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# Antigen dynamics govern the induction of CD4$^+$ T cell tolerance during autoimmunity

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ABSTRACT

Antigen-specific T cell tolerance holds great promise for the treatment of autoimmune diseases. However, strategies to induce durable tolerance using high doses of soluble antigen have to date been unsuccessful, due to lack of efficacy and the risk of hypersensitivity. In the current study we have overcome these limitations by developing a platform for tolerance induction based on engineering the immunoglobulin Fc region to modulate the dynamic properties of low doses (1 μg/mouse; ~50 μg/kg) of Fc-antigen fusions. Using this approach, we demonstrate that antigen persistence is a dominant factor governing the elicitation of tolerance in the model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), induced by immunizing B10.PL mice with the N-terminal epitope of myelin basic protein. Unexpectedly, our analyses reveal a stringent threshold of antigen persistence for both prophylactic and therapeutic treatments, although distinct mechanisms lead to tolerance in these two settings. Importantly, the delivery of tolerogenic Fc-antigen fusions during ongoing disease results in the downregulation of T-bet and CD40L combined with amplification of Foxp3$^+$ T cell numbers. The generation of effective, low dose tolerogens using Fc engineering has potential for the regulation of autoreactive T cells.



## 1. Introduction

Organ-specific autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis and type 1 diabetes mellitus represent a major cause of death in developed countries. It is well established that the aberrant activation of autoreactive CD4$^+$ T cells is a driver of autoimmune disorders. Currently approved therapies for autoimmunity that broadly target such cells include the depletion of lymphocyte subsets, the targeting of immune activation/co-stimulatory signals or the inhibition of leukocyte trafficking [1]. However, these approaches can result in adverse side effects such as systemic toxicities and increased risk for infection or cancer [1]. Consequently, a need for the development of treatments, such as tolerance induction, to selectively target autoantigen-specific T cells persists.

The induction of autoantigen-specific T cell tolerance using high doses of soluble immunodominant peptides to delete or anergize autoreactive T cells has been extensively explored [2,3]. Although such approaches, including the delivery of altered peptide ligands, have shown efficacy in reducing disease in animal models of MS and diabetes, the translation of such therapies into humans has been unsuccessful [2,4]. Further, there are significant safety concerns due to reports of fatal anaphylaxis in many animal models of MS following the delivery of relatively high doses (necessitated by rapid renal clearance [5]) of autoantigenic peptides during ongoing disease [6,7]. A longstanding, unsolved challenge is therefore to develop effective tolerizing agents that are safe for the therapy of

*Abbreviations:* APCs, antigen presenting cells; AUC, area under the curve; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; FcRn, neonatal Fc receptor; LNs, lymph nodes; MBP, myelin basic protein; MS, multiple sclerosis; WT, wild type.

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autoimmunity.

Chronic exposure to autoantigens during autoimmunity results in reduced disease severity, with mouse studies indicating that this phenomenon results from regulatory T cell (Treg) activation [8]. In addition, low dose, persistent antigen presentation during chronic viral infections can lead to $CD4^+$ T cell exhaustion or dysfunction in an antigen-specific manner [9]. We therefore reasoned that the development of delivery vehicles to enable persistence of low levels of antigen could represent an effective approach to induce antigen-specific T cell tolerance. However, the generation of antigen delivery strategies to achieve such immune homeostasis is challenging due to the limited understanding of the complex interplay between antigen longevity and intracellular trafficking behavior, which in turn determine the efficiency of antigen presentation by antigen presenting cells (APCs).

Our Fc engineering studies indicate that antigenic peptide epitopes expressed as immunoglobulin Fc-epitope fusions can be tuned to have different pharmacokinetics by modulating their binding properties for the neonatal Fc receptor (FcRn) [10]. The majority of naturally occurring antibodies of the IgG class bind to FcRn at acidic pH (pH 6.0) but with an affinity that is negligible at near neutral pH [11]. Consequently, following entry into cells bathed at pH 7.3–7.4 by fluid phase processes, IgG can bind to FcRn in early acidic endosomes and undergo recycling or transcytosis [11–13]. These endosomal sorting pathways regulate the homeostasis and transport of IgG in the body. Further, FcRn is expressed in all professional APCs and is involved in antigen presentation [14]. In the current study, this knowledge has been used to inform the design of a panel of Fc-epitope fusions comprising the N-terminal epitope of myelin basic protein (MBP1-9) linked to engineered Fc regions with the goal of defining the requirements for tolerance induction in a low antigen dose setting. Specifically, we have generated a panel of Fc-MBP fusions with different subcellular trafficking behavior and *in vivo* clearance properties. The effects of these engineered proteins on both the prophylactic blockade and treatment of disease in an experimental autoimmune encephalomyelitis (EAE) model involving the immunization of B10.PL ($H\text{-}2^u$) mice with the immunodominant epitope, MBP1-9 (with N-terminal acetylation) have been investigated.

By using Fc-engineering to tune antigen dynamics, we have established the design requirements for antigen delivery vehicles that result in T cell tolerance and amelioration of ongoing autoimmune disease. Importantly, these studies have been carried out using doses (1 μg/mouse; ~50 μg/kg) that are at least ~450-fold lower than those used previously as either soluble antigen or peptides coupled to microparticles for the treatment of autoimmunity [3,15–17], reducing the risk of anaphylactic shock. Our analyses have defined a remarkably stringent threshold of antigen persistence that is necessary to induce tolerance prior to disease induction and during ongoing disease. In these two settings, although the threshold for antigen persistence is the same, the pathways of tolerance induction are mechanistically distinct: under prophylactic conditions, antigen-specific T cells are deleted or anergized whereas during ongoing EAE, tolerance involves the downregulation of T-bet and CD40L on antigen-specific T cells, combined with the induction of regulatory $Foxp3^+$ T cells. Our studies demonstrate that the delivery of low doses of Fc-epitope fusions represents a promising strategy for the treatment of autoimmunity and other pathological, T cell-mediated conditions.

## 2. Materials and methods

### 2.1. Mice

B10.PL ($H\text{-}2^u$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice that transgenically express the 1934.4 TCR (1934.4 tg mice [18]) or clone 19 TCR ($T/R^+$ tg mice [19]) were kindly provided by Dr. Hugh McDevitt (Stanford University, CA) and Dr. Juan Lafaille (New York University School of Medicine, NY), respectively. Both the 1934.4 and clone 19 TCRs are specific for MBP1-9 complexed with $I\text{-}A^u$ [18,19] and have similar affinities for antigen [20]. Mice were bred in a specific pathogen-free facility at the University of Texas Southwestern Medical Center or Texas A&M University and were handled in compliance with institutional policies and protocols approved by the Institutional Animal Care and Use Committees. 6–10 week old male or female mice were used in experiments.

### 2.2. Peptides

The N-terminal, acetylated peptide of MBP (MBP1-9, Ac-ASQKRPSQR) and MBP1-9(4Y) (Ac-ASQYRPSQR) were purchased from CS Bio (Menlo Park, CA).

### 2.3. Production of recombinant proteins

Expression constructs for the production of full length anti-lysozyme antibodies (WT, m-set-1 and m-set-2) were generated by isolating the cDNA encoding the heavy chain and light chain from the D1.3 hybridoma (mouse IgG1, anti-hen egg lysozyme) [21]. The mutations were inserted into the WT heavy chain gene using splicing by overlap extension and cloned into pOptiVEC™-TOPO® vector (Life Technologies, Grand Island, NY) for expression. The light chain gene was cloned into pcDNA™3.3-TOPO® vector (Life Technologies, Grand Island, NY). Complete sequences of expression plasmids are available upon request. The light chain expression construct was transfected into CHO DG44 cells by electroporation. Stable clones of CHO DG44 cells were selected for light chain expression using previously described methods [22]. The light chain transfectant expressing the highest levels of recombinant protein was used as a recipient for the heavy chain constructs. Clones expressing the highest levels of anti-lysozyme antibody were selected and recombinant antibodies purified from culture supernatants using lysozyme-Sepharose [23]. Mouse IgG1 (anti-hen egg lysozyme, D1.3 [21]) was purified using lysozyme-Sepharose [23] from hybridoma culture supernatants.

Expression plasmids encoding WT or mutated (m-set-2) mouse IgG1-derived Fc-hinge connected at the C-termini through a Gly-Ser-Gly-Gly linker to codons encoding the MBP1-9(4Y) epitope or MBP1-9(4Y) epitope with residues 3 and 6 of the peptide replaced by alanine have been described previously [10]. The glycine at the N-terminus of the peptide mimics the acetyl group that is necessary for T cell recognition of the MBP epitope [24]. The m-set-1 mutations were inserted into the WT Fc-MBP fusion construct using splicing by overlap extension and designed oligonucleotide primers. All Fc-MBP fusion genes were cloned into pEF6/V5-His vector (Life Technologies, Grand Island, NY). Fc-MBP fusion constructs were transfected into CHO-S cells, stable transfectants selected and recombinant proteins purified from culture supernatants as described previously [10]. Analogous methods were used to generate Fc-hinge variants (WT, m-set-1, m-set-2) without the C-terminal MBP1-9 epitope. Complete sequences of expression constructs are available upon request.

### 2.4. Recombinant peptide-MHC complexes

Soluble, recombinant MBP1–9(4Y):$I\text{-}A^u$ complexes were generated using baculovirus-infected High Five insect cells and purified as described previously [25]. The complexes were site-specifically biotinylated and multimeric complexes ("tetramers")

were generated using PE-labeled ExtrAvidin (Sigma-Aldrich, St. Louis, MO).

### 2.5. Cell lines

The MBP1–9:I-$A^u$-specific T cell hybridoma #46 has been described previously [26]. The I-$A^u$-expressing B lymphoblastoid line PL8 was generously provided by Dr. David Wraith (University of Bristol, Bristol, U.K.). PL8:FcRn cells were generated by stably transfecting PL8 cells with an expression construct encoding mouse FcRn tagged at the C-terminus with GFP, followed by selection with G418 (600 μg/ml, Life Technologies, Grand Island, NY) [10].

### 2.6. Surface plasmon resonance analyses

Equilibrium dissociation constants of WT and mutated mouse Fc-hinge fragments (IgG1-derived) for binding to recombinant mouse FcRn were determined using surface plasmon resonance and a BIAcore 2000. Mouse Fc-hinge fragments were immobilized by amine coupling chemistry (to a density of ~250–850 RU) and BIAcore experiments carried out as described previously, using soluble mouse FcRn in Dulbecco's phosphate-buffered saline (DPBS) plus 0.01% Tween pH 6.0 or 7.4 as analyte [27]. FcRn binds to two sites on IgG that are not equivalent [27]. This results in $K_D$ estimates for two dissociation constants, and the values for the higher affinity interaction sites are presented. The data were processed as described previously [27].

### 2.7. T cell stimulation assay

Fc-MBP fusions were added to 96-well plates containing PL-8 or PL-8:FcRn cells ($5 \times 10^4$ cells/well) and MBP1–9:I-$A^u$-specific T cell hybridoma #46 cells ($5 \times 10^4$ cells/well). IL-2 levels in culture supernatants following 24 h of incubation were assessed using a sandwich ELISA with the following reagents: rat anti-mouse IL-2 capture antibody (clone, JES6-1A12; Becton-Dickinson, San Jose, CA), biotinylated rat anti-mouse IL-2 detection antibody (clone, JES6-5H4; Becton-Dickinson, San Jose, CA) and ExtrAvidin-Peroxidase (Sigma-Aldrich, St. Louis, MO).

### 2.8. Pharmacokinetic experiments

6-10 week old female B10.PL mice were fed 0.1% Lugol (Sigma-Aldrich, St. Louis, MO) in water for 72 h before i.v. injection in the tail vein with $^{125}$I-labeled IgGs or Fc-MBP fusions (10–15 μg per mouse). Levels of radioactivity in 10 μl blood samples were determined at the indicated times by gamma counting. To determine the AUC for IgGs and Fc-MBP fusion proteins, data were fitted to a bi-exponential decay model using custom software written in MATLAB (Mathworks, Natick, MA). The area under each of these bi-exponential model curves between time $t = 0$ and the time at which the extrapolated curve reaches 1% of the injected dose was calculated.

To investigate whether the Fc-MBP fusions affected the activity of FcRn in regulating the clearance rate of IgG, 6–10 week old male B10.PL mice were fed 0.1% Lugol (Sigma-Aldrich, St. Louis, MO) in drinking water for 72 h prior to i.v. injection with 10–15 μg of $^{125}$I-labeled mouse IgG1 (anti-hen egg lysozyme, D1.3). 24 h later, the mice were i.v. injected with 1 μg Fc-MBP fusion or vehicle (DPBS) control. Levels of radioactivity in 10 μl blood samples were analyzed at the indicated times by gamma counting and β-phase half-lives following injection of Fc-MBP fusion or vehicle determined as described previously [28].

### 2.9. Analyses of proliferative responses of transferred antigen-specific T cells

Antigen-specific $CD4^+$ T cells were isolated from the splenocytes of MBP1–9:I-$A^u$-specific TCR transgenic mice (1934.4 tg [18] and T/$R^+$ tg [19]) through negative selection using a MACS $CD4^+$ T cell isolation kit (Miltenyi Biotec, San Diego, CA). Female B10.PL mice were i.v. injected with 1 μg Fc-MBP fusion. One hour ('Day 0'), 3 or 5 days following Fc-MBP fusion delivery, $5 \times 10^5$ CFSE-labeled $CD4^+$ T cells were injected i.v. into the mice. Three days later, splenocytes and LN cells were isolated for flow cytometry analyses.

### 2.10. Induction of EAE

8-10 week old male B10.PL mice were immunized subcutaneously at four sites in the flanks with 200 μg acetylated MBP1-9 (CS Bio, Menlo Park, CA) emulsified with complete Freund's adjuvant (Sigma Aldrich, St. Louis, MO) containing an additional 4 mg/ml heat-inactivated *Mycobacterium tuberculosis* (strain H37Ra, Becton-Dickinson, San Jose, CA). In addition, 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) was injected i.p. on days 0 (0 h) and 2 (45 h).

Scoring of disease activity was as follows: 0, no paralysis; 1, limp tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5, quadriplegia; and 6, death due to disease. Clinical signs of EAE were assessed for up to ~30 days after immunization (prophylaxis) or disease onset (therapy).

### 2.11. Prophylactic and therapeutic treatment of mice with Fc-MBP fusions

For tolerance induction in a prophylactic setting, male B10.PL mice were injected i.v. with 1 μg Fc-MBP fusion and seven days later, immunized with MBP1-9 and treated with pertussis toxin to induce EAE. In some experiments, mice were treated with 5 doses of 1 μg Fc(v.short)-MBP (starting at 7 days prior to immunization, at 36 h intervals) or with a single dose of 5 μg Fc(v.short)-MBP delivered 7 days prior to immunization. For tolerance induction during ongoing disease, mice were injected i.v. with 1 μg Fc-MBP fusion at the onset of EAE (mean clinical score of 1–2).

### 2.12. Antibodies and flow cytometry analyses

Single cell suspensions from spleen, draining LNs (axillary, brachial and inguinal), brain and spinal cord were obtained by mechanical disruption and forcing through 70 μm cell strainers (Becton-Dickinson, San Jose, CA). For experiments involving analyses of immune cells in the CNS, mice were perfused with heparinized DPBS before collecting the organs. Splenic cell suspensions were depleted of erythrocytes using red blood cell lysis buffer.

Mononuclear cells from CNS cell suspensions were obtained using Percoll (1131 g/ml, GE Healthcare) gradients. Briefly, cells were washed with 37% Percoll and suspended in 30% Percoll which was then layered over 70% Percoll and centrifuged at 2118 g. Following centrifugation, the cells at the interface were collected, washed with DPBS and used for flow cytometry analyses.

For intracellular staining to detect Foxp3 and T-bet, cells were initially surface-stained, followed by fixation and permeabilization using Foxp3 staining buffer set (eBioscience, San Diego, CA). Permeabilized cells were incubated with fluorescently labeled anti-Foxp3 or anti-T-bet antibodies and washed with DPBS.

To detect antigen-specific $CD4^+$ T cells, single cell suspensions from spleens, LNs, brains and spinal cords were incubated with PE-labeled MBP1–9(4Y):I-$A^u$ tetramers for 90 min at 12 °C, followed by washing with DPBS.

Flow cytometry analyses were performed using a FACSCalibur (Becton-Dickinson, San Jose, CA) or LSRFortessa (Becton-Dickinson, San Jose, CA) and data analyzed using FlowJo (Tree Star, Ashland, OR). Antibodies specific for the following were purchased from either Becton-Dickinson (San Jose, CA), eBioscience (San Diego, CA) or Biolegend (San Diego, CA): CD4 (RM4-5), Foxp3 (FJK-16s), T-bet (4B10), CD40L (MR1), F4/80 (BM8), PD-1 (29F.1A12), CTLA-4 (UC10-4B9), LFA-1 (H155-78), CXCR3 (CXCR3-173), α4 (R1-2), β1 (HMβ1-1), α4β7 (DATK32) and CD45 (30-F11).

### 2.13. Statistical analyses

Tests for statistical significance for flow cytometric analyses of cell numbers and pharmacokinetic data were carried out using two-tailed Student's *t*-test in the statistics toolbox of MATLAB (Mathworks, Natick, MA). Due to the longitudinal nature of the measures of clinical scores over time, we compared the clinical score profiles between the groups of mice in disease experiments using the linear mixed effects model with AR(1) covariance structure with Statistical Analysis System software (SAS Institute Inc., Cary, NC). *p* values of less than 0.05 were taken to be significant.

## 3. Results

### 3.1. Generation of Fc-antigen fusion proteins with different in vivo dynamics

The binding of wild type (WT) mouse IgG1 or corresponding Fc fragment to mouse FcRn is highly pH-dependent, with binding at pH 5.5–6 (early-late endosomes) that becomes negligible at pH 7–7.4 [11]. Engineered IgGs with higher binding affinity than WT IgG1 for FcRn at both acidic and near-neutral pH show increased (receptor-mediated) uptake of the antibody, limited exocytic release during recycling, entry into lysosomes and reduced persistence [28,29]. Two sets of Fc mutations that alter FcRn binding were selected for this study: mutation-set (m-set)-1 (T252L/T254S/T256F/E380A/H433K/N434F) [30–32] and m-set-2 (T252Y/T256E/H433K/N434F) [29]. Based on the effects of these mutations on the equilibrium dissociation constants ($K_D$s) of the interactions of mouse IgG1-derived Fc-hinge fragments with mouse FcRn (Table 1), Fc fragments or IgG molecules harboring m-set-1 and m-set-2 mutations would be predicted to have distinct dynamic properties *in vivo* [11]. To confirm this, the pharmacokinetics of full length mouse IgG1 molecules harboring m-set-1 and m-set-2 mutations were compared with their WT counterpart in mice (Fig. 1A). The exposure to these proteins (area under the curve, or AUC, of injected dose vs. time) decreases in the following order: WT ≫ m-set-1 > m-set-2 (Fig. 1B).

We next generated Fc-MBP fusions comprising WT or mutated Fc fragments linked to MBP1-9. Although multiple studies have demonstrated that this MBP peptide requires N-terminal acetylation for T cell recognition, the replacement of the acetyl group with glycine generates an analogous epitope [24]. Further, the fusion proteins contain the '4Y' analog [MBP1-9(4Y)] of this peptide, in which lysine at position 4 is substituted by tyrosine. This analog has higher binding affinity for I-$A^u$ than its parent peptide whilst retaining recognition by autoreactive T cells [24,33]. The pharmacokinetics of the Fc-MBP fusions were analyzed in mice (Fig. 1C). Despite the lower persistence of the Fc fusions compared with the corresponding parent IgGs, most likely due to the binding of the epitope extending from the CH3 domain of the Fc fragment to the MHC Class II molecule, I-$A^u$ [34], the *in vivo* exposure (AUC) to the proteins decreased in the same order (Fig. 1C). Throughout these studies, fusion proteins containing WT or Fc fragments with m-set-1 and m-set-2 mutations were therefore designated Fc(long)-MBP, Fc(short)-MBP and Fc(v.short)-MBP, respectively. Although the difference in exposure (AUC) between Fc(short)-MBP and Fc(v.short)-MBP was significant, this difference was much lower than that for Fc(short)-MBP compared with Fc(long)-MBP (Fig. 1C). Consistent with the differences in exposure for the Fc-MBP fusions, the percentage remaining of the injected dose after 1 h was 16.33 ± 0.63% and 9.62 ± 0.28% for Fc(short)-MBP and Fc(v.short)-MBP, respectively, whereas for Fc(long)-MBP, 10.54 ± 0.5% of the injected dose remained after 118 h.

**Table 1**
Binding properties of mouse Fc fragments.

| Fc fragment | Binding to FcRn ($K_D$, nM) | |
|---|---|---|
| | pH 6.0 | pH 7.4 |
| WT | 218.2 | N.B.[a] |
| m-set-1 | 2.6 | 114.6 |
| m-set-2 | 1.1 | 20.4 |

[a] N.B. = no detectable binding.

### 3.2. Antigen persistence affects the proliferation of antigen-specific T cells in vivo

The effect of the distinct properties of the Fc-MBP fusions on the *in vivo* proliferation of MBP1-9:I-$A^u$-specific $CD4^+$ T cells was next investigated. CFSE-labeled, purified $CD4^+$ T cells isolated from MBP1-9:I-$A^u$-specific TCR ($V\beta8^+$) transgenic mice were used in adoptive transfers. Prior to T cell transfer into WT B10.PL (I-$A^u$) mice, 1 μg Fc-MBP fusion was injected into recipients on different days (day −5, −3 and 0, referring to 5, 3 and 0 days before the cell transfer, respectively, Fig. 2A). The percentage of divided $CD4^+CFSE^+V\beta8^+$ T cells was assessed in the spleens and lymph nodes (LNs) three days following T cell transfer. As a control throughout these studies, an Fc-MBP fusion in which the T cell contact residues, Gln3 and Pro6, of the MBP peptide [35] are replaced by Ala [Fc(long)-MBP(3A6A)] was used. Fc(long)-MBP induced higher levels of proliferation in the spleens and LNs than Fc(short)-MBP for all treatments (Fig. 2B). We have previously characterized the properties of Fc(v.short)-MBP in analogous assays [10], and the behavior of Fc(short)-MBP is very similar (Fig. 2B). As expected, Fc(long)-MBP(3A6A) induced no detectable proliferative response. Collectively, the data indicate that the increased affinity for FcRn at near neutral pH of Fc(short)-MBP and Fc(v.short)-MBP confers decreased *in vivo* persistence relative to Fc(long)-MBP, which in turn results in lower T cell responses *in vivo* (Fig. 2B).

### 3.3. The induction of tolerance under prophylactic conditions is regulated by antigen persistence

We next investigated the activity of low doses (1 μg/mouse; ~50 μg/kg) of the Fc-MBP fusions in inducing T cell tolerance in a prophylactic setting. These low doses of fusion protein do not affect the activity of FcRn in regulating IgG half-life (Fig. S1). B10.PL mice were pretreated with 1 μg Fc-MBP fusion and immunized 7 days later to induce EAE. Fc(long)-MBP(3A6A) was used as a control. The majority of mice developed either no, or low grade, disease following pretreatment with Fc(long)-MBP (Fig. 3A). Treatment of mice with Fc(short)-MBP was less effective in ameliorating EAE, whereas Fc(v.short)-MBP treatment had no protective effect (Fig. 3A). Thus, low dose antigen induces prophylactic tolerance, but only if antigen persists above a threshold level.

In addition to the shorter half-life of Fc(v.short)-MBP, the inability of Fc(v.short)-MBP to induce tolerance (Fig. 3A) could be due to differences between this fusion and Fc(long)-MBP in

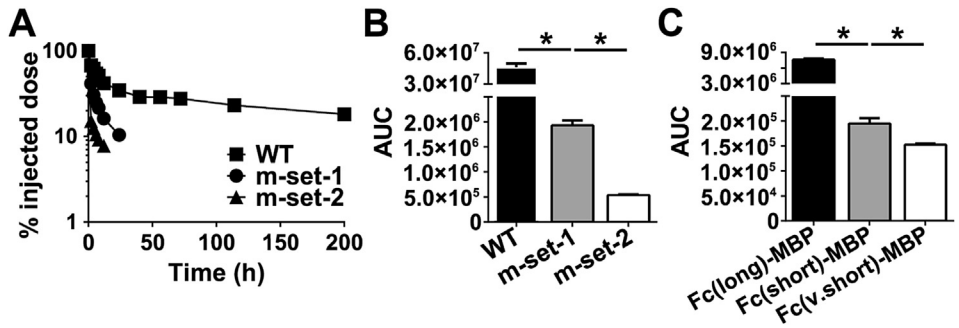


**Fig. 1.** IgGs or Fc-MBP fusions containing m-set-1 and m-set-2 mutations are cleared more rapidly in mice compared with their WT counterparts. B10.PL mice (n = 4–5 mice/group) were injected with $^{125}$I-labeled IgGs (A, B) or Fc-MBP fusions (C). (A) Remaining radioactivity levels in blood samples. (B, C) Areas under the curve (AUCs, cpm×h), calculated for fitted data following extrapolation to 1% injected dose. Error bars indicate SEM and significant differences ($p < 0.05$; two-tailed Student's *t*-test) are indicated by *.

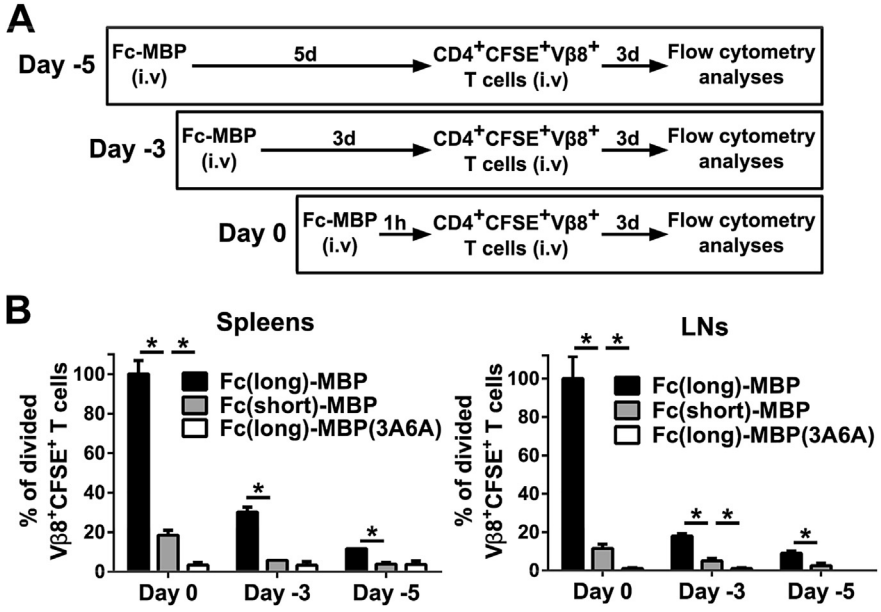


**Fig. 2.** *In vivo* persistence governs the response of cognate T cells to Fc-MBP fusions. (A) Flow chart describing the experimental design. B10.PL mice were injected with 1 μg Fc-MBP fusion 0, 3 and 5 days before the transfer of CFSE-labeled antigen-specific (Vβ8$^+$) T cells. CD4$^+$CFSE$^+$Vβ8$^+$ T cell proliferation was analyzed three days later by flow cytometry. (B) % divided Vβ8$^+$CFSE$^+$ T cells of total CD4$^+$ cells in spleens and LNs for the different treatments, normalized to the group injected with Fc(long)-MBP on day 0. Data are combined from at least two independent experiments (n = 3–4 mice/group). Error bars indicate SEM and significant differences ($p < 0.05$; two-tailed Student's *t*-test) are indicated by *.

endolysosomal trafficking behavior which influences antigen presentation by FcRn-expressing APCs [10,36]. Specifically, the binding of engineered Fc fragments to FcRn at near neutral pH results in efficient receptor (FcRn)-mediated uptake and accumulation in the endolysosomal pathway in FcRn-expressing cells, by contrast with WT Fc fragments that enter cells by fluid-phase pinocytic processes [11]. Consequently, using FcRn-transfected B lymphoblastoid (PL8:FcRn) [10] cells as APCs, Fc(short)-MBP induced significantly higher IL-2 production by cognate T cell hybridoma (#46 [26]) cells than Fc(long)-MBP (Fig. 3B), whereas in the presence of PL8 cells (that do not express FcRn), the Fc-MBP fusions induced similar levels of cytokine production (Fig. 3B; [10]). Analogously, in earlier studies we observed that Fc(v.short)-MBP stimulates T cells at around 600–3000 fold lower concentrations than Fc(long)-MBP in the presence of PL8:FcRn cells [10]. To investigate whether this behavior contributed to the inability of a single dose of Fc(v.short)-MBP to induce tolerance (Fig. 3A), we therefore compared the tolerogenic activity of five doses of 1 μg Fc(v.short)-MBP at 36 h intervals, starting at 7 days prior to immunization, with a single, equivalent bolus dose (5 μg) delivered at 7 days prior to EAE induction. Importantly, treatment with multiple doses of Fc(v.short)-MBP offered partial protection against EAE, whereas bolus administration of a five-fold higher dose of this Fc-MBP fusion did not affect disease activity (Fig. 3C). These observations indicate that antigen longevity, rather than endolysosomal trafficking behavior, is a dominant factor governing T cell tolerance. In addition, given the relatively small difference in the pharmacokinetic behavior of Fc(short)-MBP and Fc(v.short)-MBP in mice (Fig. 1C), the threshold of antigen persistence necessary for effective prophylaxis is stringent.

### 3.4. Antigen specific T cell numbers are reduced during prophylactic T cell tolerance

To investigate the mechanism of prophylactic tolerance induction, Fc-MBP fusions were delivered prophylactically and splenic antigen-specific T cells quantitated using fluorescently labeled MBP1-9(4Y)-I-A$^u$ tetramers [25] ten days following immunization with MBP1-9. Antigen-specific T cell numbers in the treated mice decreased in the order: Fc(v.short)-MBP (similar to control

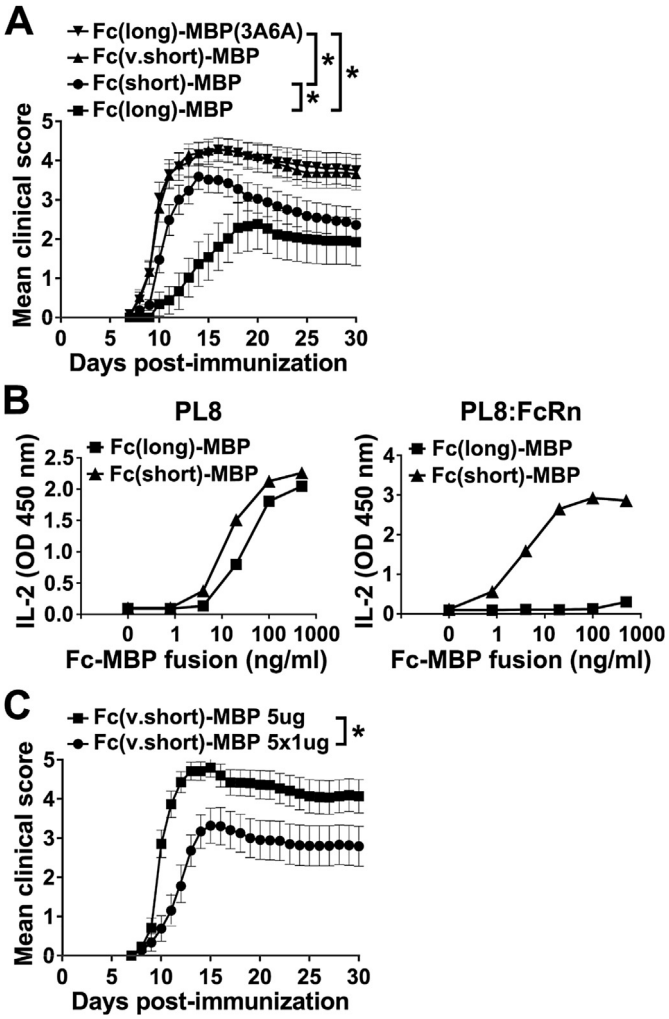


**Fig. 3.** Prophylactic tolerance induction is determined by antigen persistence. (A) B10.PL mice were pretreated with 1 μg Fc-MBP fusion and immunized seven days later with MBP1-9 to induce EAE. Mean clinical scores are shown. Data are combined from at least two independent experiments (n = 13–30 mice/group). (B) IL-2 production by antigen-specific T cell hybridoma (#46 [26]) cells in response to the Fc-MBP fusions in the presence of I-$A^u$-expressing PL8 or PL8:FcRn [10] cells. Data is representative of at least two independent experiments. (C) B10.PL mice were pretreated with either 5 doses of 1 μg of Fc(v.short)-MBP (starting at 7 days prior to immunization, at 36 h intervals) or with a single bolus dose of 5 μg of Fc(v.short)-MBP and immunized seven days later to induce EAE. Mean clinical scores are shown. Data are combined from at least two independent experiments (n = 18–26 mice/group). Error bars indicate SEM and significant differences ($p < 0.05$; linear mixed effects model) are indicated by *.

mice) > Fc(short)-MBP > Fc(long)-MBP (Fig. 4A, B). In addition, prophylactic delivery of a single dose of 5 μg Fc(v.short)-MBP resulted in higher numbers of antigen-specific T cells compared with treatment using five repeated doses (1 μg/dose) of this Fc-MBP fusion (Fig. 4C). Further, there were no significant differences between the numbers of $CD4^+Foxp3^+$ Tregs in mice treated with the different Fc-MBP fusions (Fig. S2). Consequently, there is a correlation between antigen longevity, disease blockade and reduction in antigen-specific T cell numbers.

### 3.5. Antigen persistence regulates T cell tolerance induction during ongoing disease

To assess therapeutic tolerance induction, mice were immunized with MBP1-9 to induce EAE and treated with the different fusion proteins (1 μg/mouse; ~50 μg/kg) following the onset of disease (EAE score of 1–2). Severe disease was observed in the control group of mice within 4–5 days of disease onset, whereas treatment with Fc(long)-MBP resulted in either almost complete recovery or lowered disease to a score of 1–2 following a transient increase in disease score (Fig. 5A). The therapeutic effect of Fc(short)-MBP was analogous to that of Fc(long)-MBP, whereas by analogy with prophylactic tolerance, the treatment of mice with Fc(v.short)-MBP had no effect on ongoing disease. This indicates a requirement for the Fc-MBP fusion to reach a threshold level of persistence for therapeutic tolerance, with the threshold being tightly bounded by the *in vivo* dynamics of Fc(short)-MBP and Fc(v.short)-MBP (Fig. 1C). Importantly, the delivery of a molar equivalent of MBP1-9(4Y) peptide (33 ng/mouse), which is expected to be rapidly cleared (~2–30 min [5]) by renal filtration, was less effective in treating EAE than Fc(long)-MBP (Fig. 5B).

### 3.6. The mechanisms of prophylactic and therapeutic tolerance induction are distinct

To elucidate the mechanism through which Fc-MBP fusions induce therapeutic tolerance, cells from spleens and draining LNs were analyzed in mice from Fc(long)-MBP and control treatment groups six days following treatment. Unexpectedly, and by marked contrast with the prophylactic setting, the numbers of antigen-specific $CD4^+$ T cells in the spleens and LNs of tolerized mice were approximately 10- and 4-fold higher, respectively, than in control mice (Fig. 6A). By contrast, quantitation of the antigen-specific T cells in the brain and spinal cord revealed around 10-fold lower numbers in the spinal cord of Fc(long)-MBP-treated mice, whereas similar numbers were detected in the brain (Fig. 6B). In the majority of murine EAE models, inflammation predominates in the spinal cord rather than the brain [37]. Also, MBP1-9-induced EAE in B10.PL mice is primarily Th1 cell-mediated [38,39] and it is well established that Th1 cells promote the accumulation of macrophages in the central nervous system (CNS) during EAE [40]. Consistent with the reduced T cell infiltrates in the spinal cords of tolerized mice, macrophage numbers were also decreased at this site (Fig. 6C).

The increased numbers of antigen-specific T cells in the periphery of tolerized mice, combined with their reduced numbers in the CNS, prompted us to further characterize these cells by quantitating their levels of the following markers: CXCR3, α4β1, α4β7, LFA-1, CTLA-4, PD-1 and CD40L. In addition, the intracellular levels of the master regulator of Th1 lineage development, T-bet, were analyzed. T-bet and CD40L were the only molecules that were differentially expressed between the groups. T-bet levels were significantly lower in splenic antigen-specific T cells obtained from mice treated with Fc(long)-MBP (Fig. 6D). This trend was also seen in antigen-specific T cells obtained from draining LNs (constituting only ~ 20% of the total number of antigen-specific T cells isolated from both spleen and LNs), but the difference was not statistically significant (Fig. 6D). Further, approximately threefold lower numbers of splenic antigen-specific T cells were $CD40L^{hi}$ in Fc(long)-MBP-treated mice by comparison with T cells obtained from control mice (Fig. 6E). Importantly, mice treated with Fc(long)-MBP had higher numbers of $CD4^+Foxp3^+$ Tregs in the spleen and draining LNs (Fig. 6F) which did not bind to MBP1-9(4Y):I-$A^u$ tetramers. The increase in $CD4^+Foxp3^+$ Tregs, combined with decrease in antigen-specific $CD4^+$T-$bet^+$ (Th1) T cells, resulted in higher Treg:Th1 ratios in tolerized mice (Fig. 6G). The treatment of mice with Fc(short)-MBP resulted in similar effects on splenic antigen-specific T cell numbers, their phenotype and $CD4^+Foxp3^+$ Treg numbers (Fig. S3), demonstrating antigen-specific tolerance of splenic T cells combined with the amplification of Tregs in tolerized mice.

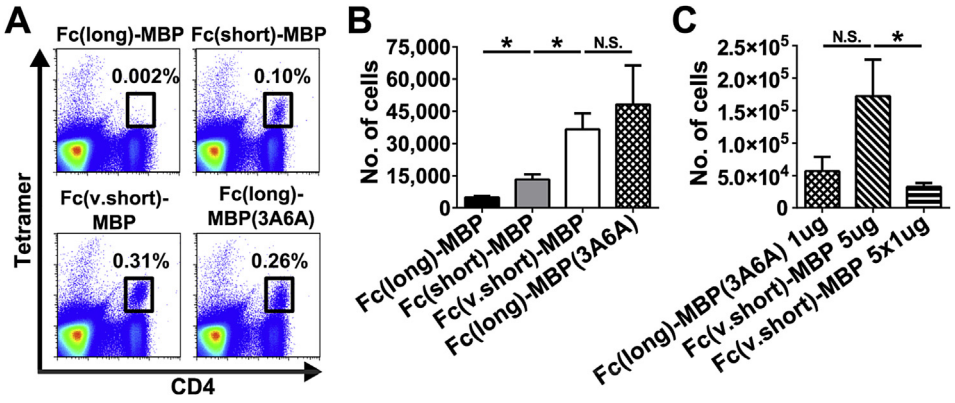


**Fig. 4.** Prophylactic tolerance induction is accompanied by lower numbers of antigen-specific T cells. (A, B, C) Quantitation of antigen-specific T cells in the spleens of mice using fluorescently labeled MBP1-9(4Y):I-$A^u$ tetramers ten days following immunization. % (boxed, A) and total numbers of $CD4^+$tetramer$^+$ T cells (B, C) are shown. Percentages (±SEM) of $CD4^+$ T cells for mice treated with the Fc-MBP fusions were: Fc(long)-MBP, 10.5 ± 0.9; Fc(short)-MBP, 11.4 ± 0.3; Fc(v.short)-MBP, 12.4 ± 0.8; Fc(long)-MBP(3A6A), 10.3 ± 0.6; 5 μg Fc(v.short)-MBP, 10.6 ± 0.7; 5 × 1 μg Fc(v.short)-MBP, 11.2 ± 0.4. Dot plots show data for one representative mouse within each group (A), and data in (B) and (C) are derived from 4-7 mice/group. Error bars indicate SEM and significant differences ($p < 0.05$; two-tailed Student's *t*-test) are indicated by *. N.S., no significant difference.

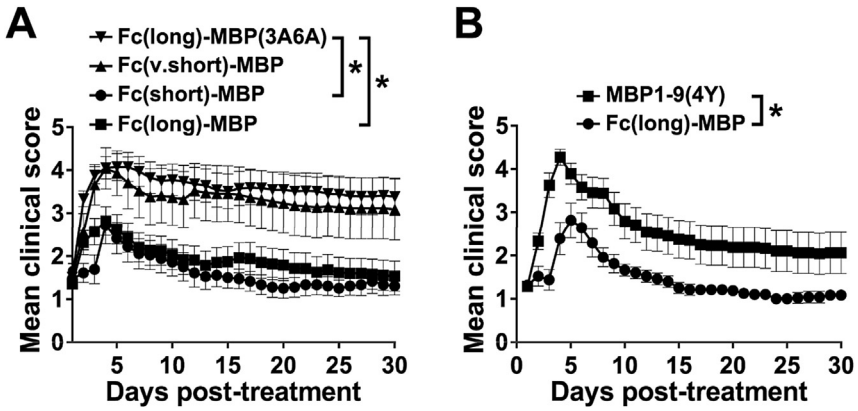


**Fig. 5.** A threshold persistence level of Fc-MBP fusion is necessary for the treatment of EAE. (A, B) B10.PL mice were immunized with MBP1-9 and treated with 1 μg Fc-MBP fusion or 33 ng MBP1-9(4Y) peptide following the onset of disease symptoms (EAE score of 1–2). Mean clinical scores are shown. Data are combined from at least two independent experiments (n = 9–26 mice/group). Error bars indicate SEM and significant differences ($p < 0.05$; linear mixed effects model) are indicated by *.

## 4. Discussion

The induction of antigen-specific T cell tolerance represents a highly specific approach for the treatment of autoimmunity. However, despite extensive preclinical analyses of the efficacy of immunodominant peptides in tolerance induction, this strategy has met with limited success in the clinic [2,41–43]. Importantly, the short half-lives of peptides necessitate the use of relatively high doses that can provoke anaphylaxis [6,7,44]. Here we have investigated the role of antigen dynamics in tolerance induction, by determining the tolerogenic activity of low doses (~50 μg/kg) of Fc fusions comprising an immunodominant MBP epitope linked to engineered Fc fragments with different binding properties for FcRn. These mutated Fc fragments are designed to endow different pharmacokinetic behavior on the appended antigen. Using this approach, we have established that the *in vivo* persistence of antigen is critical for tolerance induction and, in addition, have identified a requirement for a stringent threshold of persistence to achieve tolerance in both prophylactic and therapeutic settings.

The *in vivo* persistence of Fc-MBP fusions is governed by their interactions with FcRn in endothelial cells and/or hematopoietic cells [28]. Amongst hematopoietic cells, all professional APCs express FcRn [10,14,36,45]. Variations in interactions between Fc-MBP fusions and FcRn therefore also regulate epitope loading onto MHC class II molecules and cognate T cell activation. Consistent with our earlier study [10], Fc-MBP fusions that are recycled efficiently out of FcRn-expressing cells lead to poor antigen presentation *in vitro*, whereas fusions such as Fc(short)-MBP or Fc(v.short)-MBP that bind to FcRn with high affinity at near neutral and acidic pH accumulate to relatively high levels in APCs and are efficiently presented. However, recycled Fc-MBP fusions have prolonged *in vivo* persistence, whereas those that accumulate in FcRn-expressing cells have comparatively short half-lives. Importantly, the induction of tolerance by five doses of Fc(v.short)-MBP delivered over a seven day period prior to EAE induction, combined with the lack of efficacy of an equivalent bolus dose of this fusion protein, demonstrate that the endolysosomal trafficking properties of this protein do not mitigate tolerance induction if antigen persistence is prolonged. In addition, the lack of protection by a single dose of this Fc-MBP fusion indicates a minimum threshold of persistence of low dose antigen for tolerance induction that is tightly bounded by the pharmacokinetic behavior of Fc(short)-MBP and Fc(v.short)-MBP.

Although the benefit of tolerance induction during ongoing disease is obvious, there are also clinical situations where prophylactic T cell tolerance has potential applications such as the prevention of transplant rejection and reduction of immune responses against protein-based therapeutics [46–48]. In addition, epitope spreading has been observed in patients and animal

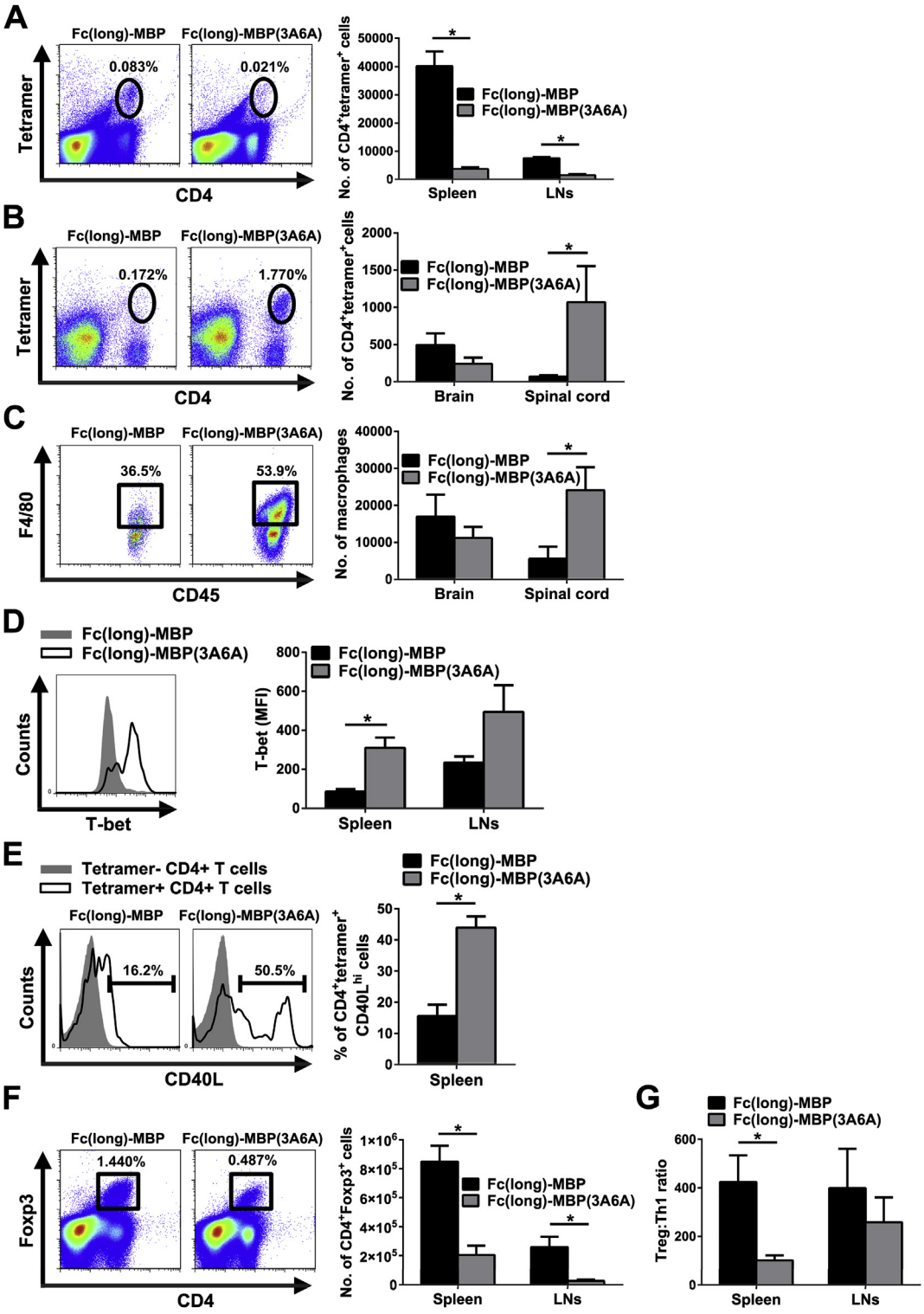


**Fig. 6.** Tolerance induction during ongoing EAE results in increased numbers of peripheral antigen-specific $CD4^+$ T cells with downregulated T-bet and CD40L levels combined with reduced inflammatory infiltrates in the CNS. B10.PL mice were immunized and treated with Fc(long)-MBP or Fc(long)-MBP(3A6A) as in Fig. 5. Six days following treatment, mice were sacrificed and tissues isolated for flow cytometry analyses to determine: (A) % (in spleens) and total numbers (in spleens, LNs) of $CD4^+$tetramer$^+$ T cells; (B, C) % (in spinal cords) and total numbers (in brains, spinal cords) of mononuclear infiltrates that are $CD4^+$tetramer$^+$ T cells (B) or $F4/80^+CD45^{hi}$ macrophages (C); (D) MFI levels for T-bet amongst $CD4^+$tetramer$^+$ T cells in spleens and LNs; (E) % $CD4^+$tetramer$^+CD40L^{hi}$ T cells in spleens; (F) % (in spleens) and total numbers (in spleens, LNs) of $CD4^+Foxp3^+$ T cells; (G) Treg ($CD4^+Foxp3^+$ T cells):Th1 ($CD4^+$tetramer$^+$T-bet$^+$ T cells) ratios in spleens and LNs. For A-F, left panels show data for one representative mouse from each group. For A-C, F, populations of interest are indicated in dot plots by solid circles or boxes. Percentages (±SEM) of $CD4^+$ T cells for mice treated with the Fc-MBP fusions were: Fc(long)-MBP, 8.8 ± 0.4 (spleens) and 34.8 ± 1.5 (LNs); Fc(long)-MBP(3A6A), 14.7 ± 0.9 (spleens) and 37.5 ± 2.4 (LNs). Data are combined from at least two independent experiments (n = 5–8 mice/group; right panels). Error bars indicate SEM and significant differences ($p < 0.05$; two-tailed Student's *t*-test) are indicated by *.

models of MS [49,50] and T cells specific for spread epitopes can induce EAE relapses [51]. Consequently, prophylactic tolerization of naïve autoreactive T cells specific to potential 'spreading' epitopes combined with tolerization of activated autoreactive T cells may result in effective treatment.

By analogy with prophylactic tolerance induction, a threshold of antigen persistence that is delimited by the behavior of Fc(short)-MBP and Fc(v.short)-MBP is also a requirement for the amelioration of ongoing disease. Analyses of the effects of Fc-MBP fusions reveal that although a fusion protein with a shorter persistence (Fc(short)-MBP) is less effective as a tolerogen in the prophylactic setting than its longer lived counterpart, Fc(long)-MBP, both fusion proteins have similar therapeutic activity during active EAE. This is possibly due to the different sensitivities of naïve and primed T cells to antigenic stimulation [52]. In addition, the mechanisms of prophylactic and therapeutic tolerance are distinct: prophylactic tolerance induction results in reduced numbers of antigen-specific $CD4^+$ T cells in the periphery, indicating T cell deletion or anergy. By contrast, in a therapeutic setting tolerance is unexpectedly accompanied by increased numbers of peripheral antigen-specific $CD4^+$ T cells. This also contrasts with the induction of T cell apoptosis in mice following the delivery of multiple high doses (400 μg/mouse) of acetylated MBP1-11 following the adoptive transfer of autoreactive $CD4^+$ T cells [3].

Importantly, we observe that the increased numbers of splenic antigen-specific T cells in the tolerized mice harbor significantly reduced levels of T-bet, which is essential for the encephalitogenicity of Th1 cells [53] and has been reported to be downregulated in tolerized Th1 cells [54]. In addition, CD40L levels are substantially lower in the majority of splenic antigen-specific T cells in the tolerized mice. Studies using both CD40L knock out mice and anti-CD40L blocking antibodies support a critical role for this molecule in T cell activation and EAE induction or progression [55,56]. Importantly, the downregulation of T-bet could be a downstream effect of reduced CD40L levels, since CD40L is required for the induction of co-stimulatory molecules such as B7.1 and B7.2 on APCs [55]. Our observation that tolerance induction during active EAE is accompanied by amplification of $CD4^+Foxp3^+$ Tregs, combined with reports that durable tolerance is dependent on the expansion of Tregs [8,57], suggest that Fc-epitope fusions will have long term effects.

Earlier studies have demonstrated that the delivery of relatively high doses of hapten-IgG conjugates can result in immunological tolerance, although the molecular mechanism was not defined [58]. More recent analyses have revealed the presence of conserved T cell epitopes, or Tregitopes, in IgGs that activate regulatory T cells [59]. Importantly, the Tregitope sequences identified to date are not altered by the Fc mutations used to generate shorter-lived Fc-MBP fusions in the current study. However, the doses of Tregitopes typically used are substantially higher than those of Fc-MBP fusions. Although we cannot exclude a contribution of the tolerogenic properties of Tregitopes to the amelioration of EAE, our results show that antigen persistence is a crucial factor for tolerance induction.

Polystyrene and poly(lactide-*co*-glycolide) microparticles coupled with antigenic peptides have also been used to induce tolerance under both prophylactic and therapeutic conditions in murine EAE [17]. The doses of peptide used in these analyses was 15–20 μg per mouse, which is ~450–600-fold higher than the peptide dose used in our studies. Further, by contrast with the inability of subcutaneously delivered microparticles to induce tolerance [17], the established use of this pathway for the delivery of therapeutic antibodies or Fc fusions [60–63] indicate that tolerogenic Fc-peptide fusions can be effectively delivered via this route. The persistence of Fc-antigen fusions can also be modulated and even increased by Fc engineering [30,32,64] to optimize tolerogenic effects, whereas for microparticles such tuning is not readily achievable.

MS is a very heterogeneous disease in terms of clinical course, the characteristics of demyelinating lesions and response to therapy [65]. Nevertheless, in the active lesions corresponding to the different disease types (pattern I–IV), T cells and macrophages predominate in the inflammatory infiltrates [66]. Hence, in the current study we employed an EAE model for which autoreactive T cells and macrophages are drivers of demyelination [38,40]. By contrast with the immunodominance of acetylated MBP1-9 in B10.PL mice [67], T cells specific for multiple neuroantigen-derived epitopes contribute to pathology in MS [49,50]. Importantly, Fc fusions harboring multiple peptides can be readily generated. In combination with the emergence of approaches to define T cell epitopes for pathological or protective immune responses in individuals [68], this provides support for the clinical translation of tolerance induction using long-lived Fc fusions.

In summary, by using Fc engineering to tune antigen dynamics, this study reveals that a stringent threshold of antigen persistence is a prerequisite for antigen-specific T cell tolerance induction. Low doses of relatively long-lived, Fc-epitope fusions are effective in ameliorating EAE in both prophylactic and therapeutic settings. Our observations not only provide mechanistic insight into tolerance induction, but also have direct relevance to the development of tunable, efficient and safer tolerogens.

## Author contributions

D.K.C., W.M. and E.S.W. designed the experiments; D.K.C., R.J.O. and E.S.W. wrote the manuscript; D.K.C., W.M. and S-T.L. performed the experiments; D.K.C., W.M., R.J.O. and E.S.W. analyzed the data.

## Competing financial interests

The authors declare no competing financial interests.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2016.05.007.

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