

On the Role of Dendritic Cells in Peripheral T Cell Tolerance and Modulation of Autoimmunity

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Abstract

Recently, it has become clear that dendritic cells (DCs) are essential for the priming of T cell responses. However, their role in the maintenance of peripheral T cell tolerance remains largely undefined. Herein, an antigen-presenting cell (APC) transfer system was devised and applied to experimental allergic encephalomyelitis (EAE), to evaluate the contribution that DCs play in peripheral T cell tolerance. The CD8 α ⁻CD4⁺ subset, a minor population among splenic DCs, was found to mediate both tolerance and bystander suppression against diverse T cell specificities. Aggregated (agg) Ig-myelin oligodendrocyte glycoprotein (MOG), an Ig chimera carrying the MOG 35–55 peptide, binds and cross-links Fc γ R on APC leading to efficient peptide presentation and interleukin (IL)-10 production. Furthermore, administration of agg Ig-MOG into diseased mice induces relief from clinical EAE involving multiple epitopes. Such recovery could not occur in Fc γ R-deficient mice where both uptake of Ig-MOG and IL-10 production are compromised. However, reconstitution of these mice with DC populations incorporating the CD8 α ⁻CD4⁺ subset restored Ig-MOG-mediated reversal of EAE. Transfer of CD8 α ⁺ or even CD8 α ⁻CD4⁻ DCs had no effect on the disease. These findings strongly implicate DCs in peripheral tolerance and emphasize their functional potency, as a small population of DCs was able to support effective suppression of autoimmunity.

Key words: autoimmunity • antigen delivery • dendritic cells • peripheral T cell tolerance • Fc γ receptors

Introduction

Dendritic cells (DCs)* represent a phenotypically heterogeneous population endowed with an important biologic function, namely, the presentation of antigen to both B and T lymphocytes (1–4). To date, it is believed that priming of naive T cells and stimulation of primary T cell responses are mainly a function of DCs with minimal contribution from other professional APCs (2, 3). Furthermore, recent evidence has indicated that different DC subsets confer discrete developmental functions to T cells (5–8). Accordingly, human DC1 and mouse CD8 α ⁺ DC subsets were

shown to promote differentiation into Th1 effectors, while human DC2 and mouse CD8 α ⁻ subsets supported development of Th2 cells (6–8). More recently, DCs have been shown to internalize apoptotic bodies and necrotic debris (9, 10) and cross-present (11) antigens exogenously acquired from the dead cells by intersecting the endogenous pathway of antigen presentation. This phenomenon has proven quite potent in cross-priming cytolytic T lymphocytes (10–13) and may provide a valuable tool for therapeutic vaccination against tumors (14–15). Our current knowledge on the roles that DCs play in T cell priming has been growing steadily due to the availability of experimental models. However, little is known about the critical contribution of DCs to central tolerance (16–18), and our knowledge on their role in peripheral tolerance is still in its infancy (19). In this report, we have devised an APC transfer system that was applied to the well-characterized experimental allergic encephalomyelitis (EAE) model of autoimmunity and evaluated the contribution of splenic DCs to peripheral T cell tolerance.

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*Abbreviations used in this paper: aa, amino acid; agg, aggregated; CNS, central nervous system; DC, dendritic cell; EAE, experimental allergic encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; PLP, myelin proteolipid protein; sol, soluble.

In prior studies, we have demonstrated that Ig-myelin proteolipid protein (PLP)1, an Ig-chimera carrying the encephalitogenic PLP1 peptide corresponding to amino acid (aa) sequence 139–151 of proteolipid protein (PLP), is presented to T cells 100-fold better than free PLP1 (20). In addition, aggregation of Ig-PLP1 facilitates cross-linking of Fc γ receptors (Fc γ R) on APCs and induces production of IL-10 by both macrophages and DCs (21) without stimulating upregulation of costimulatory molecules (22). Consequently, aggregated (agg) Ig-PLP1 induced a dramatic reduction in paralytic severity and promoted full recovery from EAE when it was administered free of adjuvant into mice with ongoing disease (21, 22). The likely mechanism underlying the effective suppression of EAE by agg Ig-PLP1 maybe a synergy between efficient peptide presentation, lack of costimulation, and IL-10-mediated bystander suppression (22). In Fc γ R-deficient (Fc γ R^{-/-}) mice, agg Ig-PLP1-mediated peptide presentation and IL-10 production by APC would be compromised. Thus, these mutant mice offer a suitable host into which to transfer wild-type DCs and evaluate their contribution to agg Ig-PLP1-mediated modulation of autoreactive T cells and maintenance of peripheral tolerance. However, due to the lack of Fc γ R^{-/-} mice on the SJL/J (H-2^s) background we opted to instead engineer the I-A^b-restricted myelin oligodendrocyte glycoprotein (MOG) aa 35–55 peptide (23) into the Ig backbone and use the resulting Ig-MOG chimera along with Fc γ R^{-/-} C57Bl/6 mice (24, 25) to evaluate the contribution of DCs to peripheral tolerance. The results outlined in this report demonstrate that agg Ig-MOG reverses clinical EAE induced in C57Bl/6 mice by injection of central nervous system (CNS) homogenate, as did agg Ig-PLP1 in the SJL/J mice. This indicates that bystander suppression of diverse T cell specificities is also operative in the Ig-MOG/C57Bl/6 system. When a similar treatment regimen with agg Ig-MOG was applied to Fc γ R^{-/-} C57Bl/6 mice recovery from disease did not occur. This result was anticipated as both agg Ig-MOG-driven peptide presentation and IL-10 production by APCs would be compromised in these Fc γ R^{-/-} mice. However, transfer of wild-type C57Bl/6 DCs into the Fc γ R^{-/-} mice before treatment with agg Ig-MOG restored Ig-MOG-mediated reversal of disease. To gain further insight into how DCs operate the modulation of autoreactive T cells, DCs were separated into subsets and tested both for cytokine production *in vitro* upon incubation with agg Ig chimeras and for restoration of agg Ig-chimera reversal of EAE in the Fc γ R^{-/-} mice. The results indicated that upon cross-linking of Fc γ R by the Ig-chimeras the CD8 α ⁻ DCs secreted IL-10 and reversed EAE in the mutant mice while the CD8 α ⁺ DCs were unable to reverse EAE and instead produced IL-12. More striking, further separation of the CD8 α ⁻ DCs into CD8 α ⁻CD4⁺ and CD8 α ⁻CD4⁻ populations indicated that IL-10 production was confined to the CD8 α ⁻CD4⁺ subset, which also supported suppression of autoreactive T cells and reversal of EAE in the Fc γ R-deficient mice.

Materials and Methods

Animals

SJL/J mice were purchased from Harlan-Sprague-Dawley and C57Bl/6 mice from The Jackson Laboratory. Fc γ 1 (Fc γ RI and III^{-/-}), Fc γ 2b (Fc γ RII^{-/-}), and Fc ϵ 1g/Fc γ 2 (Fc γ RI, II, and III^{-/-}) mice (24, 25) were purchased from Taconic. All mice were bred and maintained in our animal care facility for the duration of the experiments. All experimental procedures were performed according to the guidelines of the institutional animal care committee.

Antigens

Peptides. The peptides used in this study were purchased from Research Genetics (Huntsville, AL) and were HPLC purified to >90% purity. PLP1 peptide (HSLGKWLGHDPKF) encompasses aa residues 139–151 of PLP and is encephalitogenic in SJL/J mice (26). MOG peptide (MEVGWYRSPFSRVVHLYRNGK), encompassing aa residues 35–55 of MOG, is encephalitogenic in C57Bl/6 mice (23).

CNS Homogenate. 50 frozen unstripped rat brains (Pelfreez Biologicals) were homogenized in PBS using a Waring blender and adjusted to 300 mg/ml with PBS.

Ig-Chimeras. The Ig-PLP1 chimera harbors PLP1 peptide within the heavy chain CDR3 region and has been described previously (20–22). The Ig-MOG chimera harbors MOG 35–55 peptide within the heavy chain CDR3 region and, like Ig-PLP1, was constructed using the genes coding for the IgG2b, κ anti-arsenate antibody, 91A3 as described previously (20). In brief, the D segment was deleted from the CDR3 of the 91A3 heavy chain variable region and replaced with a nucleotide sequence that code for MOG35–55 peptide using mutagenesis procedures similar to those described for the generation of Ig-PLP1 (20). The resulting 91A3-MOG chimeric IgG2b heavy chain was cotransfected with the parental 91A3 κ chain into the non-Ig-producing SP2/0 myeloma B cell line, and the transfectoma cells producing complete Ig-MOG were selected with drugs as described previously (20). Transfection, cloning, sequencing, and purification procedures for Ig-MOG are similar to those used for Ig-PLP1 (20).

The chimeras were agg by precipitation with 50%-saturated (NH₄)₂SO₄ as has been described previously (21, 22). Since both Ig-PLP1 and Ig-MOG derive from the same Ig backbone and thereby comprise identical IgG2b isotype, their Fc-associated functions including binding and cross-linking of Fc γ R will be similar. In this respect we may refer to them indistinguishably as Ig chimeras.

Isolation of DCs

Splenic DCs were purified according to the standard collagenase/differential adherence method (27). In brief, the spleen was disrupted in a collagenase solution, and isolated DCs floated on a dense BSA gradient. Subsequently, the cells were allowed to adhere to Petri dishes for 90 min at 37°C, washed, and incubated overnight. For isolation of DC subsets, the bulk DCs were incubated with anti-CD8 α mAb coupled microbeads (Miltenyi Biotec) and separated into CD8 α ⁺ and CD8 α ⁻ populations by MACS[®] (Miltenyi Biotec). The CD8 α ⁻ fraction was repassed on magnetic columns after incubation with anti-CD8 α ⁺ mAb coupled microbeads to eliminate any residual CD8 α ⁺ cells. Subsequently, the CD8 α ⁻ cells were further purified by positive selection using anti-CD11c mAb coupled microbeads (Miltenyi Biotec). For preparation of CD8 α ⁻CD4⁺ and CD8 α ⁻CD4⁻ DC

subsets, the CD8 α ⁻ fraction was labeled with anti-CD4 mAb coupled microbeads (Miltenyi Biotec), and the subsets were separated as above. The CD4⁻ fraction was further purified by positive selection using anti-CD11c mAb coupled microbeads (Miltenyi Biotec). Each fraction was assessed for purity and no population was used if contamination was >5%.

Induction of EAE

6–8-wk-old mice were induced for EAE by subcutaneous injection in the footpads and at the base of the limbs and tail with a 200 μ l IFA/PBS (vol/vol) solution containing 6 mg CNS homogenate and 200 μ g *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). 6 h later the mice were given intravenous 200 ng of purified *Bordetella pertussis* toxin (List Biological Laboratories, Inc.). A second injection of *B. pertussis* toxin was given after 48 h. Subsequently, the mice were scored daily for clinical signs of EAE as follows: 0, no clinical score; 1, loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund or death. EAE induced by CNS homogenate manifests as a monophasic disease in C57Bl/6 mice and as a relapsing/remitting disease in SJL/J mice. For induction of EAE with peptide-pulsed DCs, the following procedure was used: purified CD8 α ⁺ or CD8 α ⁻ DCs (10⁶ cells/ml) were pulsed overnight with 50 μ g/ml PLP1 peptide, washed, resuspended in PBS, and injected subcutaneously into the hind limb footpads of mice at 3×10^5 cells per mouse. 6 and 24 h later, the mice were given intravenously 200 ng of *B. pertussis* toxin.

Treatment of EAE

Treatment with Peptide. Mice induced for EAE with CNS homogenate began receiving treatment with 100 μ g of PLP1 or MOG peptide after loss of tail tone, which occurs regularly between days 6 and 8 after disease induction. Treatment injections were given intraperitoneally in PBS on days 9, 13, and 17 as described previously (21).

Treatment with Ig-PLP1 or Ig-MOG. Mice induced for EAE with CNS homogenate began receiving treatment with 300 μ g of agg Ig-PLP1 or Ig-MOG after loss of tail tone. Treatment injections were given intraperitoneally in PBS on days 9, 13, and 17 after disease induction as described previously (21). Treatment of EAE in Fc γ R^{-/-} mice reconstituted with wild-type DCs was performed as follows: on day 5 after disease induction, the mice were adoptively transferred intraperitoneally with either 0.3 or 0.6 $\times 10^6$ purified DCs. Subsequently, the animals were treated with agg Ig-MOG on days 9, 13, and 17 as above.

Cytokine Detection

ELISA. ELISA was performed according to BD PharMingen's standard protocol. The capture Abs were as follows: rat anti-mouse IFN- γ , R4-6A2; rat anti-mouse IL-10, JES5-2A5; rat anti-mouse IL-4, 11B11; and rat anti-mouse IL-12, 9A5. The biotinylated anti-cytokine Abs were as follows: rat anti-mouse IFN- γ , XMG1.2; rat anti-mouse IL-10, JES5-16E3; rat anti-mouse IL-4, BVD6-24G2; and rat anti-mouse IL-12, C17.8. All antibodies were purchased from BD PharMingen. Assays were read on a SPECTRAMax 190 counter (Molecular Devices). Graded amounts of recombinant mouse IL-4, IFN- γ (BD PharMingen), IL-10, or IL-12 (Peprotech) were included in all experiments for construction of standard curves. The cytokine concentration in culture supernatants was interpolated from the linear portion of the standard curve.

Intracellular Cytokine Staining. CD8 α ⁺, CD8 α ⁻CD4⁺, or CD8 α ⁻CD4⁻ DCs (10⁶ cells/ml/well) were incubated with 0.6 μ M agg Ig-chimera for 24 h to allow for Fc γ R cross-linking. During the last 8 h of incubation, brefeldin A (10 μ g) (Epicentre Technologies) was added to the wells in order to block cytokine secretion. The cells were then harvested and stained with anti-CD11c-APC mAb, HL3, and FITC-labeled anti-CD8 α mAb, 53-6.7, or anti-CD4 mAb, GK1.5. Subsequently, the cells were fixed, permeabilized with 0.5% saponin (Sigma-Aldrich), and stained for intracellular IL-10 using PE-labeled anti-IL-10 mAb, JES5-16E3. The FACS[®] data were analyzed on a FACSVantage[™] Flow Cytometer (Becton Dickinson) using CELLQuest[™] software (Becton Dickinson).

Stimulation of Cytokine Production by APCs

Splenocytes and subsets of DCs from normal or Fc γ R-deficient mice were plated with graded amounts of soluble (sol) or agg Ig-PLP1, and the culture was then incubated for 24 h. Detection and quantification of cytokines was then assessed by ELISA from 100 μ l of culture supernatant as described above.

T Cells

TCC-PLP1-1B10. The generation of the PLP1-specific Th0 cell clone, TCC-PLP1-1B10, has been described previously (21). In brief, SJL mice were immunized with 100 μ g PLP1 peptide in CFA, and 10 d later the draining lymph nodes were removed and stimulated with PLP1. After three stimulation/resting cycles, the cells were cloned twice by limiting dilution, and positive clones were retested for reactivity with PLP1 peptide by both proliferation and cytokine production. Subsequently, one positive clone displaying a Th0 pattern was selected and designated TCC-PLP1-1B10.

MOG-specific T Cell Line. A similar procedure was used to generate a MOG reactive T cell line and these cells were used for testing Ig-MOG for peptide presentation.

Analysis of Cytokine Responses Upon Presentation of agg Ig-PLP1 by Subsets of DCs

Purified bulk, CD8 α ⁺, or CD8 α ⁻ DCs were plated at 5×10^4 cells/well/50 μ l and incubated with graded amounts of sol or agg Ig-PLP1 (100 μ l/well) for 1 h. Subsequently, TCC-PLP1-1B10 cells (5×10^4 cells per well per 50 μ l) were added, and the culture was continued for 24 h. Detection and quantification of cytokines were assessed by ELISA from 100 μ l of culture supernatant as described above.

Results

Broad Efficacy of agg Ig-myelin Chimeras in the Suppression of EAE. We have previously demonstrated that agg Ig-PLP1 is effective in the induction of IL-10 by APC and modulation of EAE involving diverse T cell specificities (21, 22). Furthermore this study indicated that IL-10-mediated bystander suppression plays a critical role in T cell modulation and amelioration of EAE, as administration of anti-IL-10 mAb into mice during treatment of disease with agg Ig-PLP1 restored full severity of paralysis (21, 22). This experimental model of T cell tolerance was used in conjunction with Fc γ R-deficient mice to devise an adoptive APC transfer system suitable for evaluation of the role of DCs in peripheral tolerance. Since Fc γ R-deficient mice

were not available in the H-2^s (SJL/J) background we instead chose to use C57Bl/6 (H-2^b) mice and therefore engineered an Ig-MOG chimera containing the I-A^b-restricted MOG35–55 peptide, an epitope that has been shown to induce EAE in C57Bl/6 mice (23). To ensure that the MOG peptide would indeed be processed from the chimera and presented to specific T cells, Ig-MOG was assayed for stimulation of a T cell line generated against MOG35–55 peptide. The results showed that the MOG-specific T cell line significantly proliferated when it was incubated with Ig-MOG in the presence of compatible APC, thus indicating that Ig-MOG was taken up by the APC and that a MOG peptide was generated and presented to the T cells (unpublished data).

Subsequently, Ig-MOG was tested for amelioration of EAE in C57Bl/6 mice side by side with Ig-PLP1 in the SJL strain. The results indicate that agg Ig-MOG, like agg Ig-PLP1, was able to reverse CNS homogenate induced EAE and, most likely, involved downregulation of diverse T cell specificities (Fig. 1). Indeed, while the untreated C57Bl/6 mice had a mean maximal score of 2.4 ± 0.2 , those treated with agg Ig-MOG had a mean maximal score of 1.2 ± 0.3 (Fig. 1 B). In addition, while agg Ig-MOG treated animals fully recovered from the typical monophasic EAE by day 22 after disease induction, the recovery of the untreated mice was delayed and occurred on day 47. This concurs with the SJL/Ig-PLP1 system in which the untreated mice had a mean maximal disease score of 2.8 ± 0.3 and had not recovered by day 60 post disease induction, as relapses were still observed. Those treated with agg Ig-PLP1 had a mean maximal disease score of 1.4 ± 0.3 and fully recovered

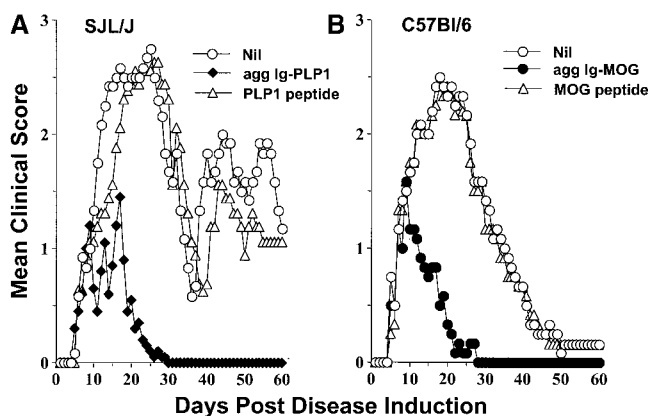


Figure 1. Agg Ig-MOG modulates disease involving diverse T cell specificities. Groups of SJL/J (A) or C57Bl/6 (B) mice were induced for EAE with 6 mg of CNS homogenate and were treated intraperitoneally with a saline solution containing 300 μ g of agg Ig-PLP1 (agg Ig-PLP1), 300 μ g agg Ig-MOG (agg Ig-MOG), 100 μ g of free PLP1 peptide (PLP1 peptide), or 100 μ g MOG peptide (MOG peptide) on days 9, 13, and 17 after disease induction. On the basis of the molecular weight of the Ig-chimeras and PLP1 and MOG peptide, we estimated that 300 μ g of free peptide contains 17- and 19-fold higher copies of peptide, respectively, than the Ig-chimera. Groups of untreated mice (Nil) were included for comparison purposes. The clinical onset of disease was at day 5–7 after disease induction in these experimental groups. Each point represents the mean clinical score of six mice.

from this relapsing/remitting disease by day 26 after induction, and relapses were not observed (Fig. 1 A). Furthermore, free PLP1 and MOG peptides used at 17- and 19-fold excess, respectively, were unable to suppress the clinical signs of EAE in their respective mouse strains (Fig. 1). These results indicate that the agg Ig chimeras display a broad effectiveness in reversing disease involving multiple epitopes and, most likely, diverse T cell specificities.

DCs Mediate the Reversal of EAE by agg Ig-myelin Chimeras. In prior studies, we demonstrated that DCs produce IL-10 upon cross-linking of their Fc γ R by agg Ig-PLP1 (21). Since neutralization of IL-10 during treatment of diseased mice with agg Ig-PLP1 restored clinical severity, it is possible that DCs play a critical role in the suppression of EAE (21). To evaluate the contribution of DCs to this form of peripheral T cell tolerance, an animal model where both antigen presentation and IL-10 secretion are compromised was needed. Since our delivery molecule belongs to the IgG2b subclass, an isotype that internalizes into APC via Fc γ R (20, 28) and induces IL-10 by the cross-linking of these receptors (21, 22), mice deficient in Fc γ R expression would provide such a model. The Fc γ R family is divided into three subfamilies that either employ an α -chain that mediates both Ig binding and signaling (Fc γ RII) or an α -chain that mediates Ig binding and a γ -chain responsible for signaling (Fc γ RI and III) (for a review, see reference 29). Mice in which the α -chain of Fc γ RII was knocked out generated animals that were deficient for Fc γ RII (Fc γ RII^{-/-}) but displayed intact Fc γ RI and III (24). Also, gene targeted mutation of the common γ -chain generated mice that were deficient in Fc γ RI and III (Fc γ RI and III^{-/-}),

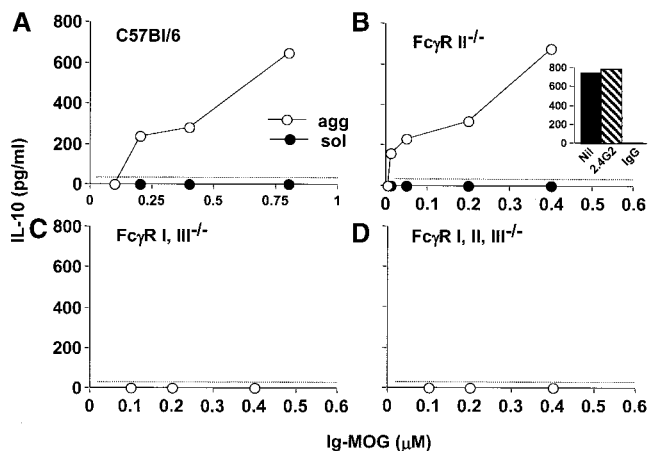


Figure 2. The induction of IL-10 production by agg Ig-chimeras is mediated primarily by Fc γ RI. Splenocytes from C57Bl/6 (A); Fc γ RII^{-/-} (B); Fc γ RI, III^{-/-} (C); and Fc γ RI, II, and III^{-/-} (D) mice were incubated with graded amounts of agg (white circles) or sol (black circles) Ig-MOG for 24 h. Subsequently, the supernatant was used to measure IL-10 production by ELISA. To determine the contribution of Fc γ RI and Fc γ RIII in mediating IL-10 production, the following experiment was performed (insert to B): DCs from Fc γ RII^{-/-} mice were incubated with 0.6 μ M of agg Ig-chimera in the presence of 20 μ g/ml 2.4G2 mAb (hatched bar) or 100 μ g/ml mouse IgG (white bar), and IL-10 production was evaluated. Each point represents the mean of triplicate wells. The dotted line indicates the lower limit of cytokine detection in this ELISA.

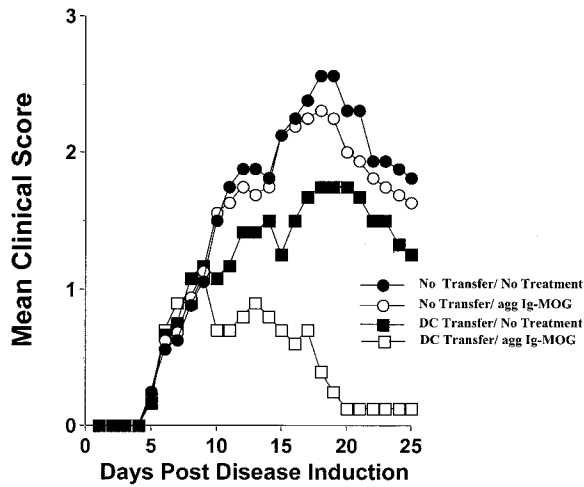


Figure 3. DCs mediate reversal of EAE by agg Ig-chimeras. Groups of Fc γ RI and III^{-/-} mice (6–8 wk of age) were induced for EAE with 6 mg of CNS homogenate, and on day 5 after disease induction, adoptively transferred with 0.6×10^6 purified C57Bl/6 DCs (squares). Subsequently, the mice were treated intraperitoneally with 300 μ g agg Ig-MOG on days 9, 13, and 17 after disease induction (white symbols). Groups of untreated mice that either received (black squares) or did not receive (black circles) DC transfer and mice that were treated with agg Ig-MOG but did not receive DC transfer (white circles) were included for comparison purposes. The clinical onset of disease was at day 5 in these experimental groups. Each point represents the mean clinical score of 6–8 mice.

but not Fc γ RII expression (25). Genetic crossing of these two mutant strains generated mice deficient for all three types of Fc γ Rs (Fc γ RI, II, and III^{-/-}) (25). Using these mutant mice, we identified Fc γ RI as the major mediator of agg Ig-chimera induced IL-10 production by APCs (Fig. 2). Indeed, splenocytes from C57Bl/6 or Fc γ RII^{-/-} mice produced IL-10 upon incubation with agg Ig-MOG (Fig. 2, A and B), while those from Fc γ RI and III^{-/-} or Fc γ RI, II, and III^{-/-} mice did not, as IL-10 production remained at the background level seen with the negative control, sol Ig-MOG (Fig. 2, C and D). Similar results were obtained

when DCs were used instead of splenocytes (unpublished data). These results indicated that Fc γ RI and/or Fc γ RIII was responsible for binding agg Ig-MOG and triggering the IL-10 production by the APC. However, blockade of Fc γ RIII with 2.4G2 antibody, which is specific for both Fc γ RII and III (30), on cells lacking Fc γ RII still allowed for equivalent IL-10 production indicating that Fc γ RI is most likely the major binder and, thus, mediator of agg Ig-MOG induced IL-10 production (Fig. 2, B insert). Therefore, Fc γ RI and III^{-/-} mice provide an appropriate model for the adoptive transfer of wild-type C57Bl/6 APC in order to investigate the role of DCs in the suppression and reversal of EAE by agg Ig-chimeras. Accordingly, Fc γ RI and III^{-/-} mice that had been induced for EAE with CNS homogenate were given C57Bl/6 DCs on day 5 after disease induction and were treated with agg Ig-MOG when EAE had become clinically apparent. As can be seen in Fig. 3, the Fc γ RI and III^{-/-} mice developed a severe disease (2.6 ± 0.2) in response to CNS homogenate (no transfer/no treatment group). In contrast to the wild-type C57Bl/6 mice, these Fc γ RI and III^{-/-} mice could not reverse their disease upon similar treatment with the agg Ig-MOG (no transfer/agg Ig-MOG group). However, if these mice were reconstituted with wild-type DCs from C57Bl/6 mice before treatment with agg Ig-MOG, the severity of EAE was significantly reduced (mean maximal score of 1.1 ± 0.2), and the animals recovered by day 20 after disease induction (DC transfer/agg Ig-MOG group). Adoptive transfer of DCs without treatment with agg Ig-MOG had a marginal effect on disease manifestation (DC transfer/no treatment group). These results indicate that DCs play a critical role in this form of peripheral tolerance and suppression of autoimmunity.

We have previously shown that modulation of disease by the Ig-chimeras is due to tolerization of myelin-specific T cells and suppression of their proliferation as well as IFN- γ production (21). In addition, the IL-10 produced by APC, upon binding of agg Ig-myelin chimeras was

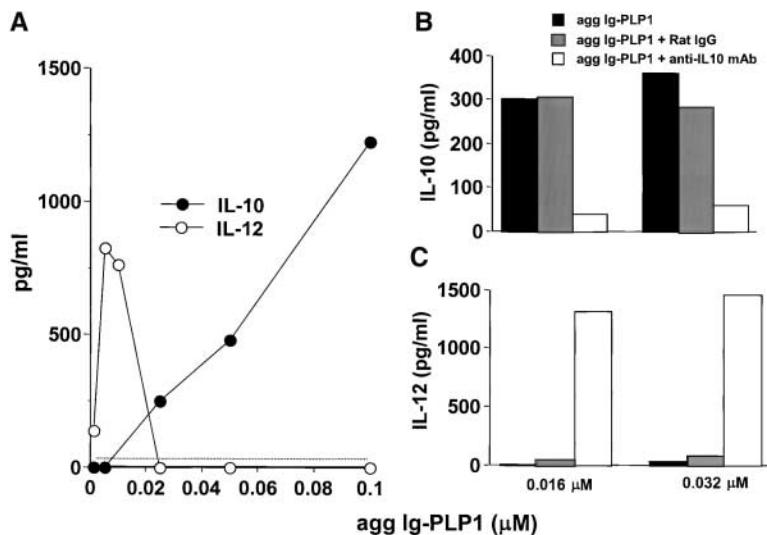


Figure 4. DCs produce IL-10 and downregulate IL-12 upon incubation with agg Ig-PLP1. 20,000 purified DCs (A) were incubated with graded amounts of agg Ig-PLP1 for 24 h, and the supernatant was then used to measure both IL-10 and IL-12 production by ELISA. In the right panel the stimulation with agg Ig-PLP1 was performed in the presence of 20 μ g/ml anti-mouse IL-10 mAb antibody (2A5) or negative control rat IgG. Subsequently IL-10 (B) and IL-12 (C) were measured by ELISA. Each point or bar represents the mean of triplicate wells. The dotted line indicates the lower limit of cytokine detection in this ELISA.

shown to play a critical role in T cell tolerization, as its neutralization by administration of anti-IL-10 antibodies restored disease severity (21). Suppression of EAE by agg Ig-myelin chimeras could operate through the direct binding of IL-10 to the autoreactive T cells leading to inhibition of their proliferation (31–33). Alternatively, IL-10 may downregulate IL-12 production by the APC and lead to defective T cell differentiation (31, 34, 35). To test this premise, we assayed DCs for production of IL-12 upon incubation with agg Ig-MOG and assessed whether any such IL-12 secretion would be subject to downregulation by IL-10. As can be seen in Fig. 4, DCs produced both IL-10 and IL-12 upon incubation with agg Ig-MOG. However, the secretion of IL-12 decreased as IL-10 production increased suggesting a regulation of IL-12 by IL-10. In fact the addition of an anti-IL-10 mAb to the culture to neutralize IL-10 supported this view and allowed for IL-12 secretion at higher concentrations of agg Ig-chimera (Fig. 4, B and C).

The CD8 α^- , but not CD8 α^+ , DC Population Is Responsible for IL-10 Production and Reversal of EAE upon Treatment with agg Ig-MOG. While all DCs defined to date function as APC, they are quite heterogeneous in their surface phenotypes (36, 37). Recently, it has become clear that the different DC subtypes display different trafficking patterns (38) and contribute distinct developmental functions in T cell priming (7, 8). Since DCs produce IL-10 and mediate reversal of EAE upon treatment with agg Ig-MOG, this model offers an opportunity to investigate the role of the DC subsets in peripheral T cell tolerance. Initially, DCs were separated into CD8 α^+ and CD8 α^- populations and tested for both IL-10 and IL-12 production upon incubation

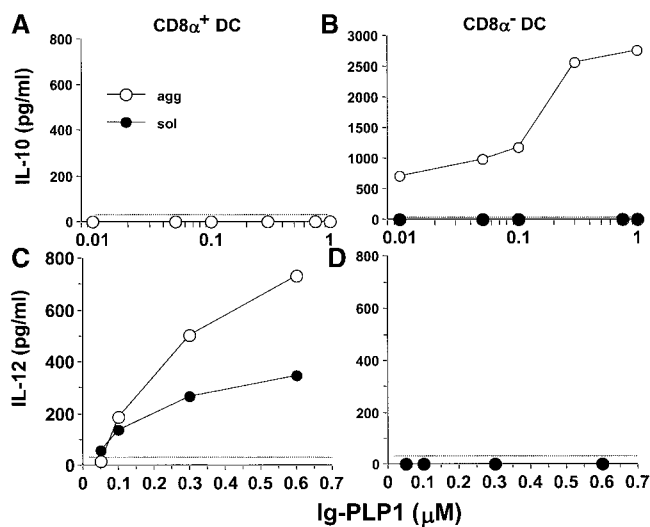


Figure 5. CD8 α^- DCs produce IL-10, while CD8 α^+ DCs secrete IL-12 in response to agg Ig-PLP1. 50,000 purified CD8 α^+ (A and C) or CD8 α^- (B and D) DCs were incubated with graded amounts of agg (white circles) or sol (black circles) Ig-PLP1 for 24 h. Subsequently, the supernatant was used to measure both IL-10 (A and B) and IL-12 (C and D) production by ELISA. Each point represents the mean of triplicate wells. The results shown are representative of four experiments. The dotted line indicates the lower limit of cytokine detection in this ELISA.

tion with agg Ig-MOG. As illustrated in Fig. 5, CD8 α^- DCs produced IL-10, while CD8 α^+ DC secreted IL-12, upon cross-linking of their Fc γ Rs by agg Ig-PLP1. The production of IL-12 by CD8 α^+ DCs was also inducible by sol Ig-PLP1, possibly indicating that binding of monomeric IgG to Fc γ Rs is sufficient to trigger IL-12 secretion. Alternatively, some sol Ig-PLP1 might have agg during culture at 37°C. If this is the case, IL-12 induction requires only a small amount of agg Ig relative to that required for the induction of IL-10 (Fig. 4).

Since the CD8 α^- DCs produced IL-10 upon incubation with agg Ig-PLP1, T cells engaged by these APCs through peptide presentation should be tolerized rather than stimulated (31, 32). To test this premise, the PLP1-specific Th0 clone, TCC-PLP1-1B10 (21), was incubated with CD8 α^- DCs in the presence of sol or agg Ig-PLP1 and both IL-10 production by the APC and IFN- γ secretion by the T cells were measured. For comparison purposes, bulk and CD8 α^+ DCs were included in these experiments. As can be seen in Fig. 6, A and B, CD8 α^+ DCs, which do not produce IL-10 upon incubation with agg or sol Ig-PLP1, activated the Th0 clone to produce IFN- γ through presentation of PLP1 peptide. In contrast, CD8 α^- DCs, which produce IL-10 upon incubation with agg Ig-PLP1 (Fig. 6 C), were not able to drive production of IFN- γ by the Th0 clone (Fig. 6 D). Surprisingly, incubation with sol Ig-PLP1,

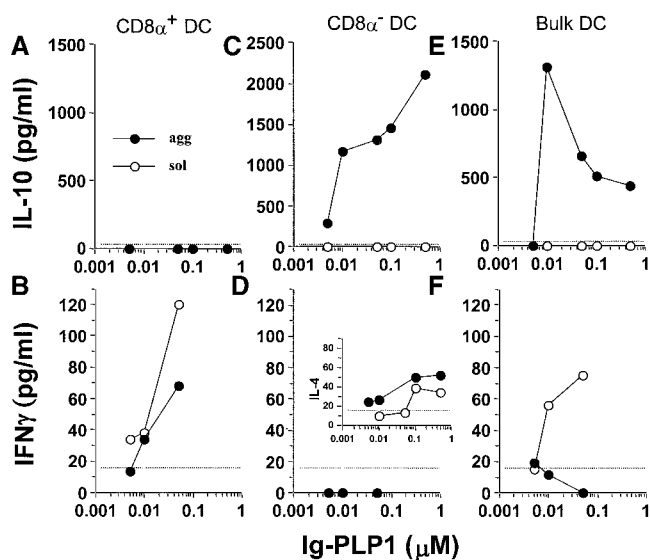


Figure 6. CD8 α^- DCs are unable to promote the production of IFN- γ by T cells. Purified CD8 α^+ (A and B), CD8 α^- (C and D), and unseparated CD11c $^+$ (E and F) DC (5×10^4 cells per well) were incubated with graded amounts of agg (black circles) or sol (white circles) Ig-PLP1 for 1 h. Subsequently, TCC-PLP1-1B10 Th0 cells (5×10^4 cells per well) were added, and the incubation was continued for 24 h. IL-10 (A, C, and E) and IFN- γ (B, D, and F) production in the same culture well were then measured by ELISA from 100 μ l of culture supernatant. The insert in D, measuring IL-4 secretion, indicates that the absence of IFN- γ production by the T cells upon stimulation with CD8 α^- DCs and Ig-PLP1 was not due to inadequate antigen presentation. Each point represents the mean of triplicate wells. The dotted line indicates the lower limit of cytokine detection in this ELISA.

which does not cross-link Fc γ R or trigger IL-10 production by the CD8 α ⁻ DCs (Fig. 6 C), was unable to stimulate the Th0 clone for IFN- γ production (Fig. 6 D). The lack of IFN- γ was not due to defective presentation of sol Ig-PLP1 as the Th0 clone was able to produce IL-4 in response to such stimulation (Fig. 6, D insert). Nevertheless, the IL-10 produced by the CD8 α ⁻ subset within the bulk DC population (Fig. 6 E) suppressed the capacity of the CD8 α ⁺ subset to drive IFN- γ production by the T cells (Fig. 6 F), a phenomenon that further argues for the tolerogenic function of CD8 α ⁻ DCs. Overall, the results of this *in vitro* experiment with a Th0 clone demonstrate that CD8 α ⁻ DCs do not support the polarization of T cells into IFN- γ -producing cells and can actively antagonize the activation of Th1 cells through IL-10 production.

The ability of CD8 α ⁻ DCs to mediate T cell tolerance *in vivo* was then analyzed. To this end, CD8 α ⁻ DCs were adoptively transferred into Fc γ RI and III^{-/-} mice and tested for reversal of EAE upon treatment with agg Ig-MOG. The results illustrated in Fig. 7 indicate that transfer of either 3 or 6 $\times 10^5$ C57Bl/6 CD8 α ⁻ DCs into Fc γ RI and III^{-/-} mice with ongoing clinical EAE allowed for the reversal of paralysis when the mice were treated with agg Ig-MOG. However, mice adoptively transferred with CD8 α ⁺ DCs, instead of the CD8 α ⁻ population, were unable to suppress the disease and had clinical scores similar to mice that did not receive any cell transfer. These results indicate that CD8 α ⁻ DCs are responsible for tolerization of pathogenic myelin-specific Th1 T cells and, thus, play a critical role in this form of peripheral tolerance.

It has been previously shown that DCs loaded with antigenic peptide induce immune responses without the re-

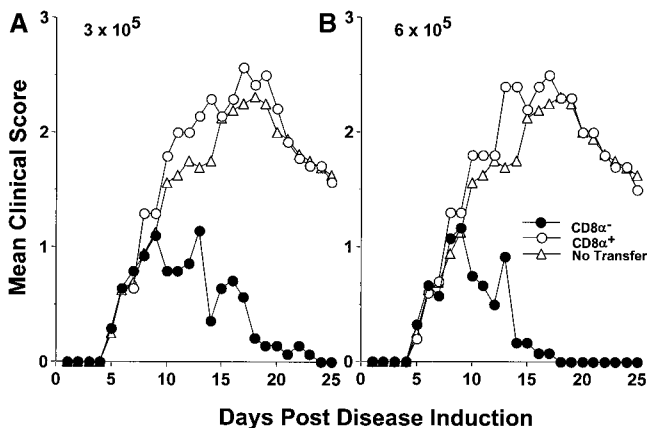


Figure 7. CD8 α ⁻ wild-type DCs restore agg Ig-MOG mediated reversal of EAE in Fc γ RI and III^{-/-} mice. Groups of Fc γ RI and III^{-/-} mice (6–8 wk of age) were induced for EAE with 6 mg of CNS homogenate. On day 5 after disease induction, the mice were given intraperitoneally 3 $\times 10^5$ (A) or 6 $\times 10^5$ (B) purified C57Bl/6 CD8 α ⁻ (black circles) or CD8 α ⁺ DCs (white circles). Subsequently, the mice were treated intraperitoneal with 300 μ g agg Ig-MOG on days 9, 13, and 17 after disease induction. A group of mice that were treated with agg Ig-MOG but did not receive cell transfer (white triangles) was included for comparison purposes. The clinical onset of disease was at day 5 in these experimental groups. Each point represents the mean clinical score of 6–8 mice.

quirement for adjuvant (3). This regimen provided a useful approach to test the intrinsic tolerogenic function of the CD8 α ⁻ DCs. Accordingly, CD8 α ⁻ and CD8 α ⁺ DCs were loaded with an excess of PLP1 peptide *in vitro* and injected into mice according to a regimen defined to induce EAE (39). Daily analysis of paralytic scores indicated that CD8 α ⁺, but not CD8 α ⁻ DCs, induced EAE (Fig. 8). Again, these results argue that PLP1-reactive T cells were available but were unresponsive to PLP1 peptide when it was presented by these CD8 α ⁻ DCs as activation and differentiation into Th1 effectors that cause disease did not occur (7, 8).

CD8 α ⁻CD4⁺, but not CD8 α ⁻CD4⁻, DCs Produce IL-10 and Mediate Reversal of EAE upon Treatment with Ig-MOG. The CD8 α ⁻ subset of splenic DCs is comprised of two phenotypically different populations based upon the expression of CD4 (37). However, whether these subsets, CD8 α ⁻CD4⁺ and CD8 α ⁻CD4⁻, display distinct biologic functions has not yet been clarified. Herein, the two subsets were separated from splenic DCs and tested for IL-10 production as well as reversal of EAE upon treatment with agg Ig-MOG. The results illustrated in Fig. 9 indicate that CD8 α ⁻CD4⁺, but not CD8 α ⁻CD4⁻ DCs, produce IL-10 upon incubation with agg Ig-MOG as tested by intracellular cytokine staining (Fig. 9, A and B). IL-10 production was not observed with the CD8 α ⁺ subset that was used as a negative control (Fig. 9 C). Subsequently, the two subsets were adoptively transferred into Fc γ RI and III^{-/-} mice with ongoing clinical EAE and tested for reversal of paralysis upon treatment with agg Ig-MOG. The results indicated that CD8 α ⁻CD4⁺, but not CD8 α ⁻CD4⁻, DCs were able to mediate tolerance and reverse the disease upon treatment with agg Ig-MOG (Fig. 9, D and E). Indeed, the mice transferred with 3 or 6 $\times 10^5$ CD8 α ⁻CD4⁺ DCs had a mean maximal disease severity of 0.9 \pm 0.3 and fully recovered by day 18 after disease induction. Those transferred with equivalent numbers of CD8 α ⁻CD4⁻ DCs had a mean maximal disease severity of 2.6 \pm 0.3 and did not recover from their paralysis during the 21-d period of clinical assessment. The reversal of EAE requires treatment with agg Ig-MOG because adoptive transfer of 6 $\times 10^5$

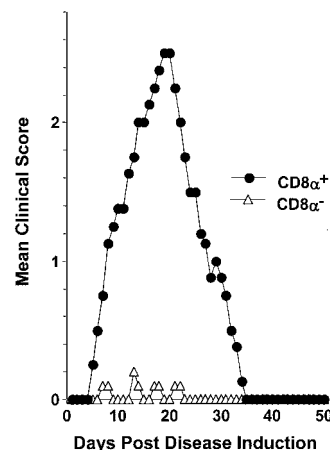


Figure 8. CD8 α ⁺, but not CD8 α ⁻, DCs promote the development of EAE. Purified CD8 α ⁺ (black circles) or CD8 α ⁻ (white triangles) DCs were pulsed overnight with 50 μ g/ml PLP1 peptide, washed, resuspended in PBS, and injected (3 $\times 10^5$ cells per mouse) into the hind limb footpads of SJL/J mice. 6 and 24 h later, the mice were given intravenous 200 ng of *B. pertussis* toxin. The clinical onset of disease was at day 6 in these experimental groups, and each point represents the mean clinical score of five mice.

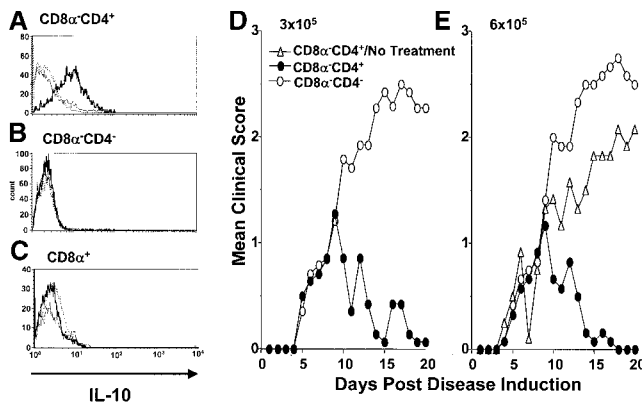


Figure 9. CD8 α ⁻CD4⁺ DCs produce IL-10 and mediate reversal of EAE. IL-10 production by purified (A) CD11c⁺CD8 α ⁻CD4⁺, (B) CD11c⁺CD8 α ⁺CD4⁻, and (C) CD11c⁺CD8 α ⁺ DCs was measured by intracellular cytokine staining. Accordingly, DCs (10⁶ cells per well) were incubated with 0.6 μ M agg Ig-PLP1 for 24 h and brefeldin A (10 μ g) was added to the wells during the last 8 h of incubation, in order to block cytokine secretion. Subsequently, the cells were harvested and stained with anti-CD11c-APC and FITC-labeled anti-CD8 α or anti-CD4 mAb. The cells were then fixed, permeabilized, and intracellularly stained with PE-conjugated anti-IL-10 (thick lines) or isotype-matched (dotted lines) mAb. Cells incubated with media without the addition of Ig-PLP1 (thin lines) and intracellularly stained with anti-IL-10-PE mAb were included for control purposes. Shown are histogram plots representing the intensity of IL-10 expression by the indicated DC population. The results shown are representative of four experiments. In D and E, groups of Fc γ RI and III^{-/-} mice (6–8 wk of age) were induced for EAE with 6 mg of CNS homogenate and on day 5 the mice were given intraperitoneally 3 × 10⁵ (D) or 6 × 10⁵ (E) purified C57Bl/6 CD8 α ⁻CD4⁺ (black circles) or CD8 α ⁺CD4⁻ (white circles) DCs. Subsequently, the mice were treated intraperitoneally with 300 μ g agg Ig-MOG on days 9, 13, and 17 after disease induction. A group of untreated mice that received 0.6 × 10⁶ CD8 α ⁻CD4⁺ DC (white triangles) was included for comparison purposes. The clinical onset of disease was at day 5 in these experimental groups. Each point represents the mean clinical score of 6–8 mice.

CD8 α ⁻CD4⁺ DCs did not confer reduction of the severity of disease or recovery from paralysis when the animals were not given agg Ig-MOG. These results identify the CD8 α ⁻CD4⁺ DC subset as the tolerogenic population among mature splenic DCs.

Discussion

The results presented in this report show that C57Bl/6 mice recover from EAE when treated with agg Ig-MOG as did SJL/J mice after treatment with agg Ig-PLP1 (Fig. 1). However, C57Bl/6 mice deficient for Fc γ RI and III could not resolve EAE upon similar treatment with agg Ig-MOG unless reconstituted with DCs from wild-type C57Bl/6 mice before the treatment (Fig. 3). Previously, we have shown that APCs internalize Ig-chimeras of the same IgG2b backbone as Ig-MOG and Ig-PLP1 mainly via Fc γ Rs leading to processing of the chimeras within endosomes and efficient loading of the incorporated peptide onto newly synthesized MHC molecules (40). Aggregation of the Ig-chimeras adds another feature to the Ig delivery system, namely the cross-linking of Fc γ R and production of IL-10 by the APC (21, 22). Fc γ RI was

identified as the main target for Ig-MOG and mice lacking such a receptor could not resolve EAE (Fig. 3) most likely due to compromised presentation of Ig-MOG and lack of IL-10 production (Fig. 2). Furthermore, this report shows that DCs produce IL-12 upon cross-linking of their Fc γ R but that the simultaneous production of IL-10 negatively regulates such IL-12 secretion (Fig. 4). This observation prompted us to determine whether the two cytokines were produced by the same or by separate subsets of DCs and whether there was a correlation between a specific population of DCs and recovery from disease. The findings indicated that the IL-10 production was mediated by CD8 α ⁻ DCs and IL-12 by the CD8 α ⁺ population (Fig. 5). While this result is in good agreement with data obtained recently with pansomycin (killed *S. aureus*) stimulated DCs (41), it is also supported by the observation that CD8 α ⁻ but not CD8 α ⁺ DCs mediate recovery from EAE in the Fc γ RI and III^{-/-} mice upon treatment with Ig-MOG (Fig. 7). Although different subsets of DCs may display different patterns of trafficking (38) and migration issues could be at play in this form of peripheral tolerance, a number of observations argue instead that these results represent intrinsic functions of the two populations. Fig. 6 shows that upon incubation with agg Ig-PLP1 the CD8 α ⁺ DCs produce IL-12, present the PLP1 peptide, and stimulate the PLP1-specific Th0 cells to produce IFN- γ . Therefore, these cells most likely would not support reversal of EAE upon encounter with pathogenic T cells when properly located. However, CD8 α ⁻ DCs produced IL-10, did not stimulate IFN- γ production by the same Th0 cells (Fig. 6), and therefore not surprisingly supported the reversal of EAE (Fig. 7). Thus, in this in vitro T cell regulation system, where migration issues should not be at play, the DC subsets displayed functions that parallel the results obtained in vivo. Furthermore, when the two subpopulations were loaded with free peptide in vitro and then used to induce EAE, only the CD8 α ⁺ DCs produced paralysis. This observation suggests that both naive self-reactive T cells were available and that CD8 α ⁺ DCs were able to encounter these cells and drive their activation and induction of paralysis. However, the CD8 α ⁻ DCs population seems to mediate unresponsiveness as they did not activate the T cells to produce EAE despite their ability to migrate through the circulation, encounter the T cells and mediate reversal of EAE as shown in Fig. 7. Thus, the intrinsic function of these cells most likely dictates the type of contribution that a DC subset displays in this model of peripheral tolerance. However, we cannot currently rule out that differential trafficking patterns between the two populations influence their functions.

In recent years reports have been made indicating that human immature DCs cross-presenting viral (42) or allogeneic (43) antigens induce IL-10-producing regulatory T cells that could potentially sustain peripheral tolerance. In addition, immature DCs were found to internalize apoptotic bodies generated from normal cells dying as a consequence of physiologic turnover, and transport the associated self-antigens to nearby lymph nodes populated with T

cells (10, 44). Since T cell responses to those self-antigens were undetectable it was presumed that such transport served to maintain peripheral T cell tolerance (19, 44). The contribution of DCs to peripheral tolerance, however, remains largely undefined. Lately, it has been shown that repetitive injections of TNF- α matured peptide-loaded DCs can protect against EAE presumably via induction of IL-10-producing regulatory T cells (45). Moreover, peptide loading into the endosomal compartment of DCs in the absence of inflammation also supports T cell down-regulation and likely maintains peripheral tolerance (46). The Ig-chimeras drive both peptide loading into endosomes without causing inflammation, and induce IL-10 production by APC aligning powerful factors against the self-reactive T cells. Interestingly, among the mature splenic DCs, the CD8 α ⁻ CD4⁺ subset seems to drive such tolerance as they produce IL-10 and support reversal of EAE in the Fc γ RI and III^{-/-} mice (Fig. 9). Given the small size of the CD8 α ⁻ CD4⁺ DCs population we suspect that such potency emanated from coordinated inhibitory functions. IL-10 produced by the CD8 α ⁻ CD4⁺ DCs could inhibit the function of T cells (47) directly by alteration of CD28 expression (33), and/or indirectly by downregulation of IL-12 from the CD8 α ⁺ DCs (Figs. 4 and 6). Furthermore, we believe that the CD8 α ⁻ DCs and more specifically the CD8 α ⁻ CD4⁺ subpopulation display tolerizing effects on Th1 cells as they were unable to drive IFN- γ production by the Th0 T cell clone even upon stimulation with sol Ig-PLP1, a chimera which does not induce IL-10 production (Fig. 6). The observation that CD8 α ⁻ DCs loaded with free PLP1 peptide (hence no IL-10 involvement) were unable to induce EAE, while CD8 α ⁺ DCs did, (Fig. 8) further supports the idea that CD8 α ⁻ DCs are tolerogenic against Th1 cells.

CD8 α ⁻ DCs, which proved tolerogenic against Th1 responses in this model, have previously been shown to promote the development of Th2 cells (7, 8). Thus, the question that arises is whether the same DCs display a functional plasticity and prime Th2 responses while tolerizing for Th1 immunity, or if each function is confined to a distinct subset within the CD8 α ⁻ population. The latter was confirmed for the tolerizing function as the CD8 α ⁻ CD4⁺, but not the CD8 α ⁻ CD4⁻, subset was found to produce IL-10 and reverse EAE upon treatment with agg Ig-MOG (Fig. 9). Therefore, we believe the CD8 α ⁻ CD4⁺ subset of mature splenic DCs displays intrinsic tolerizing properties complemented with the ability to produce IL-10 and play a critical role in peripheral tolerance against self-reactive T cells. Whether or not these cells represent the described previously (6, 19, 48) mature DC subset specialized in T cell tolerance is not clear at this time.

Our prior studies of disease reversal using agg Ig-PLP1 in the SJL system have not been able to detect regulatory T cells (21). Since regulatory T cells usually adopt a cycling pattern (49) and may be subject to self-limitation upon modulation of the pathogenic T cells, they may be difficult to detect subsequent to recovery from disease. If this is the case the possibility remains that CD8 α ⁻ CD4⁺ DCs are able

to induce regulatory T cells, particularly that they produce IL-10, a growth factor for regulatory T cells (34). Whatever the mechanism might be, these cells seem to be very potent, as tolerance has occurred in two different antigenic systems using two different mouse strains and the disease was reversed even in SJL mice where the autoreactive T cell repertoire encompasses an usual high frequency of self-reactive T cells (50).

Overall, these investigations have identified a DC subset that displays powerful modulatory effects on autoreactive T cells and provides a DC candidate that could sustain peripheral tolerance against self-antigens.

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