Peripheral Tolerance Induction Using Ethylenecarbodiimide-Fixed APCs Uses both Direct and Indirect Mechanisms of Antigen Presentation for Prevention of Experimental Autoimmune Encephalomyelitis

Danielle M. Turley and Stephen D. Miller

*J Immunol* 2007; 178:2212-2220; doi: 10.4049/jimmunol.178.4.2212

http://www.jimmunol.org/content/178/4/2212

References

This article cites 43 articles, 24 of which you can access for free at: http://www.jimmunol.org/content/178/4/2212.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Peripheral Tolerance Induction Using Ethylenecarbodiimide-Fixed APCs Uses both Direct and Indirect Mechanisms of Antigen Presentation for Prevention of Experimental Autoimmune Encephalomyelitis

Danielle M. Turley and Stephen D. Miller

MHC class II (MHC II)-restricted T cell responses are a common driving force of autoimmune disease. Accordingly, numerous therapeutic strategies target CD4+ T cells with the hope of attenuating autoimmune responses and restoring self-tolerance. We have previously reported that i.v. treatment with Ag-pulsed, ethylenecarbodiimide (ECDI)-fixed splenocytes (Ag-SPs) is an efficient protocol to induce Ag-specific tolerance for prevention and treatment of experimental autoimmune encephalomyelitis (EAE). Ag-SPs coupled with peptide can directly present peptide:MHC II complexes to target CD4+ T cells in the absence of costimulation to induce anergy. However, Ag-SPs coupled with whole protein also efficiently attenuates Ag-specific T cell responses suggesting the potential contribution of alternative indirect mechanisms/interactions between the Ag-SPs and target CD4+ T cells. Thus, we investigated whether MHC II compatibility was essential to the underlying mechanisms by which Ag-SP induces tolerance during autoimmune disease. Using MHC-deficient, allogeneic, and/or syngeneic donor Ag-SPs, we show that MHC compatibility between the Ag-SP donor and the host is not required for tolerance induction. Interestingly, we found that ECDI treatment induces apoptosis of the donor cell population which promotes uptake and reprocessing of donor cell peptides by host APCs resulting in the apparent MHC II-independent induction of tolerance. However, syngeneic donor cells are more efficient at inducing tolerance, suggesting that Ag-SPs induce functional Ag-SP tolerance via both direct and indirect (cross-tolerance) mechanisms leading to prevention and effective treatment of autoimmune disease. The Journal of Immunology, 2007, 178: 2212–2220.

Experimental autoimmune encephalomyelitis (EAE) is a CD4+ T cell-mediated disease of the CNS and a well established animal model of human multiple sclerosis (MS) (1). As the etiology of MS is unknown, current therapies are not Ag specific and are only partially effective, often having considerable side effects associated with long-term use as they are nonspecifically immunosuppressive. Since the CD4+ T cells that confer protection against microbial pathogens are also most often the cells mediating autoimmune disease, there is a desperate need for therapies that specifically target autoreactive CD4+ T cells in the absence of global immunosuppression.

Department of Microbiology-Immunology and the Interdepartmental Immunobiology Center, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

Received for publication September 8, 2006. Accepted for publication November 22, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by the United States Public Health Service, National Institutes of Health Grants NS-026543, NS-030871-13, and NS-048411-02, National Multiple Sclerosis Society Grant RG 3793-A-7, and by support from the Myelin Repair Foundation.

2 Address correspondence and reprint requests to Dr. Stephen D. Miller, Department of Microbiology-Immunology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611. E-mail address: s-d.miller@northwestern.edu

3 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; 7-AAD, 7-aminoactinomycin D; Ag-SP, Ag-coupled splenocyte; BM-DC, bone marrow-dendritic cell; CITIA, class II transcriptional activator; DAPI, 4',6'-diamidino-2-phenylindole; DTH, delayed-type hypersensitivity; ECDI, ethylenecarbodiimide; LN, lymph node; MBP, myelin basic protein; MHC I, MHC class I; MHC II, MHC class II; MOG, myelin oligodendrocyte protein; MS, multiple sclerosis; MSCH, mouse spinal cord homogenate; PLP, proteolipid protein; PTx, pertussis toxin; Tg, transgenic.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/82.00

www.jimmunol.org
interactions between MHC II-peptide complexes and the TCR expressed by target CD4+ T cells (14, 15). However, alternative mechanisms may contribute to the induction of functional tolerance by Ag-SP as in addition to peptides, both whole protein (e.g., proteolipid protein (PLP) and myelin basic protein) and mouse spinal cord homogenate (MSCH) also efficiently induce tolerance in CD4+ T cells and prevent EAE induced with defined encephalitogenic peptides when coupled to ECDI-fixed spleen cells (16–18). In light of the dependence on and potential of bystander costimulatory signals to bypass tolerance and result in T cell activation, a more complete understanding of the mechanism(s) by which Ag-SPs confer tolerance in host T cells is required to aid future therapeutic treatment designs.

In this study, we set out to establish whether the ECDI-fixed splenocytes serve directly as APCs (donor MHC II-restricted) for the responding T cells, and/or if there was a secondary mechanism of tolerance induction in which the Ag-SPs serve simply as an indirect Ag carrier that require uptake, reprocessing and representation by the host APCs which then interact with the host T cells to render them tolerant. While syngeneic donor Ag-SPs are more efficient in inducing unresponsiveness, we report here that donor Ag-SPs are not required to directly function as APCs to the host T cell. Splenic cells derived from both allogeneic and MHC II-deficient mice are able to function efficiently as donor carrier cell sources. ECDI appears to nonspecifically bind Ag to the donor cells while simultaneously inducing apoptosis allowing the donor coupled cells to be reprocessed leading to peptide presentation by host APCs in a “tolerogenic” fashion. The Ag specificity of coupled cell tolerance induction is maintained regardless of the donor cell source. We also demonstrate that naive bone marrow-derived dendritic cells (BM-DCs) are able to uptake coupled cells in vitro providing a mechanism of cross-tolerance involving the representation of the myelin specific Ags by host APCs to host T cells. Our findings thus support the possibility that donor Ag-coupled cells derived from syngeneic donors maintain the ability to directly interact with host T cells when using peptides. However when allogeneic donors or donor cells lacking MHC are used to induce coupled cell tolerance, reprocessing, and representation of the donor Ag-coupled cells by host APCs is the primary mechanism by which Ag-SPs render autoreactive T cells tolerogenic.

Materials and Methods

Mice

Wild-type C57BL/6 (I-Aq), B10.S (I-Aq), and BALB/c (I-Ad) female mice, 5–6 wk old, were purchased from The Jackson Laboratory. Female S1I/J (I-Aq) mice, 5–7 wk old, were purchased from Harlan Laboratories. C57Bl/6Inder/or-mice were purchased from Taconic Farms. I-Aq-Era murine donor spleens were provided by Dr. L. Ignatowicz (Medical College of Georgia, Augusta, GA). C57Bl/6Inder/or, and transgenic (Tg) mice were bred by homozygous brother-sister mating. All mice were housed under specific pathogen-free conditions (viral Ab free) in the Northwestern University Center for Comparative Medicine and maintained according to protocols approved by the Northwestern University Animal Care and Use Committee.

Peptides and reagents

Synthetic peptides MOG13–25 (MEYGWYRSFPSVRYVLYRNGK), PLP130–143 (HSLGWKLHGPDKF), and E26–48 (ASPEAQGALANIA VDKA) were purchased from Genemed Synthesis. PLP139–151 (HSLGKWLGHPDKF), and E26–48 coupled cell tolerance

Tolerance was induced by i.v. injection of chemically treated Ag-coupled splenocytes, as described previously. Briefly, spleens were removed from naive female mice, and the RBCs were lysed. The splenocytes were incubated with ECDI (150 mg/3 x 106 cells; Calbiochem) and peptide (1 mg/ml) on ice, shaking for 1 h. The coupled cells were washed, centrifuged, and filtered to remove cell clumps. The coupled cells (Ag-SPs) were resuspended at 250 x 106 cells/ml in PBS. Each mouse received 50 x 106 Ag-SPs unless otherwise indicated in 200 μl of PBS given by i.v. injection 7 days before disease induction or at peak of disease in actively induced EAE, or 2 days postadoptive transfer.

In vitro proliferation, delayed-type hypersensitivity (DTH), and ELISPOT assays

DTH and ELISPOT assays were performed as reported previously (19). For proliferation assays, draining lymph nodes (LNIs) (axillary, brachial, and inguinal) were harvested from naive mice or primed mice at indicated days following disease induction, counted, and cultured in 90-well microtiter plates at a density of 5 x 105 cells/well in a total volume of 200 μl of HL-1 medium (BioWhittaker; 1% penicillin/streptavidin and 1% glutamine). Cells were cultured at 37°C with medium alone or with different concentrations of peptide Ag for 72 h. During the last 24 h, cultures were pulsed with 1 μCi/well [3H]Tdr, and uptake was detected using a Top-count microplate scintillation counter and results are expressed as A mean of triplicate cultures.

Immunohistochemistry

Mice were anesthetized and perfused with PBS on day +30 postimmunization. Spinal cords were removed by dissection, and 2- to 3-mm lower lumbar spinal cord (approximately L2-L3) blocks were immediately frozen in OCT (Miles Laboratories) in liquid nitrogen. The blocks were stored at −80°C in plastic bags to prevent dehydration. Six-micrometer thick cross-sections from the lumbar region were cut on a cryostat microtome, collected in 0.1 M sodium cacodylat buffer, fixed in 2% paraformaldehyde at room temperature, and rehydrated in 1× PBS. Nonspecific staining was blocked using anti-CD16/CD32, (FcIII/IIR, 2.4G2; BD Pharmingen) and an avidin/biotin blocking kit (Vector Laboratories) in addition to the blocking reagent provided by the Tyramide Signal Amplification Direct kit (NEN) according to the manufacturer’s instructions. Lumbar sections from each group were thawed, air-dried, fixed in 2% parafomaldehyde and rehydrated in 1× PBS. Nonspecific staining was blocked using anti-CD16/CD32, (FcIII/IIR, 2.4G2; BD Pharmingen) and an avidin/biotin blocking kit (Vector Laboratories). Slides were examined and images were acquired using a Lica DM5000B fluorescent microscope and Advanced SPOT software. Eight serial lumbar sections from each sample per group were analyzed at ×10 magnification.

Apoptosis assays

Apoptosis was assessed by the appearance of DNA laddering, phosphatidylserine flipping, and by caspase activity. For all assays naive spleen were left untreated, ECDI fixed, fixed with 2% paraformaldehyde, and/or irradiated (3000 rad). Cells were washed in PBS and apoptotic detection assays were conducted. To measure caspase activity, the Guava Multicaspase Detection kit (Guava Technologies) was used according to the manufacturer’s instructions. A total of 5 x 106 cells/ml was plated per test. To assess DNA laddering, 105 treated cells were washed once with ice-cold PBS and resuspended in 100 μl of lysis buffer (200 mM Tris (pH 8.0), 200 mM NaCl, 10 mM EDTA, and 0.4% SDS) by gentle vortexing, and 0.1 mg/ml protease K was added. The cell lysates were incubated at 55°C for 2 h. Samples were spun briefly to pellet any further cell debris and the supernatant was collected. Phenol/chloroform extraction was done, and DNA samples were washed once with absolute ethanol, spun down once with 70% ethanol, and then resuspended in 100 μl of TE buffer (10 mM Tris (pH 8.0) and 1 mM EDTA). Four microliters of 6X DNA loading dye was mixed in the lysate (the final volume of each sample was ~40 μl). The genomic DNA was electrophoresed on a 2% agarose gel containing 0.2 mg/ml ethidium bromide at 100 V and then visualized under UV light. DNA marker used was the 1-kb DNA ladder from Invitrogen Life Technologies. Phosphatidylserine flipping was indicated by positive staining for...
annexin V, which is a Ca$^{2+}$-dependent phospholipid-binding protein (30–36 kDa) that has a high affinity for phosphatidylserine (Kd, 5 × 10^{-5}). Fluorescein-bound annexin V (BD Pharmingen) serves as a fluorescence probe for apoptotic cells by binding to cells with exposed phosphatidylserine. The staining was performed according to manufacturer’s instructions on freshly isolated spleen cells, ECDI-fixed cells, 2% paraformaldehyde-fixed cells, and cells that had been irradiated (3000 rad). 7-Aminoactinomycin D (7-AAD; BD Pharmingen), a nucleic acid dye, was also added to detect nonviable cells. Apoptosis was quantified by flow cytometric analysis of annexin V-positive staining and 7-AAD exclusion.

**Phagocytosis of apoptotic cells**

BM-DCs were generated by removing BM from femurs of naive SJL/J mice. Briefly, femur bones were removed from mice, and BM was flushed out with HBSS. Cells were spun down, RBCs were lysed with Tris ammonium chloride lysis buffer (Tris-NaCl), pelleted, and then washed twice in HBSS and then resuspended in RPMI 1640 medium containing 10% FBS, 1 mM glutamine, 1% penicillin-streptomycin, 1 mM nonessential amino acids. The staining was performed according to manufacturer’s instructions on freshly isolated spleen cells, ECDI-fixed cells, 2% paraformaldehyde-fixed cells, and cells that had been irradiated (3000 rad). 7-Aminoactinomycin D (7-AAD; BD Pharmingen), a nucleic acid dye, was also added to detect nonviable cells. Apoptosis was quantified by flow cytometric analysis of annexin V-positive staining and 7-AAD exclusion (data not shown).

**Results**

**Cognate interactions between Ag-SPs and target CD4$^+$ T cells are not critical to successful tolerance induction**

Since peptides, whole protein and MSCH all serve as efficient sources of Ag for Ag-SP-induced tolerance for protection from peptide-induced EAE, we first investigated the requirement of cognate interactions between the target and donor cells during Ag-SP tolerance. The efficiency of donor cells isolated from mice of various MHC haplotypes, as well as from mice lacking MHC class I (MHC I) and/or MHC II molecules, in mediating Ag-SP-induced tolerance was tested. We first examined the ability of donor Ag-SP lacking self MHC II to induce tolerance in recipient mice by treating naive wild-type C57BL/6 (B6) mice with PLP178–191/coupled cells (PLP178–191-SP), on day 7 before active EAE induction with PLP178–191/CFA on day 0.
Interestingly, each group of donor cells conferred a significant level of tolerance against Ag-specific autoreactive T cell activation and effector function regardless of the MHC haplotype or level of MHC II expression on donor SP cells as measured by clinical disease severity (Fig. 1A). Ag-specific in vivo DTH (Fig. 1B) and in vitro proliferation (Fig. 1C). Peptide-specific Th1 responses, as measured by the frequency of PLP\textsubscript{178–191}-specific IFN-\(\gamma\)-producing cells (Fig. 1D), were also significantly reduced in PLP\textsubscript{178–191}-SP-treated mice compared with Sham-SP controls. Interestingly, cells deficient for both MHC I and II expression retained the ability to successfully serve as donor cells during Ag-SP tolerance, but required ~2-fold the number of donor cells to induce a state of tolerance equivalent to that of MHC-intact control treatments. In agreement with protection from clinical disease progression, tolerance with each group of donor cells also significantly decreased the extent of CD4\(^+\) T cell CNS infiltration (Fig. 2).

We previously established that syngeneic Ag-SP-tolerance induction is dependent on the quantity of Ag-SP given to recipients (18) and increasing the number of donor cells appears to compensate for the lack of MHC II expression on donor cells during Ag-SP-induced tolerance (Fig. 1). To test the dose dependence of tolerance induced with allogeneic coupled splenocytes, we titrated the number of donor Ag-SP given to recipients (Fig. 3). Naive SJL mice were tolerized with an increasing number of PLP\textsubscript{139–151}-SP derived from either syngeneic SJL donors or allogeneic BALB/c donors in comparison to OVA\textsubscript{323–339}-SP (sham) controls. Recipient mice were then primed on day 0 with 50 \(\mu\)g of PLP\textsubscript{139–151}/CFA and received PTx on days 0 and +2. Recall responses were determined by DTH to PLP\textsubscript{178–191} challenge on day +10. Asterisks denote a significant reduction in DTH responses (\(*, p < 0.0001; \bullet, p < 0.0005\)) as compared with Sham-SP controls.
Ag-SPs. A similar dose dependency was found in naive B6 recipients tolerized with donor cells derived from syngeneic B6 or allogeneic SJL donors (Fig. 3B).

Taken together, the efficacy of Ag-SP-mediated tolerance appears to be directly related to the number of donor Ag-SP cells. Since cognate interactions cannot occur between donor Ag-SPs of mismatched MHC haplotypes or donor Ag-SPs lacking MHC I and II expression, these data suggest that MHC II restriction and cognate interactions are not an absolute requirement of Ag-SP-induced tolerance establishing the possibility that the Ag-SP cells may simply act as tolerogenic Ag carriers. Under this scenario, it would seem likely that host APCs may play a critical role in underlying mechanisms of Ag-SP tolerance potentially via reprocessing and representation of Ag delivered by Ag-SPs.

Allogeneic Ag-SPs ameliorate both adoptively induced and ongoing clinical disease

Since the etiology of MS is currently unknown, potential therapeutic interventions must be able to efficiently treat established clinical disease. Therefore, we next tested the ability of allogeneic Ag-SP cells to inhibit the clinical progression of EAE at times following T cell activation. This was accomplished by injecting Ag-SP cells either two days following disease induction via adoptive transfer of previously activated PLP\textsubscript{139–151}-specific T cells (Fig. 4, A and B) or at a time corresponding with peak clinical disease in the active peptide-induced model of EAE (Fig. 4, C and D). Importantly, injection of either syngeneic SJL PLP\textsubscript{139–151}-SP or allogeneic B6 PLP\textsubscript{139–151}-SP significantly attenuated the clinical progression of EAE in SJL mice in both model systems (Fig. 4, A and C). In agreement with these findings, SJL-SP and B6-SP treatment also significantly decreased in vivo Ag-specific T cell effector DTH responses (Fig. 4, B and D). Taken together, these data demonstrate that both syngeneic and allogeneic Ag-SPs efficiently protect against ongoing clinical disease supporting the potential clinical application of this treatment and obviating the need for autologous donor cells.

We have previously reported that i.v. injection of Ag-SP cell induces a state of functional tolerance (16), and it was assumed...
that Ag-SP-induced tolerance was dependent upon cognate inter-
actions between APCs and target CD4<sup>+</sup> T cells. However, in light
of our current findings demonstrating the efficacy of allogeneic
Ag-SPs in protecting against clinical disease progression, it is im-
portant to revisit the specificity of the treatment to ensure that
allogeneic Ag-SP treatment does not induce a state of general im-
une suppression. To revisit this issue, naive SJL mice were toler-
ized on day −7 with PLP<sub>139–151</sub> or OVA<sub>323–339</sub> coupled Ag-SP
derived from either syngeneic SJL or allogeneic B6 donor mice.
Mice were then primed with a combination of PLP<sub>139–151</sub> and
OVA<sub>323–339</sub> emulsified in CFA. On day +7, DTH responses were elicited to 10
μg of E<sub>62–68</sub> and PLP<sub>178–191</sub> in the right and left ears, respectively. Re-
sults shown are the mean ear swelling of five mice per group ± SEM.
Asterisks indicate a significant difference in DTH responses as compared
with internal Sham controls (*, p < 0.05; **, p < 0.005; ***, p < 0.0005).

![Graph](http://www.jimmunol.org)
these findings reinforce the conclusion that Ag-SP treatment induces a specific state of functional tolerance in the absence of both general immune suppression and the requirement for direct cognate interactions between the donor and target cell populations.

**Donor Ag-SPs can interact with host T cells and be represented by host APCs**

A possible explanation for the ability for Ag-SP treatment to induce specific tolerance in the absence of cognate interactions is that the peptide may be shed from the surface of Ag-SPs at times following i.v. injection and be reprocessed and presented by host tolerogenic APCs. To test this, we used donor cells isolated from I-A<sup>B</sup>-E<sub>Ae</sub> Tg (YAe) mice in which 100% of the MHC II contain covalently linked E<sub>α52–68</sub> peptide (22), removing the possibility of peptide shedding and representation by host APC. Splenocytes isolated from either B6 or YAe mice were ECDI coupled with PLP<sub>178–191</sub> (B6-178, YAe-178) or E<sub>α52–68</sub> peptide (B6-Eae) peptide and injected into naive B6 recipients. Seven days later, recipient mice were immunized with a combination of 200 μg of PLP<sub>178–191</sub> and 200 of μg E<sub>α52–56</sub> in CFA, and the degree of Ag-specific tolerance was assessed by DTH (Fig. 6). As expected, PLP<sub>178–191</sub>-specific responses were significantly attenuated in B6-178-treated mice, but E<sub>α52–56</sub>-specific responses were unaffected by the treatment. Importantly, B6-Eae-treated mice displayed attenuated E<sub>α52–56</sub>-specific responses, but normal PLP<sub>178–191</sub>-specific responses and mice treated with YAe-178 Ag-SP cells displayed reduced responses to both PLP<sub>178–191</sub> and E<sub>α52–68</sub>. These findings demonstrate that Ag-SP treatment successfully induces functional tolerance under conditions in which peptide is either covalently linked to or excluded from the MHC binding groove, suggesting the potential contribution of at least two distinct mechanisms to Ag-SP-induced tolerance.

**ECDI fixation induces apoptosis in donor cells**

To further investigate the independence of Ag-SP tolerance on cognate interactions between the donor and target cell populations, we next tested the hypothesis that ECDI-mediated fixation induced apoptosis of the donor cells, which would facilitate the ability of host APCs to reprocess and represent Ag in a nonimmunogenic manner (i.e., indirect or cross-tolerance). Other groups have previously demonstrated that ECDI-treated BM-DCs up-regulate annexin V 24 h post-ECDI treatment (23). To test this in our model system, which uses unsorted spleen cells to induce tolerance, we measured caspase activity, DNA fragmentation, and annexin V staining in donor cells at various times following fixation by a number of techniques. Naive SJL splenocytes were either ECDI-fixed, irradiated, or treated with 2% paraformaldehyde for 1 h and then incubated at 37°C for 0, 4, 8, or 10 h before analysis. All treatments induced a significant level of apoptosis by 4 h as determined by intracellular caspase activity (Fig. 7, A–C) and ECDI fixation also significantly unregulated the level of annexin V expression with the same kinetics (Fig. 7, D–F). Similar annexin V expression was observed in irradiated cells but not in untreated cells (data not shown). DNA fragmentation analysis confirmed these findings as no laddering was observed in the paraformaldehyde-treated (Fig. 7G, lanes 2–4) group at any of the time points; however, fragmentation was observed in both the irradiated cells (Fig. 7G, lanes 5–7) and the ECDI-treated cells (Fig. 7G, lanes 8–10) at 4 and 10 h after treatment. Taken together, these data demonstrate that ECDI fixation actively induces apoptosis in donor splenocytes shortly after treatment.

**Uptake of ECDI-Ag-SPs by BM-DCs in vitro**

In light of our findings that ECDI-fixation induces apoptosis of the Ag-SP cells, we next examined whether host APCs were capable of phagocytizing the Ag-SP cells as a potential mechanism of representation of the peptide Ag for induction of cross-tolerance. Naive BM-DCs were labeled with PKH67 membrane dye and incubated for 2 h with PKH26-labeled Ag-SPs at either 37 or 4°C. Cells were then examined by flow cytometry to assess uptake of Ag-SP by BM-DCs. Approximately 44% of the BM-DCs were able to internalize apoptotic ECDI-Ag-SP after incubation at 37°C (Fig. 8A); however, this internalization was decreased to only 4% at 4°C. Cytospin preparations of the BM-DC and Ag-SP confirmed internalization of the Ag-SPs by the BM-DCs after incubation for 2 h at 37°C (Fig. 8B). Incubation at 4°C or with the addition of cytochalasin D (CytoD) or incubation at 4°C. Cytospins were counterstained with DAPI (blue).

**Discussion**

We and others (5, 13) have previously demonstrated the exquisite ability of ECDI Ag-coupled (Ag-SP) cells to restore self tolerance and prevent disease progression during EAE. Not surprisingly, co-stimulatory signals appear to play a dogmatic role in the regulation of Ag-SP tolerance induction. For example, low B7 expression on the donor-coupled cells is necessary for tolerance induction, while
CTL-A-4 is important for the maintenance phase of Ag-SP tolerance upon secondary encounter with Ag on stimulatory APCs expressing up-regulated levels of costimulatory molecules (14, 15, 24). Also, we have demonstrated that a chemical fixative, such as ECDI, is required during the coupling process (24, 25). Taken together, these observations support the idea that i.v. injection Ag-SP induces a state of Ag-specific immune tolerance via the delivery of signal one (TCR cross-linking) in the absence of signal two (costimulation) following direct interactions between the injected Ag-SPs and target CD4+ T cells (4, 5, 26). However, in light of inability of the splenic APCs to process Ag during the coupling procedure which is conducted for 60 min on ice, the above proposed mechanism fails to explain the ability of splenocytes coupled with whole protein Ags to induce self-tolerance to peptide epitopes during Ag-SP treatment.

In this study, we set out to better understand the mechanisms by which injection of Ag-SP cells restores a state of self-tolerance during autoimmune disease. It is well established that cognate interactions between APCs and CD4+ T cells requires recognition in the context of MHC II (27). Interestingly, both peptide (PLP139-151) and whole protein (PLP or MSCH) successfully induce tolerance in the SJL model of EAE following ECDI-coupling to spleen cells (5). Thus, we first investigated whether MHC II compatibility between the tolerogenic Ag-SP and the host was necessary for successful tolerance induction. Importantly, allogeneic and syngeneic donor-derived spleen cells appear to be roughly equivalent in their ability to induce Ag-specific tolerance as measured by EAE progression, DTH, and in vitro T cell assays (Fig. 1). There was also decreased infiltration of inflammatory cells (Fig. 2) in the CNS of tolerized mice regardless of donor Ag-SP origin. These findings suggest the potential contribution/existence of an indirect mechanism of tolerance that does not require cognate interactions between APCs and the target CD4+ T cells and are supported by another recent study (28). Underscoring this potential alternative mechanism was the finding that donor cells isolated from Aβ/β2M mice, deficient for both MHC I and II, retained the ability to induce tolerance (Fig. 1, A and B).

One plausible explanation for the lack of requirement for MHC II on the Ag-SPs for successful tolerance induction is peptide may either simply detach from the donor cells or is enzymatically cleaved by host proteases upon i.v. injection of the coupled cells both allowing peptide to be exchanged between donor and host APCs. To test this hypothesis, we isolated donor cells from I-Aα-Eα Tg mouse (YAe), which has the Eo52-68 peptide covalently bound into the MHC II (22). As expected, the I-Aα-Eα splenocytes coupled to PLP178-191 were able to tolerate the recipient mice to both Eo52-68 and PLP178-191 (Fig. 6). This tolerance was as effective, although not as robust, as tolerizing to the individual Ags alone; however, it still significantly inhibited DTH recall responses compared with sham-tolerized controls. Collectively, these data thus support the potential contribution of two distinct mechanisms to Ag-SP tolerance, direct tolerance between the donor Ag-SP and host T cell, and indirect “cross-tolerance” that requires reprocessing and representation of the Ag by host APCs.

DCs are important regulators of tolerance and immunity; however, relatively little is known about the mechanisms used to maintain this balance. Naïve immature DCs have been shown to be able to phagocytize apoptotic cells and obtain antigenic peptides for cross-presentation by both MHC I and II (29). Much has been studied about the ability of immature DCs to phagocytize apoptotic cells and cross-present Ag to MHC I-restricted CD8+ T cells to induce tolerance (30–33); however, less is known about cross-presentation to CD4+ T cells (34, 35). Since ECDI has been shown to induce apoptosis in cultured DCs used to prolong organ graft survival (23), we also looked to see whether ECDI induced apoptosis in the donor spleen cells in our tolerance model system to encourage host APCs to reprocess Ag from the injected Ag-SP population. As expected, ECDI treatment induced a significant level of apoptosis within 4 h in donor cells (Fig. 7). As immature DCs are known to phagocytize apoptotic cells (29), we next tested the ability of naïve BM-DCs to uptake the apoptotic Ag-SP donor cells in vitro. BM-DCs were found to successfully internalize apoptotic Ag-SPs (Fig. 8), further supporting the potential contribution of host cell reprocessing of Ag-SPs to induce indirect tolerance. Our preliminary results indicate that labeled Ag-SPs are also phagocytized by a splenic APC subpopulation following i.v. injection. We are currently performing experiments to uncover the identity of the in vivo resident APC population capable of reprocessing allogeneic Ag-SPs, and the functional ability of these cells to induce tolerance upon adoptive transfer is currently being investigated. This is especially interesting as we previously reported that only i.v., but not i.p. or s.c., administration of Ag-SP inhibited the induction of relapsing-remitting EAE (18), suggesting that uptake of the Ag-SP by a resident splenic APC population possibly requires immature DCs for successful tolerance.

One hallmark of the ECDI-Ag-coupled-cell tolerance regimen is its exquisite Ag specificity when used in both preventative and treatment modes in relapsing-remitting EAE (5, 12, 13, 36). In light of the potential contribution of indirect tolerance to Ag-SP in our model system, we revisited the specificity of Ag-SP tolerance under conditions favoring indirect tolerance. Similar to previous investigations of direct tolerance, Ag-SP treatment exhibits Ag specificity regardless of the syngeneic or allogeneic nature of the donor carrier cells (Fig. 5). Our current studies also indicate multiple peptides can be coupled to the donor cells to simultaneously induce tolerance to multiple encephalitogenic epitopes (C. Smith and S. Miller, submitted for publication).

In the case of EAE, Ag-SP tolerance can be used to successfully target the primary immunogenic peptide, as well as newly emerging epitopes involved in epitope spreading (37). Given that immunodominant myelin epitopes have been identified in MS patients (38), this further supports the therapeutic potential of Ag-SPs for treatment of human autoimmune disease. Coupled-cell tolerance has shown to be nontoxic and well tolerated by treated animals at all stages of disease and more effective than oral or i.v. tolerance with soluble peptide (12, 39). Unlike soluble i.v. tolerance induction in which the tolerizing Ag can induce an anaphylactic response resulting in death of treated mice (40), Ag-SP tolerance did not induce an allergic response regardless of the Ag used or the time of treatment (39) demonstrating its effectiveness at both preventing and treating ongoing disease (40, 41). The potential dangers in therapies using soluble peptides were illustrated in a recent clinical trial using a myelin basic protein85-99 altered peptide that were terminated due to systemic hypersensitivity reactions (42). As the etiology of MS is unknown, there is currently no way to predict the identity of the initiating autoepitope or other specificities that may be activated following myelin destruction. Current therapies for autoimmune disease are primarily anti-inflammatory and non-Ag specific in design. Glatiramer acetate, a random polymer of four amino acids that mimics myelin proteins, is the only approved MS therapy that acts in a semi-Ag-specific manner but is only beneficial to a minority of MS patients (43). Thus, tolerance induced with Ag-SP coupled with a mixture of encephalitogenic myelin epitopes may provide a safe and Ag-specific treatment option for treatment of MS and other autoimmune diseases with known dominant immunogenic epitopes.

Collectively, the current findings support the conclusion that the ECDI-Ag-coupled cells may induce Ag-specific tolerance via two
distinct mechanisms. Donor cells derived from a syngeneic source may induce direct tolerance/energy in host T cells. However, since ECDI fixation induces apoptosis, the donor cells may also serve as Ag carriers to be reprocessed and represented by host APCs in a tolerogenic fashion to host T cells, resulting in indirect or cross-tolerance. It is very likely that these two mechanisms of Ag-specific tolerance are not mutually exclusive and may both contribute to the therapeutic mechanisms of self-tolerance restoration. Given our results, the use of Ag-SP tolerance is an attractive potential specific therapy for the treatment of human MS using either autologous or heterologous donor carrier cells.

Acknowledgment
We thank Adam Kohm for critical reading of the manuscript preparation and for technical assistance.

Disclosures
The authors have no financial conflict of interest.

References