Differential induction of IL-17, IL-10, and IL-9 in human T helper cells by B7h and B7.1.

Differential induction of IL-17, IL-10, and IL-9 in human T helper cells by B7h and B7.1

Riccardo Mesturini, Casimiro L. Gigliotti, Elisabetta Orilieri, Giuseppe Cappellano, Maria F. Soluri, Elena Boggio, Abiy Woldetsadik, Chiara Dianzani, Daniele Sblattero, Annalisa Chiocchetti, Junji Yagi, Josè M. Rojo, Umberto Dianzani

Abstract

ICOS and CD28 are expressed by T cells and are involved in costimulation of cytokine production in T helper (TH) cells. ICOS binds B7h expressed by several cell types, whereas CD28 binds B7.1 and B7.2 expressed by activated antigen presenting cells. This work investigated the role of B7h and B7.1 in TH17 and TH9 cell differentiation by assessing activity of recombinant B7h-Fc and B7.1-Fc on human naïve TH cells activated in the presence of different combinations of exogenous cytokines. In the presence of TGF-β1 and IL-1β (TH17 promoting condition), B7h-Fc was more effective than B7.1-Fc in inducing IL-17A and IL-10 secretion, whereas B7.1-Fc was more effective in inducing IL-17F. Dual costimulation with B7h-Fc and B7.1-Fc displayed an intermediate pattern with predominance of IL-17F over IL-17A, secretion of high levels of IL-10, and secretion of IL-9 levels lower than those induced by B7.1-Fc alone. In the presence of TGF-β1 and IL-4 (TH9 promoting condition), B7h-Fc induced IL-17A only, whereas B7.1-Fc induced also IL-17F, IL-10, and high levels of IL-9. Experiments on memory TH cells showed that B7h-Fc mainly supported secretion of IL-17A and IL-10, whereas B7.1-Fc supported secretion of IL-17A, IL-17F, IL-10, and IL-9. These data indicate that B7h and B7.1 play different roles in modulation of TH17 and TH9 differentiation. This plasticity might be important in the immune response to pathogens and tumors, and in the development of autoimmune diseases, and should be taken in consideration in designing of immunotherapeutic protocols triggering ICOS or CD28.

Abbreviations

TH, T helper; APC, antigen-presenting cells; TCR, T cell receptor; mAbs, monoclonal antibody; PBMC, peripheral blood mononuclear cells; ICOS, inducible costimulator; TGF-β, transforming growth factor-β; IL-, interleukine-; INF-γ, interferon-γ; PB,peripheral blood; Ig, immunoglobulin

1. Introduction

Activation and differentiation of naïve T helper (TH) cells requires three signals from antigen-presenting cells (APC). The first is delivered through the T cell receptor (TCR) upon recognition of the antigenic peptide presented by the appropriate MHC molecule, the second by T cell costimulatory molecules engaged by their ligands expressed on APC, and the third by cytokines in the microenvironment [1] and [2].

The best known TH cell costimulatory molecule is CD28, which is constitutively expressed by all TH cells, binds B7.1 (CD80) and B7.2 (CD86) on APC, and promotes cell proliferation and cytokine secretion [3], [4] and [5]. Another TH costimulator is ICOS (CD278), which belongs to the CD28 family and is selectively expressed by activated T cells [6], [7] and [8]. It binds B7h (CD275, B7H2,

B7-RP1, ICOSL, GL50) expressed by both haematopoietic and non-haematopoietic cells. B7h is constitutively expressed by B cells, macrophages, and dendritic cells, but it is also expressed by vascular endothelial cells, epithelial cells, and fibroblasts; ICOS-mediated triggering of B7h modulates adhesiveness of vascular endothelial cells to inflammatory and tumor cells [9] and [10]. This expression pattern suggests that the ICOS:B7h interaction has a role in both T cell activation in lymphoid organs and the control of T cell function at sites of inflammation[11]. In TH cells, ICOS function appears to modulate cytokine secretion by acting on recently activated/memory cells, whereas it is less effective in naïve TH cells, whose activation seems to depend primarily on CD28 costimulation [12], [13], [14], [15],[16] and [17].

To date, four types of effector TH cells are generally recognized, namely TH1, TH2, TH17, and Treg cells, characterized by secretion of different patterns of cytokines [18]. Under certain circumstances, these patterns may switch from that of one lineage toward another suggesting that TH cells are plastic depending on the inflammatory conditions[19]. Their differentiation from naïve TH cells depends on the cytokine environment during activation, but a role may also be played by the costimulatory receptors delivering the “second signal”. For instance, CD28 costimulation induces secretion of high amounts of IL-2 supporting most types of T cells, whereas ICOS costimulation is incapable to induce IL-2 but potently induces IL-10 secretion. Moreover, substantial differences have been reported in humans and mice since ICOS triggering induces secretion of high amounts of IL-4 in mice but IFN-γ in humans, which are associated with TH2 and TH1 cells respectively [20], [21], [22], [23], [24] and [25]. These differences might be partly due to the capacity of the human but not the mouse B7h to weakly interact with CD28 and CTLA4, using binding sites different from that used by ICOS [26].

Recent data showed that ICOS and CD28 may also play a different role in differentiation of TH17 cells, which are involved in the immune response against certain parasites and in some autoimmune diseases [27], [28], [29] and [30]. In humans, they are characterized by the production of IL-17A, IL-17F, IL-21, IL-22, and IL-26 and their differentiation is dependent on the transcription factor retinoic acid-related orphan nuclear receptor C2 (RORC2) [31]. TH17 differentiation is controlled by several cytokines and is inhibited under TH1 and TH2 polarizing conditions, i.e. presence of IFN-γ and IL-4, respectively. In mice, TH17 differentiation requires TGF-β1 and IL-6, is amplified in an autocrine fashion by IL-21, and is further promoted by IL-23 [32]. In humans, TH17 differentiation involves the same cytokines, but their relative contributions are debated [31], [33], [34], [35],[36] and [37].

Early reports suggested that both ICOS and CD28 were involved in TH17 differentiation in mice [28] and [38]. More recently, studies using ICOS-deficient mice indicated that ICOS was dispensable for TH17 differentiation, but it was necessary for TH17 cells to attain full effector functions [39]. In humans, ICOS was found to be more effective than CD28 in inducing IL-17A and IL-17F secretion in peripheral blood (PB) “memory” TH17 ($CCR4^{+}CCR6^{+}$), Treg ($CD25^{+}CD127^{lo}FoxP3^{+}$), and TFH ($CXCR5^{+}CD45RO^{+}$) cells, but not in TH1 ($CXCR3^{+}CCR4^{-}CCR6^{+}$) and TH2 ($CCR4^{+}CXCR3^{-}CCR6^{-}$) cells [40]. Moreover, in long-term cultures, ICOS but not CD28 triggering was capable to support development of TH17 cells from umbilical cord blood naïve TH cells ($CD4^{+}CD45RA^{+}CD25^{-}$) but only in the presence of a TH17-polarizing milieu containing TGF-β1, IL-6, IL-1β, IL-23, and neutralizing antibodies to IFN-γ and IL-4 [40].

TH cells generated under TH17-promoting conditions comprises not only IL-17 secreting cells, but also cells secreting IL-9, exerting its activity on several cell types including T cells, mast cells, eosinophils, and epithelial cells. IL-9 secretion has been initially ascribed to TH2 cells, but recent

reports described a distinct subset of TH9 cells characterized by secretion of IL-9 and IL-10, lacking secretion of IFN-γ, IL-4, and IL-17, and involved in development of allergic airway disease and in the response to parasitic infections [19]. However, IL-9 production is not unique to such cells since it may also be expressed by Tregs and TH17 cells [18]. TH9 cell differentiation is supported by CD28 costimulation and requires presence of TGF-β1 and IL-4, whereas no data are available on the role of ICOS.

This work compared the activity of fusion proteins of the extracellular portion of human B7h or B7.1 fused to the human IgG1 Fc (i.e. B7h-Fc and B7.1-Fc) and dissected the role of the cytokine microenvironment in induction of TH17 and TH9 cytokine secretion in short term cultures of PB naïve TH cells from adult donors. The aim was to provide information helping to understand the variable effects that these reagents have when used as systemic or local immune modulators in vivo. Results showed that B7h-Fc was more effective than CD28 in induction of IL-17A and IL-10, whereas B7.1-Fc was more effective in induction of IL-17F and IL-9. The minimal cytokine microenvironment to obtain these responses was presence of TGF-1β plus either IL-1β or IL-4, but IL-6 and IL-21 further supported production of these cytokines.

2. Materials and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation from buffy coats provided by the local Blood Transfusion Service (Novara, Italy). Naïve $CD4^+$ T cells were purified by panning to remove $CD11b^+$, $CD45RO^+$, $CD25^+$, $CD19^+$, and $HLA\text{-}DR^+$ cells with the appropriate monoclonal antibody (mAbs), followed by use of the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec) or directly with $CD4^+$ T naïve Cell Isolation Kit II (Miltenyi Biotec). This approach provided >97% cells displaying the phenotype $CD3^+CD4^+CD45RA^+CD14^-CD16^-$, as assessed by direct immunofluorescence and flow cytometry.

$CD4^+CCR6^+$ were purified by using the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec) followed by positive selection with a PE-conjugated anti-CCR6 mAb and anti-PE magnetic beads (Miltenyi Biotec). $CD4^+CD45RO^+$ were purified by using the Memory $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec).

2.2. TH cell differentiation assay

Round-bottom 96-well plates were coated with 100 µl of anti-CD3 mAb (OKT3) overnight at 4 °C. To stimulate ICOS or CD28, plates were washed with PBS and further coated with B7h-Fc (5 µg/ml; R&D System) or B7.1-Fc (5 µg/mL; R&D System) for 2 h at room temperature.

Plates were then washed with PBS and purified naïve $CD4^+$ T cells were seeded at 10^5 cells/well in triplicate in 200 µl of in RPMI 1640 (Gibco) plus 10% FCS, or EX-VIVO 20 Serum Free (Lonza). Where indicated, cultures were performed in the presence of the following recombinant cytokines: IL-1β and IL-4 (10 ng/ml; PeproTech), IL-6 (20 ng/ml; PeproTech), IL-21 (100 ng/ml; Immunotools), IL-23 (20 ng/ml; R&D Systems), and TGF-β1 (5 ng/ml; R&D Systems). In some experiments, neutralizing antibodies against TGF-β1 (10 µg/ml; R&D Systems), IL-1β (4 µg/ml; R&D Systems), IL-4 (4 µg/ml; R&D Systems), and IFN-γ (8 µg/ml; R&D Systems) were added to the cultures. To

analyze cytokine secretion, supernatants were collected at day 5 of culture and standard enzyme-linked immunosorbent assays (ELISA) were used to evaluate secretion of IL-17A (R&D Systems), IL-17F, IL-17A/F, IL-9 (eBioscience), and IL-10 (Biolegend).

2.3. Real-time PCR

Total RNA was extracted and transcribed to cDNA with Cells-to-cDNA II Kit (Ambion) from cells collected at day 5 of culture after re-stimulation with PMA (50 ng/mL, Sigma–Aldrich) plus Ionomycin (1 μg/mL, Sigma–Aldrich) for the last 5 h. Transcripts were quantified by real-time PCR on an ABI PRISM 7000 sequence detector (Applied Biosystems) with Applied Biosystems predesigned TaqMan Gene Expression Assays in duplicate for each sample. Relative quantification was performed using the comparative ΔΔCT method.

The following probes were used (Applied Biosystems assay identification numbers in parentheses): IL-17A (Hs00174383_m1), IL-17F (Hs00369400_m1), RORc (Hs01076112_m1), IL-26 (Hs00218189_m1), PU.1 (Hs02786711_m1). For each sample, mRNA abundance was normalized to the amount of ribosomal protein HPRT (Hs99999909_m1).

2.4. Intracellular staining

Cytokine-producing cells were stained after stimulation with PMA (20 ng/mL, Sigma–Aldrich) plus Ionomycin (2 μg/mL, Sigma–Aldrich) for 6 h in the presence of Brefeldin A (10 μg/ml, Sigma–Aldrich). After fixation with 2% paraformaldehyde (Sigma–Aldrich), cells were permeabilized with PBS/1% Saponin (Sigma–Aldrich) and stained using anti-IL-17A mAb (IgG1) conjugated with APC (eBioscience), anti-IL-17F mAb (IgG1) conjugated with PE (eBioscience), anti-IL-10 mAb (IgG1) conjugated with PE (Miltenyi Biotec), anti-IFNγ mAb (IgG2b) conjugated with FITC (eBioscience), and ROR-γt mAb (IgG2b) conjugated with APC (eBioscience), and the isotype-matched Ig controls (eBioscience).

2.5. Statistical analysis

The non-parametric paired Wilcoxon test was used to compare differences in all experiments. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Differential effect of costimulation mediated by B7h-Fc and B7.1-Fc on expression of TH17-related cytokines

To investigate the cytokine milieu necessary to support IL-17A secretion in naïve TH cells, we analyzed IL-17A secretion induced by B7-Fc in the presence of different combinations of recombinant cytokines. Experiments were performed in the absence of neutralizing antibodies to IL-4 and IFN-γ, often used to increase IL-17 secretion, to allow these cytokines to endogenously influence the cell response. Purified PB naïve TH cells were incubated in serum free medium in wells pre-coated with anti-CD3 mAb in the presence and absence of B7h-Fc and different combinations of exogenous TGF-β1, IL-1β, IL-6, IL-21, and IL-23. Fig. 1 shows the data obtained in the presence of B7h-Fc. It shows that IL-17A secretion was always undetectable in the absence of TGF-β1. In the presence of TGF-β1, IL-1β or IL-21 were able to induce IL-17A, with a higher induction observed in the presence of both the exogenous cytokines. In contrast, IL-6 and IL-23 did not induce IL-17A secretion even in the presence of TGF-β1 and did not increase secretion induced by TGF-β1 + IL-21, but they increased secretion induced by TGF-β1 + IL-1β (Fig. 1). B7h-

Fc was crucial for this response since IL-17A secretion was never detected when cells were activated using the anti-CD3mAb alone (data not shown).

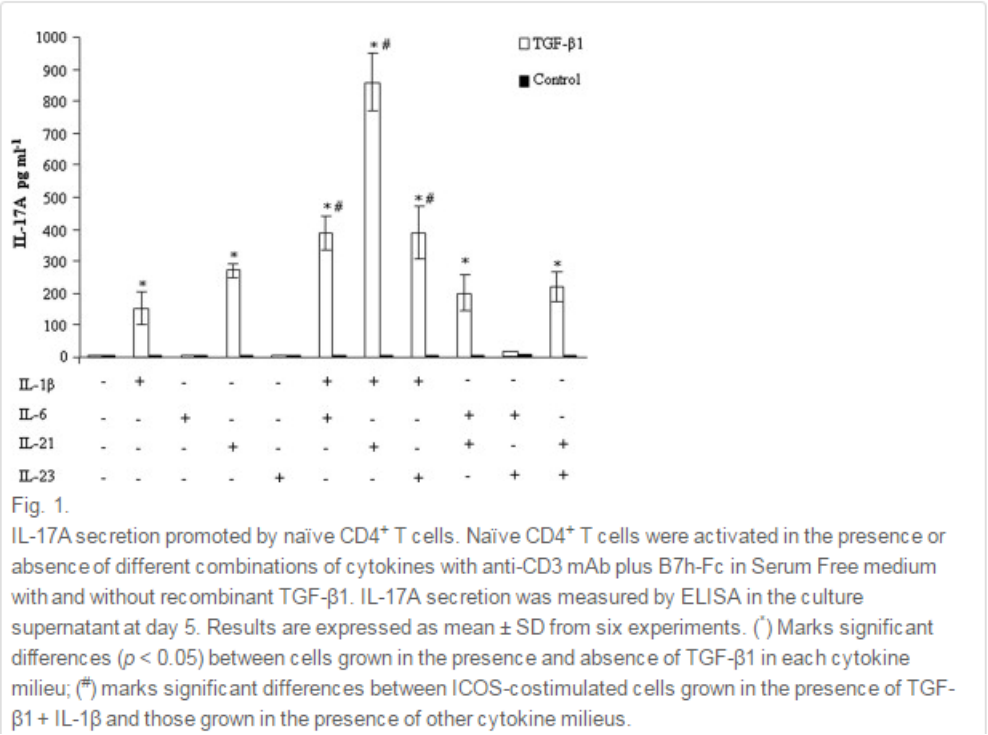

Fig. 1.
IL-17A secretion promoted by naïve CD4+ T cells. Naïve CD4+ T cells were activated in the presence or absence of different combinations of cytokines with anti-CD3 mAb plus B7h-Fc in Serum Free medium with and without recombinant TGF-β1. IL-17A secretion was measured by ELISA in the culture supernatant at day 5. Results are expressed as mean ± SD from six experiments. (*) Marks significant differences ($p < 0.05$) between cells grown in the presence and absence of TGF-β1 in each cytokine milieu; (#) marks significant differences between ICOS-costimulated cells grown in the presence of TGF-β1 + IL-1β and those grown in the presence of other cytokine milieus.

Then, we compared the effect of B7h-Fc and B7.1-Fc on IL-17A secretion in PB naïve TH cells and we extended the analysis to IL-17F, the IL-17A/F heterodimer, and IL-10, which can be secreted by TH17 cells. Naïve CD4+ T cells were activated with anti-CD3 mAb plus either B7h-Fc or B7.1-Fc and were cultured in the presence or absence of TGF-β1 + IL-1β together with either IL-6, or IL-21, or IL-23, or the three cytokines combined. ELISA analysis of the culture supernatants showed that B7h-Fc was more efficient than B7.1-Fc in inducing IL-17A (Fig. 2A) and IL-10 (Fig. 2C) secretion in the presence of TGF-β1 + IL-1β with or without the other cytokines. In B7h-Fc costimulated cells, IL-10 secretion was substantially increased by addition of either IL-6 or IL-21 to the culture medium. Conversely, IL-17F secretion was induced to higher levels by B7.1-Fc than by B7h-Fc provided that TGF-β1 and IL-1β were supplemented with at least one of the other cytokines (Fig. 2B). By contrast, secretion of the IL-17A/IL-17F heterodimer was undetectable in all culture conditions (data not shown).

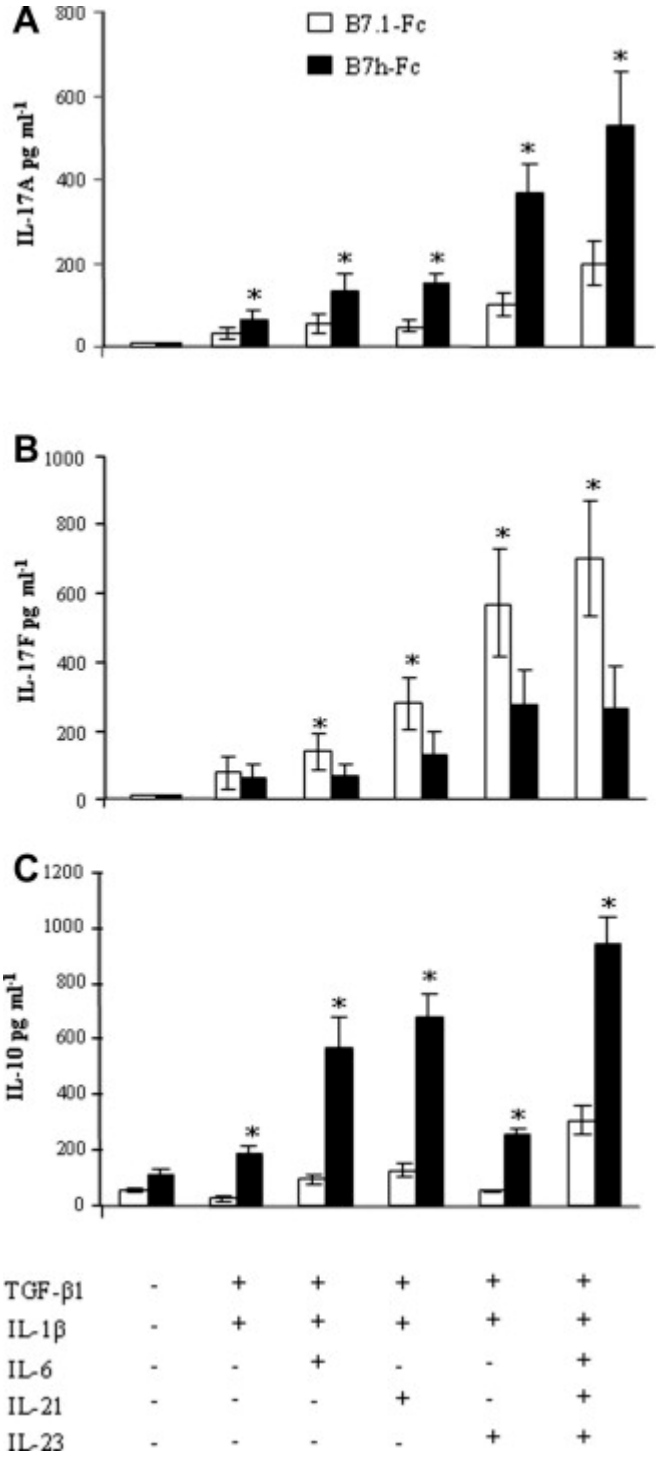

Fig. 2.

Secretion of IL-17A, IL-17F, and IL-10 induced in naïve $CD4^+$ T cells by costimulation with B7h-Fc or B7.1-Fc. Naïve $CD4^+$ T cells were stimulated with anti-CD3 plus either B7h-Fc or B7.1-Fc in the presence of different combinations of TGF-β1, IL-1β, IL-6, IL-21, and IL-23. Secretion of IL-17A (A), IL-17F (B) and IL-10 (C) was assessed by ELISA in the supernatants at day 5. Results are expressed as mean ± SD from six experiments. (*) Marks significant differences ($p < 0.05$) between B7h-Fc and B7.1-Fc costimulated cells in each cytokine milieu.

To determine if these effects were manifested at transcriptional level, we evaluated expression of the IL-17A and IL-17F mRNA by real time PCR in cells activated for 5 days using the conditions above followed by reactivation with PMA + Ionomycin for 5 h. This analysis was also extended to the IL-26 mRNA which is also expressed by human TH17 cells. IL-17A mRNA analysis (Fig. 3A) was largely in agreement with the protein data since B7h-Fc significantly induced higher levels of IL-17A mRNA than B7.1-Fc in the presence of TGF-β1 + IL-1β with either IL-6, or IL-21, or IL-23. IL-17F mRNA analysis (Fig. 3B) paralleled the protein data since B7.1-Fc significantly induced higher levels than B7h-Fc under all culture conditions where TGF-β1 + IL-1β were present. IL-26 mRNA was induced to relatively low levels, but induction was higher with B7.1-Fc than with B7h-Fc. Moreover, the IL-26 mRNA was induced at the highest levels when all recombinant cytokines were present in both B7h- and B7.1-costimulated cells (Fig. 3C).

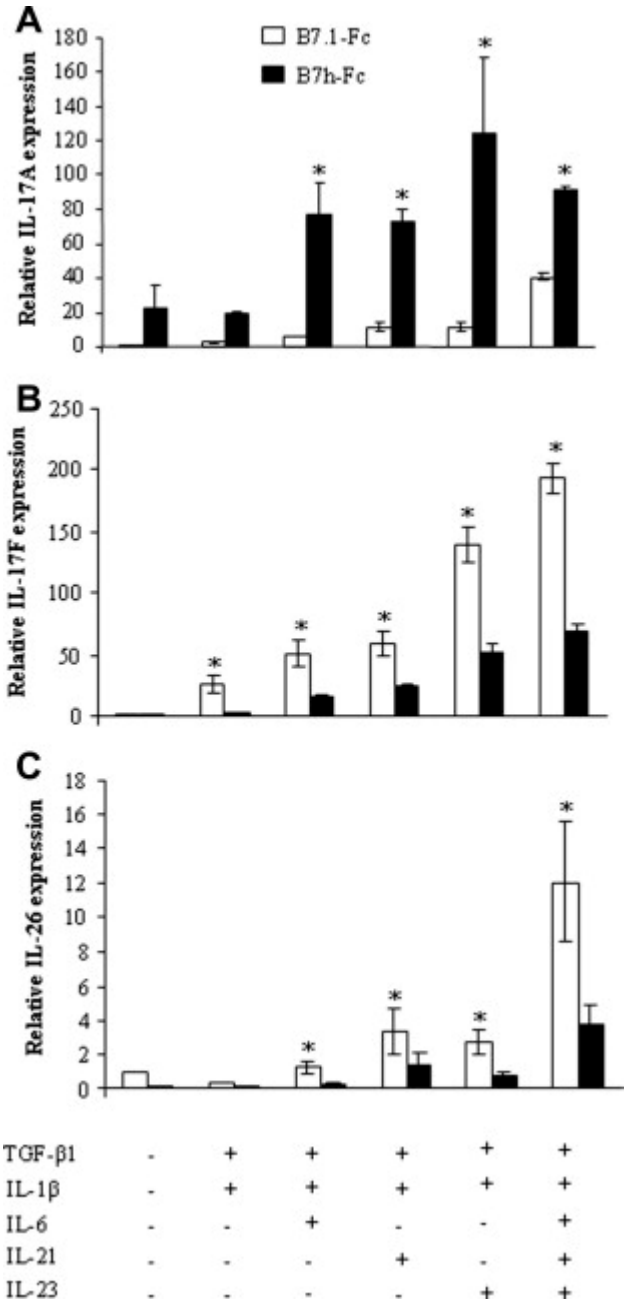

Fig. 3.

Expression of the IL-17A, IL-17F, and IL-26 mRNA induced in naïve $CD4^+$ T cells by costimulation with B7h-Fc or B7.1-Fc. Naïve $CD4^+$ T cells were stimulated as in Fig. 2 and mRNA encoding for IL-17A (A), IL-17F (B), and IL-26 (C) was evaluated by real time PCR at day 5 after re-stimulation with PMA/Ionomycin for the last 5 h; cycling threshold values were normalized to those of mRNA encoding HPRT, and expression detected in B7.1-Fc-treated cells was set as 1.0. Results are expressed as mean ± SD from six experiments. (*) Marks significant differences ($p < 0.05$) between B7h-Fc and B7.1-Fc costimulated cells in each cytokine milieu.

3.2. Differential effect of B7h-Fc and B7.1-Fc on expression of TH9-related cytokines

Differentiation of TH9 cells requires presence of TGF-β1 + IL-4, but IL-9 secretion can also be induced, at lower levels, in the presence of TGF-β1 + IL-1β [19]. Therefore, we compared ability of B7h-Fc and B7.1-Fc to induce secretion of IL-9, IL-10, IL-17A, and IL-17F in the presence of either

TGF-β1 + IL-1β or TGF-β1 + IL-4. Moreover, we assessed the effect of the dual costimulation with B7h-Fc + B7.1-Fc in the same conditions to assess the reciprocal interference of the two costimuli. PB naïve $CD4^+$ T cells were activated with anti-CD3 mAb plus B7h-Fc and/or B7.1-Fc and cultured with either TGF-β1 + IL-1β (TH17 promoting conditions) or TGF-β1 + IL-4 (TH9 promoting conditions) in the presence and absence of IL-6 + IL-21 supporting both TH17 and TH9 cells [35],[41] and [42]. Secretion of IL-17A, IL-17F, IL-10, and IL-9 was then assessed by ELISA in the culture supernatants (Fig. 4).

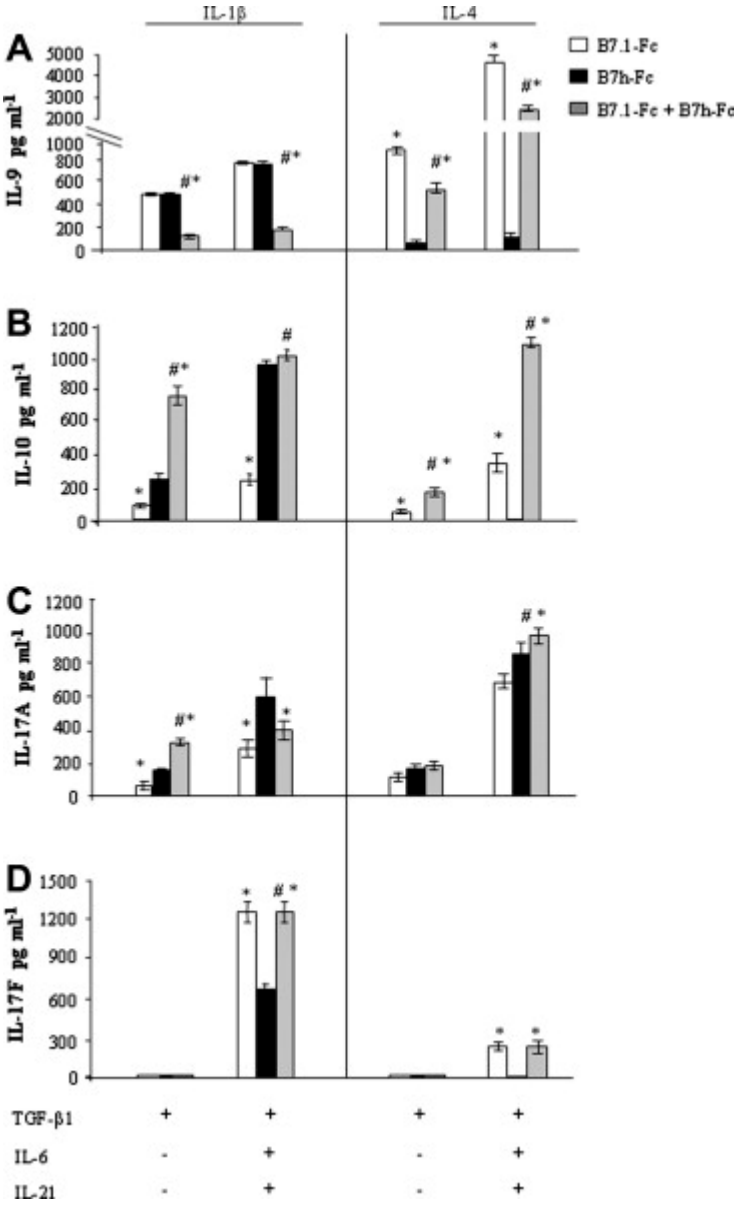

Fig. 4.

Expression of IL-9, IL-10, IL-17A, and IL-17F induced in naïve $CD4^+$ T cells by costimulation with B7h-Fc and/or B7.1-Fc in TH17- and TH9-promoting conditions. Naïve $CD4^+$ T cells were stimulated with anti-CD3 plus either B7h-Fc and/or B7.1-Fc in the presence of different combinations of TGF-β1, IL-1β or IL-4, IL-6, and IL-21. Cytokine secretion was evaluated by ELISA after 5 days of culture. Results are expressed as mean ± SD from six experiments. (*) Marks significant differences from costimulation of B7h-Fc alone; ($^\#$) marks significant differences from costimulation of B7.1-Fc alone.

In the presence of TGF-β1 + IL-1β, B7h-Fc induced levels of IL-9 secretion that were similar to those induced by B7.1-Fc. Secretion of IL-17A, IL-17F, and IL-10 induced by B7h-Fc or B7.1-Fc were in line with those shown in the previous experiments. The dual use of B7h-Fc + B7.1-Fc increased secretion of IL-10 and, at a lesser extent, IL17A, but decreased secretion of IL-9 compared with the effect exerted by either B7h-Fc or B7.1-Fc. Addition of IL-21 + IL-6 did not change the pattern of IL-9 secretion, and the effect exerted on secretion of IL-17A, IL-17F, and IL-10 was in line with that shown in the previous experiments. In these conditions, the dual use B7h-Fc + B7.1-Fc induced high secretion of IL-10 and IL-17F, and low secretion of IL-17A similar to those induced by B7.1-Fc, and decreased secretion of IL-9 compared with the effect exerted by either B7h-Fc or B7.1-Fc.

In the presence of TGF-β1 + IL-4, B7h-Fc induced secretion of lower levels of IL-9 and IL-10 than B7.1-Fc, whereas IL-17A secretion was similarly induced by each costimulus; dual use of B7h-Fc + B7.1-Fc decreased secretion of IL-9, but increased secretion of IL-10 compared to the effect exerted by B7.1-Fc alone; IL-17F was almost undetectable with any stimulus. When also IL-21 + IL-6 were added to the cultures, B7h-Fc still induced secretion of minimal levels of IL-9, IL-10, and IL-17F, but it induced similar levels of IL-17A as B7.1-Fc; by contrast, B7.1-Fc induced substantial levels of all cytokines; dual use of B7h-Fc + B7.1-Fc increased secretion of IL-10 and, at a lesser extent, IL-17A but it decreased secretion of IL-9 and had no effect on secretion of IL-17F compared to the effect exerted by B7.1-Fc alone.

To further investigate the influence of the cytokine milieu, we assessed the effect of neutralizing antibodies to IL-4, IFNγ, IL-1β, and TGF-β1 on secretion of IL-17A, IL-17F, IL-9, and IL-10 by cells activated with the different costimuli in the presence of exogenous TGF-β1 + IL-1β + IL-6 + IL-21. Fig. 5 shows that neutralization of either TGF-β1 or IL-1β abrogated secretion of IL-17A, IL-17F, and IL-9 and decreased secretion of IL-10 in all costimulation settings. Neutralization of IL-4 + IFNγ substantially increased secretion of IL-17A and inhibited secretion of IL-10 in all costimulation settings but especially in those costimulated with B7h-Fc alone; moreover, it slightly increased secretion of IL-17F in the cells costimulated with both costimuli, whereas it had no effect on secretion of IL-9 in any costimulation setting.

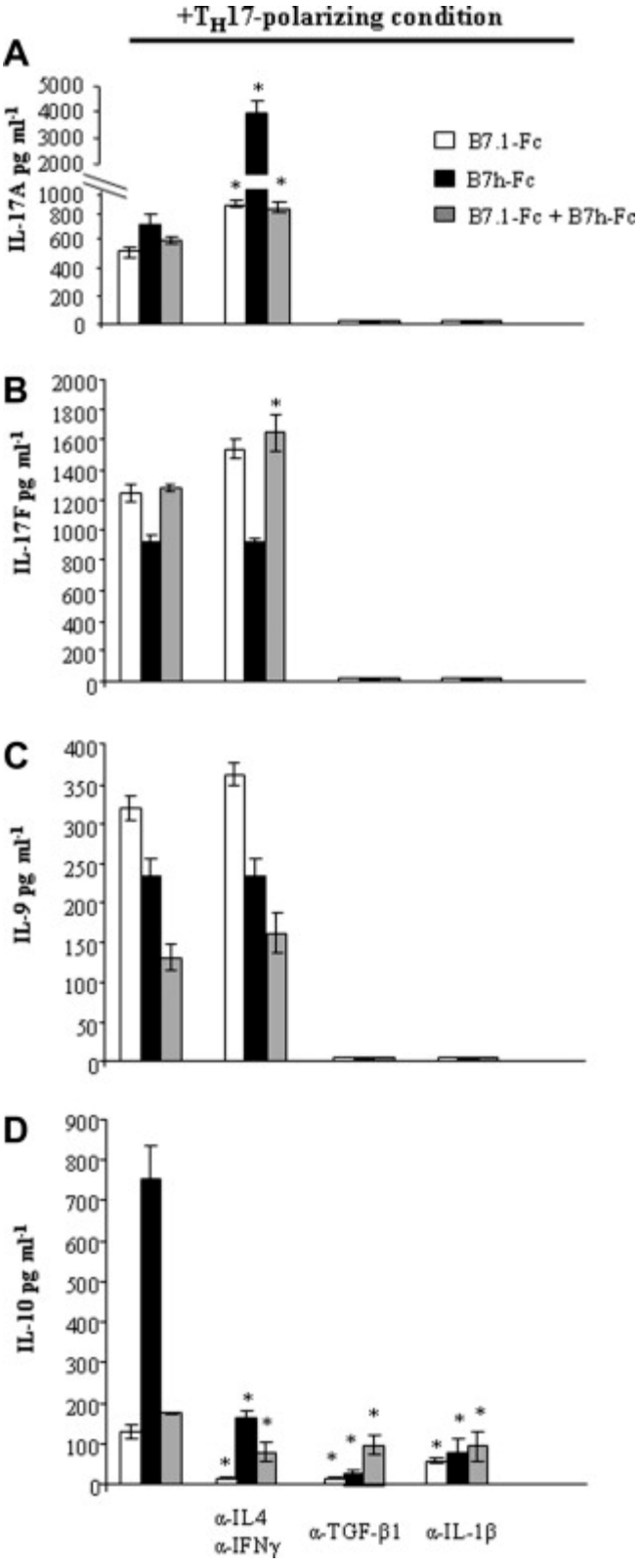

Fig. 5.

Effect of neutralizing antibodies to IL-4, IFN-γ, IL-1β, and TGF-β1 on secretion of IL-17A, IL-17F, IL-9, and IL-10. Naïve CD4$^+$ T cells were activated with the different costimuli in the presence of exogenous TGF-β1 + IL-1β + IL-6 + IL-21 and in the presence or absence of neutralizing antibodies to either TGF-β1, or IL-1β, or IL-4 + IFNγ. Cytokine secretion was evaluated by ELISA after 5 days of culture. Results are expressed as mean ± SD from six experiments. (*) Marks significant differences from the corresponding costimulation setting in the absence of neutralizing antibodies.

3.3. Induction of the RORC2 and PU.1 transcription factors

The transcription factors RORC2/ROR-γt and PU.1 are involved in differentiation of TH17 and TH9 cells respectively. To further address the effect of B7h-Fc in TH17 and TH9 differentiation, we analyzed its capacity to support expression of *Rorc2* and *PU.1*mRNA in naïve TH cells activated in TH17 and TH9 conditions as described above.

In the presence of TGF-β1 + IL-1β, B7h-Fc induced similar levels of the *Rorc2* mRNA as B7.1-Fc, and copresence of both costimuli substantially increased these levels. In the further presence of IL-6 + IL-21, B7h-Fc induced similar levels of the *Rorc2* mRNA as B7.1-Fc, but copresence of both costimuli decreased these levels (Fig. 6A), which was in line with the decreased IL-17A secretion detected in this condition (Fig. 4C). By contrast, the *PU.1* mRNA was always barely detectable (data not shown).

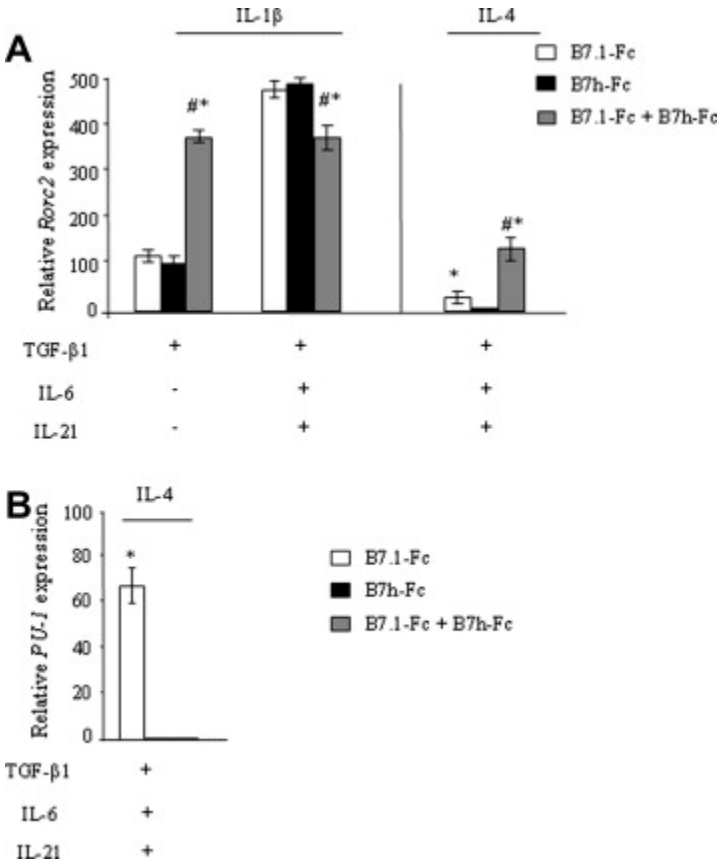

Fig. 6.

Rorc2 and *PU-1* mRNA expression induced by naïve $CD4^+$ T cell costimulation with B7h-Fc or B7.1-Fc. Experiments were performed as in Fig. 3 and levels of mRNA encoding for RORC2 and PU.1 were evaluated by real time PCR. Results are expressed as mean ± SD from six experiments. (*) Marks significant differences from costimulation of B7h-Fc alone; ($^\#$) marks significant differences from costimulation of B7.1-Fc alone.

In the presence of TGF-β1 + IL-4, both the *Rorc2* and the *PU.1* mRNAs were detectable only in the further presence of IL-6 + IL-21.

In these conditions, *Rorc2* was induced at higher levels by B7.1-Fc than by B7h-Fc and these levels were substantially increased by costimulation of both receptors (Fig. 6A), which was in line with the increased IL-17A secretion detected in this condition (Fig. 4C); *PU.1* was detectable only using B7.1-Fc (Fig. 6B), which was in line with high IL-9 secretion detected in this condition (Fig. 4A).

3.4. Effect of B7h-Fc and B7.1-Fc in memory T cells

To evaluate the effects of B7h-Fc and B7.1-Fc in memory T cells, we used two T cell preparations: $CD4^+CCR6^+$ T cells which comprise memory TH cells producing IL-17 [43], and $CD4^+CD45RO^+$ TH cells which comprise whole TH memory cells. Cells were activated as described above and cultured with a cytokine cocktail composed of TGF-β1, IL-6, and IL-21 supplemented with either IL-1β (TH17 promoting conditions) for $CD4^+CCR6^+$ cells, or IL-4 (TH9 promoting conditions) for $CD4^+CD45RO^+$ cells. Secretion of IL-17A, IL-17F, IL-10, IL-9, and the IL-17A/F heterodimers was then evaluated by ELISA in the supernatants.

In $CD4^+CCR6^+$ cells, B7h-Fc induced higher amounts of IL-10 and lower amounts of IL-17F, IL-17A/F and IL-9 than B7h-Fc. Dual use of B7h-Fc + B7.1-Fc increased secretion of IL-10, whereas secretion of IL-9 was intermediate between those induced by each costimulus alone; secretion of IL-17F and the IL-17A/F heterodimer was similar to that induced by B7.1-Fc. Secretion of IL-17A was induced at similar levels by all costimuli (Fig. 7).

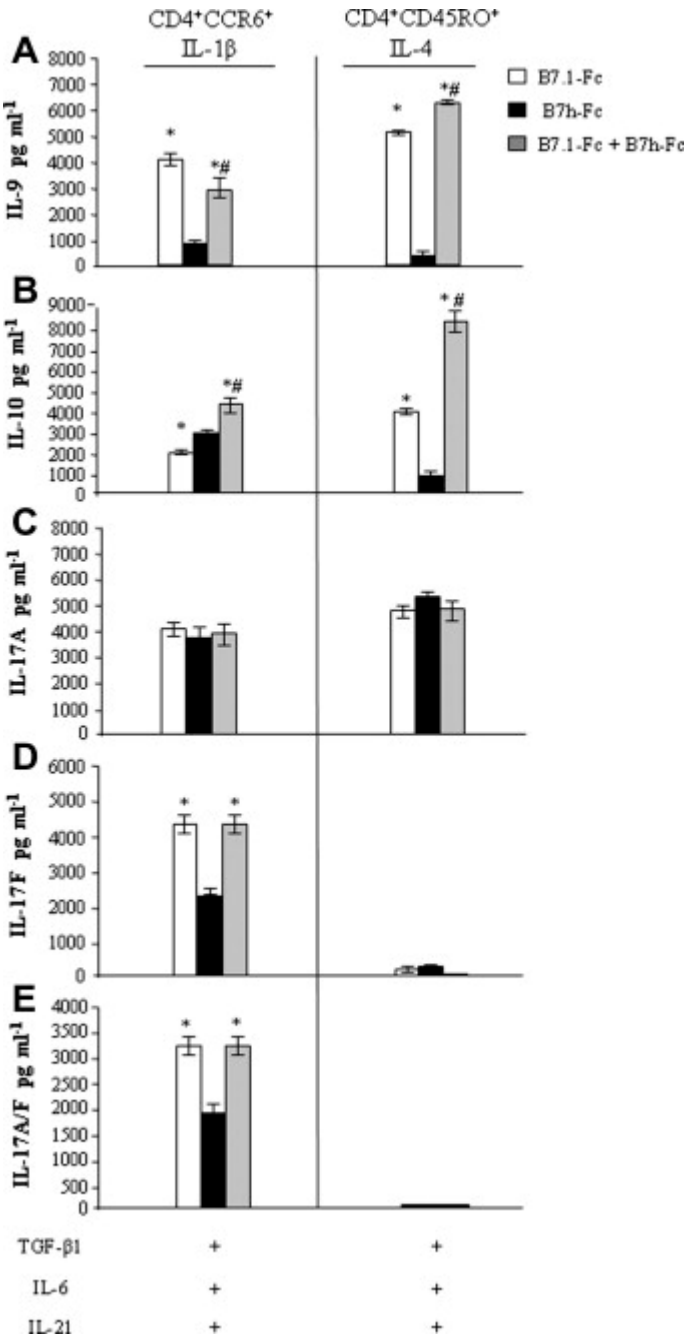

Fig. 7.

Secretion of IL-17A, IL-17F, IL-17A/F, IL-9 and IL-10 induced in "memory" TH cells by costimulation with B7h-Fc or B7.1-Fc. $CD4^+CCR6^+$ and $CD4^+CD45RO^+$ "memory" T cells were stimulated as in Fig. 2 in the presence and absence of a cytokine cocktail of TGF-β1, IL-1β, IL-6, IL-21, and IL-23; secretion of IL-9 (A), IL-10 (B), IL-17A (C), IL-17F (D) and IL-17A/F (E) was assessed by ELISA in the supernatants at day 5. Results are expressed as mean ± SD from six experiments. (*) Mark significant differences from costimulation of B7h-Fc alone; ($^\#$) mark significant differences from costimulation of B7.1-Fc alone

In $CD4^+CD45RO^+$ T cells, B7.1-Fc induced higher levels of IL-9 and IL-10 than B7h-Fc and these levels were further increased by use of both costimuli. All costimuli induced similar high levels of IL-17-A and minimal levels of IL-17F and IL-17A/F (Fig. 7).

3.5. Single cell analysis of IL-17A, IL-17F, and IL-10 production

To assess whether IL-17A was produced by the same cells producing IL-10 or IL-17F, we performed a two-color intracellular staining and cytofluorimetric analysis in both naïve TH cells and "memory" $CD4^+CCR6^+$ TH cells. B7h-Fc and B7.1-Fc costimulated cells were cultured for 5 days in the presence of a cytokine-cocktail containing TGF-β1, IL-1β, IL-6, IL-21, and IL-23. Cells were then re-activated with PMA and Ionomycin in the presence of Brefeldin A and stained for intracellular IL-17A and either IL-17F or IL-10.

Analysis of naïve TH cells showed that B7h-Fc induced higher proportion of $IL\text{-}17A^+$ cells and lower proportions of $IL\text{-}17F^+$ cells than B7.1-Fc (Fig. 8A). B7h-Fc and B7.1-Fc induced comparable proportions of $IL10^+$ single positive cells (Fig. 8B). The small proportions of positive cells detected in these experiments are in line with those reported by other authors [36]. Minimal proportions of cells were either $IL\text{-}17A^+/IL\text{-}17F^+$ or $IL\text{-}17A^+/IL\text{-}10^+$ double positive in all costimulation settings. Moreover, these cells were also stained for intracellular expression of either IFN-γ or ROR-γt, which showed that both B7h-Fc and B7.1-Fc induced high expression of ROR-γt (Fig. 8E) and no production of IFN-γ (Fig. 8D). The discrepancy between the high proportions of $ROR\text{-}\gamma t^+$ cells and the low proportions of $IL\text{-}17A^+$ or $IL\text{-}17F^+$ cells is not surprising since $ROR\text{-}\gamma t^+$ expression may not be sufficient for TH17 cell differentiation which also involves other transcription factors, such as RORa [44], IRF4 [45], and Batf [46]. Analysis of $CD4^+CCR6^+$ cells showed that B7h-Fc induced higher proportion of $IL\text{-}17A^+$ cells and lower proportions of $IL\text{-}17F^+$ cells than B7.1-Fc. A substantial population of $IL\text{-}17A^+/IL\text{-}17F^+$ double positive cells were induced by both B7h-Fc and B7.1-Fc (Fig. 8G). The proportion of $IL\text{-}10^+$ cells was low and it was similar in the cells costimulated with either B7h-Fc or B7.1-Fc; $IL\text{-}17A^+/IL\text{-}10^+$ double positive cells were almost absent (Fig. 8H).

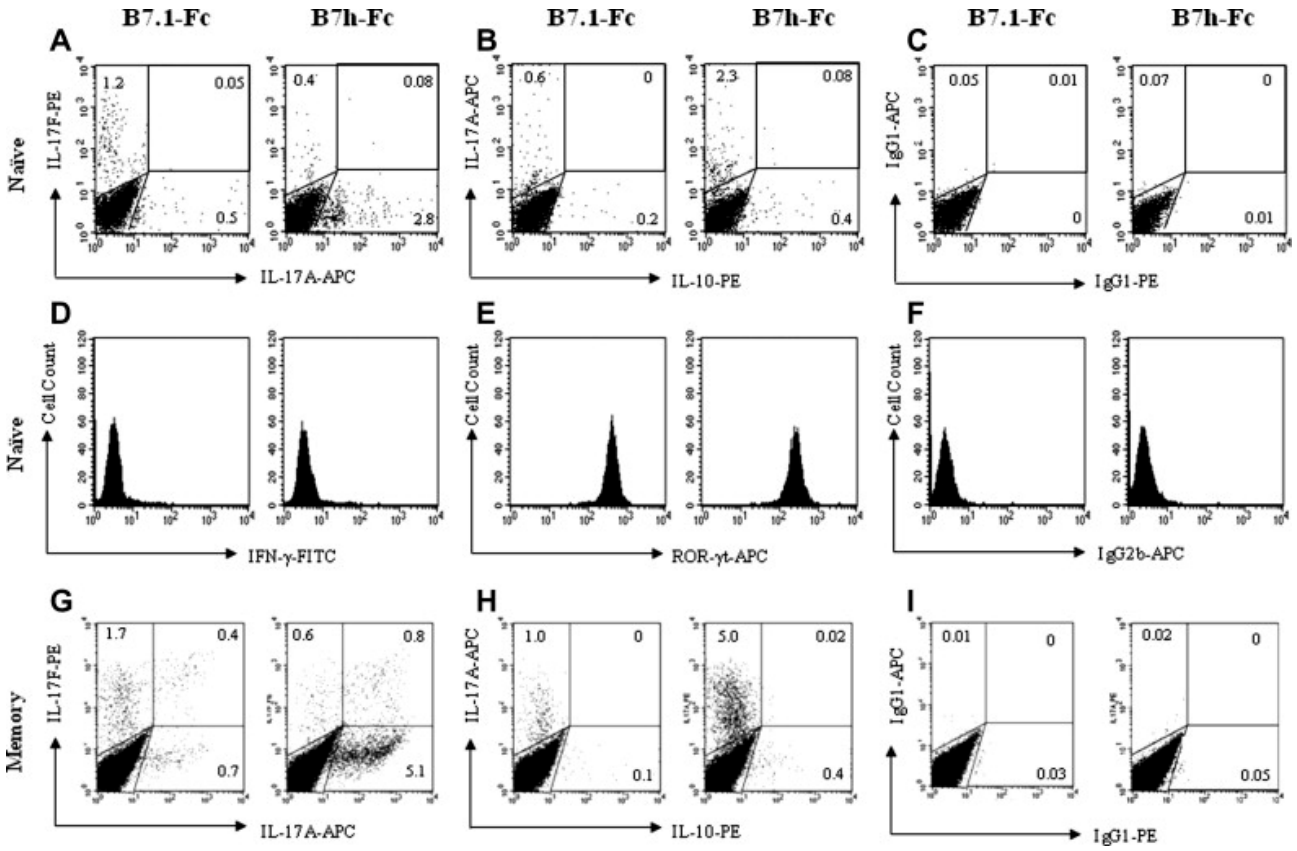

Fig. 8.

Cytofluorimetric analysis of intracellular expression of IL-17A, IL-17F, IL-10, IFN-γ, and ROR-γt. Naïve $CD4^+$ T cells (A, B, D, and E), and $CCR6^+CD4^+$ T cells (G and H) were cultured as described in Fig. 3 in the presence of a cytokine cocktail of TGF-β1, IL-1β, IL-6, IL-21, and IL-23 for 5 days; $IL\text{-}17A^+$ and either $IL\text{-}17F^+$ or $IL\text{-}10^+$ cells were evaluated by two-color intracellular staining after a 6 h re-stimulation with PMA/Ionomycin in the presence of Brefeldin A. Control staining with the isotype-matched control Ig is shown in C, F, and I. The proportion of each population are indicated within the quadrants. Data are from one representative experiment out of four experiments.

4. Discussion

This work shows that costimulation with B7h-Fc or B7.1-Fc have different effects on secretion of TH17 and TH9 cytokines in naïve TH cells. The overall data indicate that B7h-Fc is more selective than B7.1-Fc since it exerts a preferential effect on IL-17A and IL-10, whereas B7.1-Fc is highly effective on IL-17F and IL-9 too.

In naïve TH cells, the minimal cytokine milieu to induce secretion of both TH17 and TH9 cytokines included TGFβ1 + IL-1β, whose effect was potentiated by a cocktail composed of IL-6 and IL-21. In these conditions, B7h-Fc induced secretion of IL-17A and IL-10 more efficiently than B7.1-Fc, which in turn induced higher secretion of IL-17F and expression of the IL-26 mRNA; by contrast, secretion of IL-9 was similarly induced by the two costimuli. Secretion of IL-17A and IL-10 were differentially sensitive to TH1- and TH2-polarizing cytokines since neutralization of IFN-γ and IL-4 strikingly increased secretion of IL-17A and decreased secretion of IL-10, especially in the cells costimulated with B7h-Fc. When IL-1β was substituted with IL-4, the most striking response was the secretion of high levels of IL-9 induced by B7.1-Fc but not by B7h-Fc, which in turn displayed a selective effect in induction of IL-17A. By contrast, B7.1-Fc induced also secretion of IL-10, IL-17A, and low levels of IL-17F.

Memory TH cells, too, responded differently to B7h-Fc and B7.1-Fc, and B7h-Fc was more selective than B7.1-Fc in inducing cytokine secretion. In $CD4^+CCR6^+$ cells cultured in TH17 promoting conditions, B7h-Fc induced similar levels of IL-17A as B7.1-Fc, but it induced higher levels of IL-10 and lower levels of IL-17F and IL-9 than B7.1-Fc. In $CD4^+CD45RO^+$ cells cultured in TH9 promoting conditions, B7h-Fc induced high levels of IL-17A only, whereas B7.1-Fc induced high levels of IL-9 and IL-10 too.

Dual costimulation with B7h-Fc + B7.1-Fc influenced these patterns in a complex manner and displayed different effects in different cytokine milieus. In most culture conditions, it increased secretion of IL-10 and decreased secretion of IL-9, whereas the effect on secretion of IL-17A was variable in the different conditions.

Secretion of IL-17F was more dependent on the cytokine milieu than that of IL-17A since it required that the minimal milieu, containing either TGFβ1 + IL-1β or TGFβ1 + IL-4, was enriched with at least IL-6 and/or IL-21. Moreover, presence of exogenous IL-4 substantially decreased secretion of IL-17F in all culture conditions, whereas it had no substantial effect on secretion of IL-17A.

In naïve TH cells, neither B7h-Fc nor B7.1-Fc induced secretion of IL-17A/F heterodimers even when substantial amounts of both IL-17A and IL-17F were induced. In contrast, especially when costimulated with B7.1-Fc, substantial amounts of the heterodimer were produced by “memory” $CCR6^+$ TH17 cells. These data suggest that IL-17A and IL-17F were produced by distinct cell subsets in the naïve TH cell cultures, whereas memory TH cells comprised substantial amounts of

cells producing both cytokines. In line with this possibility, IL-17A$^+$/IL-17F$^+$ double positive cells were almost absent in the naïve TH cell cultures, whereas they were present in the “memory” CD4$^+$CCR6$^+$ T cell cultures. Moreover, the single cell analyses showed that also IL-17A and IL-10 were produced by distinct subsets of cells, since IL-17A$^+$/IL-10 double-positive cells were always almost absent.

IL-17A and IL-17F are homodimers sharing about 50% sequence identity and several biological activities including the induction of proinflammatory cytokines and antimicrobial peptides, neutrophil recruitment, mucosal immunity against extracellular pathogens, and an involvement in several autoimmune diseases [29] and [30]. However, certain features distinguish these two cytokines. Specifically, the ability to upregulate transcription factors and proinflammatory molecules appears lower for IL-17F than for IL-17A. Furthermore, IL-17A but not IL-17F appears to play a role in development of collagen-induced arthritis, contact hypersensitivity, and delayed-type hypersensitivity[47]. In the mouse model of OVA-alum induced asthma, IL-17A promotes the TH2 response, whereas IL-17F has a regulatory role in restricting allergic asthma development. In contrast, in dextran sulfate sodium-induced colitis, IL-17A played a protective role, whereas IL-17F appeared to exacerbate the intestinal inflammation. In multiple sclerosis lesions, IL-17A but not IL-17F was detected [48]. Finally, in gastric adenocarcinoma, IL-17A induced activation of MAPK, AP-1, and NF-κB, and secretion of high amounts of IL-8, whereas IL-17F induced activation of NF-κB alone and secretion of low amounts of IL-8 [48]. IL-17 receptor usage, too, has been reported to be partly different since IL-17A seemed to preferentially interact with IL-17RA, whereas IL-17F with IL-17RC [49]. Plasmon resonance experiments showed that IL-17A, IL-17F, and IL-17A/F heterodimers bound to IL-17RC with comparable affinities, but with different affinities to IL-17RA where the affinity for IL-17A highest, IL-17F lowest, and intermediate for the heterodimer. Moreover, biological activity of IL-17A was primarily inhibited by soluble IL-17RA, IL-17F by soluble IL-17RC, and IL17A/F heterodimer by both receptors[50].

Paulos et al. [40] reported that ICOS triggering is a potent inducer of IL-17A secretion in umbilical cord blood naïve TH cells in the presence of a TH17-polarizing milieu containing IL-1β, IL-6, IL-23, neutralizing antibodies to IFN-γ and IL-4. By contrast, triggering of CD28 and dual costimulation of CD28 + ICOS were poorly effective in inducing IL17A secretion. Moreover, IL-10 secretion was induced at comparable levels by each costimulus. The inconsistencies with our results may partly depend on the different TH cell source since naïve cord blood TH cells include high proportions of recent thymic emigrant cells, which may influence the response and are rare in adult PBMC [51]; moreover, their stimulation protocols were different from ours since they triggered CD28 and ICOS by means of either mAb or cells transfected with the costimulatory ligands and expanded cells in long term (15 days) cultures using serum-containing medium.

Both CD28 and ICOS signaling uses PI3 K but their different signaling outcomes may be due to the ability of CD28, but not ICOS, to bind the Grb-2/GADS adaptor proteins and the Itk and Lck kinases, and their differential usage of PI3 K subunits [52]. Moreover, ICOS costimulation has been reported to induce higher levels of NFATc1 than CD28 costimulation [21] which is intriguing at the light of the notion that NFATc1 activity is involved in induction of IL-17A, but not IL-17F [53].

5. Conclusion

The overall data suggest that costimulation mediated by B7.1 triggers distinct effector functions depending on the cytokine microenvironment, i.e. it induces IL-17F secreting cells in the presence of TGFβ1 + IL-1β, and IL-9 secreting cells in the presence of TGFβ1 + IL-4. By contrast,

costimulation mediated by B7h is generally biased toward induction of IL-17A secreting cells and supports cell plasticity by favouring contemporary secretion of IL-10, which substantially modulates TH17 cell function [54]. IL-10 can be produced by several cell types, including TH1 and TH17 cells, where it negatively regulates their function and can be induced by strong TCR stimulation leading to sustained Erk activation [18]. This suggests that IL-10 serves to control the magnitude of many types of immune responses and to prevent tissue damage. This plasticity might be important in the immune response to pathogens and tumors, and in the development of autoimmune diseases, and should be taken in consideration in designing of immunotherapeutic protocols triggering ICOS and/or CD28.

Conflict of interest

The authors have no financial conflict of interest.

References

1.
   - D.J. Lenschow, T.L. Walunas, J.A. Bluestone, B. Patel, A. Koons, H.Y. Qin, *et al.*
   - **CD28/B7 system of T cell costimulation**
   - Annu Rev Immunol, 14 (1996), pp. 233–258

2.
   - R.H. Schwartz
   - **T cell anergy**
   - Annu Rev Immunol, 21 (2003), pp. 305–334

3.
   - C.A. Chambers, J.P. Allison
   - **Costimulatory regulation of T cell function**
   - Curr Opin Cell Biol, 11 (1999), pp. 203–210

4.
   - C.A. Janeway Jr, R. Medzhitov
   - **Innate immune recognition**
   - Annu Rev Immunol, 20 (2002), pp. 197–216

5.
   - R.J. Greenwald, G.J. Freeman, A.H. Sharpe
   - **The B7 family revisited**

- Annu Rev Immunol, 23 (2005), pp. 515–548

6.

- D. Buonfiglio, M. Bragardo, V. Redoglia, R. Vaschetto, F. Bottarel, S. Bonissoni, *et al.*
- **The T cell activation molecule H4 and the CD28-like molecule ICOS are identical**
- Eur J Immunol, 30 (2000), pp. 3463–3467

7.

- V. Redoglia, U. Dianzani, J.M. Rojo, P. Portolés, M. Bragardo, H. Wolff, *et al.*
- **Characterization of H4: a mouse T lymphocyte activation molecule functionally associated with the CD3/T cell receptor**
- Eur J Immunol, 26 (1996), pp. 2781–2789

8.

- A. Hutloff, A.M. Dittrich, K.C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, *et al.*
- **ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28**
- Nature, 397 (1999), pp. 263–266

9.

- M.M. Swallow, J.J. Wallin, W.C. Sha
- **B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFa**
- Immunity, 11 (1999), pp. 423–432

10.

- C. Dianzani, R. Minelli, R. Mesturini, A. Chiocchetti, G. Barrera, S. Boscolo, *et al.*
- **B7h triggering inhibits umbilical vascular endothelial cell adhesiveness to tumor cell lines and polymorphonuclear cells**
- J Immunol, 185 (2010), pp. 3970–3979

11.

- S.K. Yoshinaga, J.S. Whoriskey, S.D. Khare, U. Sarmiento, J. Guo, T. Horan, *et al.*
- **T-cell co-stimulation through B7RP-1 and ICOS**
- Nature, 402 (1999), pp. 827–832

12.

- C. Dong, A.E. Juedes, U.A. Temann, S. Shresta, J.P. Allison, N. Ruddle, *et al.*
- **ICOS co-stimulatory receptor is essential for T cell activation and function**
- Nature, 409 (2001), pp. 97–101

13.

- A. Tafuri, A. Shahinian, F. Bladt, S.K. Yoshinaga, M. Jordana, A. Wakeham, *et al.*
- **ICOS is essential for effective T-helper-cell responses**
- Nature, 409 (2001), pp. 105–109

14.

- A.J. McAdam, R.J. Greenwald, M.A. Levin, T. Chernova, N. Malenkovich, V. Ling, *et al.*
- **ICOS is critical for CD40-mediated antibody class switching**
- Nature, 409 (2001), pp. 102–105

15.

- V. Ling, P.W. Wu, H.F. Finnerty, K.M. Bean, V. Spaulding, L.A. Fouser, *et al.*
- **Cutting edge: identification of GL50, a novel B7-like protein that functionally binds to ICOS receptor**
- J Immunol, 164 (2000), pp. 1653–1657

16.

- S. Wang, G. Zhu, A.I. Chapoval, H. Dong, K. Tamada, J. Ni, *et al.*
- **Costimulation of T cells by B7–H2, a B7-like molecule that binds ICOS**
- Blood, 96 (2000), pp. 2808–2813

17.

- R.I. Nurieva, X.M. Mai, K. Forbush, M.J. Bevan, C. Dong
- **B7h is required for T cell activation, differentiation, and effector function**
- Proc Natl Acad Sci USA, 100 (2003), pp. 14163–14168

18.

- J. Zhu, W.E. Paul
- **Heterogeneity and plasticity of T helper cells**
- Cell Res, 20 (2010), pp. 4–12

19.

- M. Stassen, E. Schmitt, T. Bopp
- **From interleukin-9 to T helper 9 cells**
- Ann NY Acad Sci, 1247 (2012), pp. 56–68

20.

- J. Yagi, Y. Arimura, U. Dianzani, T. Uede, T. Okamoto, T. Uchiyama
- **Regulatory roles of IL-2 and IL-4 in H4/inducible costimulator expression on activated CD4$^+$ T cells during TH cell development**
- J Immunol, 171 (2003), pp. 783–794

21

- R.I. Nurieva, J. Duong, H. Kishikawa, U. Dianzani, J.M. Rojo, I. Ho, *et al.*
- **Transcriptional regulation of TH2 differentiation by inducible costimulator**
- Immunity, 18 (2003), pp. 801–811

22

- O. Akbari, G.J. Freeman, E.H. Meyer, E.A. Greenfield, T.T. Chang, A.H. Sharpe, *et al.*
- **Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity**
- Nat Med, 8 (2002), pp. 1024–1032

23

- L. Wassink, P.L. Vieira, H.H. Smits, G.A. Kingsbury, A.J. Coyle, M.L. Kapsenberg, *et al.*
- **ICOS expression by activated human TH cells is enhanced by IL-12 and IL-23: increased ICOS expression enhances the effector function of both TH1 and TH2 cells**
- J Immunol, 173 (2004), pp. 1779–1786

24

- P.L. Vieira, L. Wassink, L. Smith, S. Nam, G.A. Kingsbury, J.C. Gutierrez-Ramos, *et al.*
- **ICOS-mediated signaling regulates cytokine production by human T cells and provides a unique signal to selectively control the clonal expansion of TH2 helper cells**
- Eur J Immunol, 34 (2004), pp. 1282–1290

25

- R. Mesturini, S. Nicola, A. Chiocchetti, I.S. Bernardone, L. Castelli, T. Bensi, *et al.*
- **ICOS cooperates with CD28, IL-2, and IFN-gamma and modulates activation of human naïve CD4+ T cells**
- Eur J Immunol, 36 (2006), pp. 2601–2612

26

- S. Yao, Y. Zhu, G. Zhu, *et al.*
- **B7–h2 is a costimulatory ligand for CD28 in human**
- Immunity, 34 (2011), pp. 729–740

27

- L.E. Harrington, R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, *et al.*

- **Interleukin-17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages**
- Nat Immunol, 6 (2005), pp. 1123–1132

28

- H. Park, Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, *et al.*
- **A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin-17**
- Nat Immunol, 6 (2005), pp. 1133–1141

29

- F.L. van de Veerdonk, M.S. Gresnigt, B.J. Kullberg, J.W. van der Meer, L.A. Joosten, M.G. Netea
- **TH17 responses and host defense against microorganisms: an overview**
- BMB Rep, 42 (2009), pp. 776–787

30

- F. Sallusto, A. Lanzavecchia
- **Human TH17 cells in infection and autoimmunity**
- Microbes Infect, 11 (2009), pp. 620–624

31

- N. Manel, D. Unutmaz, D.R. Littman
- **The differentiation of human TH17 cells requires transforming growth factor-b and induction of the nuclear receptor RORct**
- Nat Immunol, 9 (2008), pp. 641–649

32

- C. Dong
- **TH17 cells in development: an updated view of their molecular identity and genetic programming**

- Nat Rev Immunol, 8 (2008), pp. 337–348

33

- E.V. Acosta-Rodriguez, G. Napoletani, A. Lanzavecchia, F. Sallusto
- **Interleukins 1b and 6 but not transforming growth factor-b are essential for the differentiation of interleukin 17-producing human T helper cells**
- Nat Immunol, 8 (2007), pp. 942–949

34

- N.J. Wilson, K. Boniface, J.R. Chan, B.S. McKenzie, W.M. Blumenschein, J.D. Mattson, *et al.*
- **Development, cytokine profile and function of human interleukin 17-producing helper T cells**
- Nat Immunol, 8 (2007), pp. 950–957

35

- L. Yang, D.E. Anderson, C. Baecher-Allan, W.D. Hastings, E. Bettelli, M. Oukka, *et al.*
- **IL-21 and TGF-beta are required for differentiation of human T(H)17 cells**
- Nature, 454 (2008), pp. 350–352

36

- E. Volpe, N. Servant, R. Zollinger, S.I. Bogiatzi, P. Hupé, E. Barillot, *et al.*
- **A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses**
- Nat Immunol, 9 (2008), pp. 650–657

37

- F. Annunziato, L. Cosmi, F. Liotta, E. Maggi, S. Romagnani
- **The phenotype of human TH17 cells and their precursors, the cytokines that mediate their differentiation and the role of TH17 cells in inflammation**
- Int Immunol, 20 (2008), pp. 1361–1368

38

- R.I. Nurieva
- **Regulation of immune and autoimmune responses by ICOS-B7h interaction**
- Clin Immunol, 115 (2005), pp. 19–25

39

- A.T. Bauquet, H. Jin, A.M. Paterson, M. Mitsdoerffer, I.C. Ho, A.H. Sharpe, *et al.*
- **The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells**
- Nat Immunol, 10 (2009), pp. 167–175

40

- C.M. Paulos, C. Carpenito, G. Plesa, M.M. Suhoski, A. Varela-Rohena, T.N. Golovina, *et al.*
- **The inducible costimulator (ICOS) is critical for the development of human T(H)17 cells**
- Sci Transl Med, 27 (2010), pp. 55ra–78

41

- L. Zhou, I.I. Ivanov, R. Spolski, R. Min, K. Shenderov, T. Egawa, *et al.*
- **IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways**
- Nat Immunol, 8 (2007), pp. 967–974

42

- P. Putheti, A. Awasthi, J. Popola, W. Gao, T.B. Strom
- **Human CD4 memory T cells can become CD4+ IL-9+ T cells**
- PLoS One, 5 (2010), p. e8706

43

- E.V. Acosta-Rodriguez, L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, *et al.*
- **Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells**
- Nat Immunol, 8 (2007), pp. 639–646

44

- X.O. Yang, B.P. Pappu, R. Nurieva, A. Akimzhanov, H.S. Kang, Y. Chung, *et al.*
- **T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma**
- Immunity, 28 (2008), pp. 29–39

45

- A. Brüstle, S. Heink, M. Huber, C. Rosenplänter, C. Stadelmann, P. Yu, *et al.*
- **The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4**
- Nat Immunol, 8 (2007), pp. 958–966

46

- B.U. Schraml, K. Hildner, W. Ise, W.L. Lee, W.A. Smith, B. Solomon, *et al.*
- **The AP-1 transcription factor Batf controls T(H)17 differentiation**
- Nature, 460 (2009), pp. 405–409

47

- C.J. Haines, T.D. Giffon, L.S. Lu, X. Lu, M. Tessier-Lavigne, D.T. Ross, *et al.*
- **Human CD4+ T cell recent thymic emigrants are identified by protein tyrosine kinase and have reduced immune function**
- J Exp Med, 206 (2009), pp. 275–285

48

- H. Ishigame, S. Kakuta, T. Nagai, M. Kadoki, A. Nambu, Y. Komiyama, *et al.*
- **Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses**
- Immunity, 30 (2009), pp. 108–119

49

- S.H. Chang, C. Dong
- **IL-17F: regulation, signaling and function in inflammation**
- Cytokine, 46 (2009), pp. 7–11

50

- R.E. Kuestner, D.W. Taft, A. Haran, C.S. Brandt, T. Brender, K. Lum, *et al.*
- **Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F**
- J Immunol, 179 (2007), pp. 5462–5473

51

- J.F. Wright, F. Bennett, B. Li, J. Brooks, D.P. Luxenberg, M.J. Whitters, *et al.*
- **The human IL-17F/IL-17A eterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex**
- J Immunol, 181 (2008), pp. 2799–2805

52

- Y.Y. Acosta, M.P. Zafra, G. Ojeda, I.S. Bernardone, U. Dianzani, P. Portolés, *et al.*
- **Biased binding of class IA phosphatidyl inositol 3-kinase subunits to inducible costimulator (CD278)**
- Cell Mol Life Sci, 68 (2011), pp. 3065–3079

53

- J. Gomez-Rodriguez, N. Sahu, R. Handon, T.S. Davidson, S.M. Anderson, M.R. Kirby, *et al.*
- **Differential expression of interleukin-17A and -17F is coupled to T cell receptor signaling via inducible T cell kinase**
- Immunity, 31 (2009), pp. 587–597

54

- M.J. McGeachy, K.S. Bak-Jensen, Y. Chen, C.M. Tato, W. Blumenschein, T. McClanahan, *et al.*
- **TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology**
- Nat Immunol, 8 (2007), pp. 1390–1397