; 1 oz. pack

glass slides, 25 x 75 x 1 mm
Fisher; Cat. #: 12-544-7; gross of 144

grade 1 filter paper, 15.0 cm diameter
Whatman; Cat. #: 1001-150; pack of 100

Hema 3 staining solutions, 3x 500 mL
Protocol; Cat. #: 122-911C; pack of 3

HEPES, 1 M, 100 mL
Mediatech Cellgro; Cat. #: 25-060-CI; pack of 6

histopaque, 100 mL
Sigma; Cat. #: 10771-100ML; 100 mL

incandescent projector lamp bulb; BLC, INC 120V, 30W
Ushio; Cat. #: 1000060; each
for light microscope in TC room

L-Glutamine, 200 mM, 100 mL
Mediatech Cellgro; Cat. #: 25-005-CI; pack of 6

laboratory labeling tape, yellow 3/4" width x 500" length
VWR; Cat. #: 89097-996; pack of 4 rolls

laboratory wipes, 4.4"x8.4"
Kimwipe; Cat. #: 34155; pack of 60

laboratory wipes, 4.5"x8.4"
VWR; Cat. #: 82003-820; case of 60

large nitrile gloves, pack of 100
Fisher; Cat. #: 19-130-1597D; case of 10

Lipofectamine 2000 transfection reagent
Invitrogen / Life Technologies; Cat. #: 11668-027; 0.75 mL

medium nitrile gloves, pack of 100
Fisher; Cat. #: 19-130-1597C; case of 10

medium nitrile gloves, pack of 100
Denville Scientific; Cat. #: G4162; case of 10

microcentrifuge tubes, 1.8 mL, assorted, 500/pack
Axygen; Cat. #: MCT-150-A; case of 10

Mouse T Cell Nucleofector Kit
Lonza; Cat. #: VPA-1006; 25 reactions

Natelson blood collecting tubes
Kimble Chase; Cat. #: 42F604; pack of 100

Natelson blood collecting tubes
Fisher; Cat. #: 02-668-15; pack of 100

pasteur pipets, glass
Fisher; Cat. #: 13-678-8A; pack of 250

PBS, 500 mL, cell culture grade
Mediatech; Cat. #: 21-040-CV; pack of 6

PCR tubes, 0.2 mL, thin wall, clear
Axygen; Cat. #: PCR-02-C; pack of 1,000

penicillin-streptomycin, 100 mL
Mediatech Cellgro; Cat. #: 30-002-CI; pack of 6
100x; 10,000 I.U./mL penicillin; 10,000 µg/mL streptomycin

Permount mounting medium
Fisher; Cat. #: SP15-100; 100 mL

petri dishes, 100 x 20 mm
Corning; Cat. #: 430167; case of 500

pipet basins, 50 mL capacity, bulk packaging
Fisher; Cat. #: 13-681-500; case of 100

pipet basins, 50 mL capacity, bulk packaging
VWR; Cat. #: 89094-684; case of 100

Platinum Taq DNA Polymerase High Fidelity
Invitrogen; Cat. #: 11304-011; 100 rxns

Precision Plus Protein Dual Color Standard
Bio-Rad; Cat. #: 161-0374; 500 µL
50 applications

ProLong anti-fade mounting medium
Invitrogen; Cat. #: P7481; 1 kit

Protein G Sepharose 4 Fast Flow Media
GE Healthcare; Cat. #: 17-0618-01; 5 mL

red biohazard bags, 25” x 35”
Fisher; Cat. #: 01-828D; pack of 200

RPMI-1640, w/o L-Glutamine, 500 mL
Mediatech Cellgro; Cat. #: 15-040-CV; pack of 6

scintillation vials, borosilicate glass, 20 mL
VWR; Cat. #: 66022-060; case of 500

single cytology funnels with cards
Biomedical Polymers, Inc.; Cat. #: BMP-CYTO-S50; pack of 50

small nitrile gloves, pack of 100
Fisher; Cat. #: 19-130-1597B; case of 10

Stopcock one-way luer lock
Promega; Cat. #: A7261; 10 each
for vacuum manifold

SuperSignal West Pico ECL substrate
Pierce; Cat. #: 34080; 500 mL

SuperSignal West Pico ECL substrate
Pierce; Cat. #: 34077; 100 mL

syringe filters, 0.22 *μ*m
Nalgene; Cat. #: 190-2520; case of 50

syringes, 1mL
BD; Cat. #: 309602; pack of 100

transfer pipets, 1 mL, bulk packaging
Fisher; Cat. #: 13-711-9D; case of 500

transfer pipets, 1 mL, individually wrapped
Fisher; Cat. #: 13-711-20; case of 500

UltraClean 15 UltraBind
Mo Bio Laboratories; Cat. #: 121003001; each

UltraClean 15 UltraSalt
Mo Bio Laboratories; Cat. #: 121003002; each

UltraClean 15 UltraWash
Mo Bio Laboratories; Cat. #: 121003003; each

VaporGuard charcoal filter
Vet Equip; Cat. #: 931401; each

water, 500 mL, cell culture grade
HyClone / Thermo Scientific; Cat. #: SH30529.02; each

weighing paper, 3”x3”
VWR; Cat. #: 12578-121; pack of 500

weighing paper, 3”x3”
Fisher; Cat. #: 09-898-12A; pack of 500

Wizard Maxipreps DNA Purification Resin
Promega; Cat. #: A7401; 500 mL

Wizard MegaColumns
Promega; Cat. #: A7421; 50 each

Wizard MiniColumns
Promega; Cat. #: A7211; 250 each

X-large nitrile gloves, pack of 100
Fisher; Cat. #: 19-130-1597E; case of 10

X-small nitrile gloves, pack of 100
Fisher; Cat. #: 19-130-1597A; case of 10

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