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Oligodendrocytes Enforce Immune Tolerance of the Uninfected Brain by Purging the Peripheral Repertoire of Autoreactive CD8$^+$ T Cells

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SUMMARY

Myelin-specific CD8$^+$ T cells are thought to contribute to the pathogenesis of multiple sclerosis. Here we modeled this contribution in mice with CD8$^+$ T cells recognizing ovalbumin (OVA) expressed in oligodendrocytes (ODCs). Surprisingly, even very high numbers of activated OVA-reactive CD8$^+$ T cells failed to induce disease and were cleared from the immune system after antigen encounter in the central nervous system (CNS). Peripheral infection with OVA-expressing *Listeria* (Lm-OVA) enabled CD8$^+$ T cells to enter the CNS, leading to the deletion of OVA-specific clones after OVA recognition on ODCs. In contrast, intracerebral infection allowed OVA-reactive CD8$^+$ T cells to cause demyelinating disease. Thus, in response to infection, CD8$^+$ T cells also patrol the CNS. If the CNS itself is infected, they destroy ODCs upon cognate antigen recognition in pursuit of pathogen eradication. In the sterile brain, however, antigen recognition on ODCs results in their deletion, thereby maintaining tolerance.

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease characterized by accumulation of lymphocytes and macrophages in the central nervous system (CNS), with localized inflammation and demyelination of axons. Depending on the subtype of MS, multiple immune cells are detected in the lesions, including CD4$^+$ and CD8$^+$ T cells (Brück et al., 2002). A pathogenic role of the latter is supported by several observations: acute and chronic lesions of MS patients contain more CD8$^+$ than CD4$^+$ T cells (Crawford et al., 2004; Tsuchida et al., 1994), expanded myelin-reactive cytotoxic T lymphocyte (CTL) clones are found in the CNS and the periphery of MS patients (Babbe et al., 2000), and depletion of CD4$^+$ T cells in MS patients fails to improve relapse rates whereas global T cell depletion leads to their reduction (Coles et al., 1999; Hohlfeld and Wiendl, 2001).

In the CNS, antigen recognition by CD8$^+$ T cells is hampered by low expression of major histocompatibility complex (MHC) class I antigens on neurons, oligodendrocytes (ODCs), and astrocytes, suggesting microglia and resident dendritic cells (DCs) as prime targets for antigen recognition by CD8$^+$ T cells. However, MHC I expression is upregulated by inflammation, and ODC-derived myelin antigens have indeed been identified as targets for autoreactive CD8$^+$ T cells in both humans (Jurewicz et al., 1998) and mice (Saxena et al., 2008). Accordingly, the clonal CD8$^+$ T cell expansions in MS are likely to reflect myelin-specific autoimmunity.

In experimental autoimmune encephalomyelitis (EAE), a group of animal models for MS, disease is classically induced by peripheral immunization with myelin antigens in proinflammatory adjuvants or by transfer of encephalitogenic CD4$^+$ T cells (Gold et al., 2006). In recent years, however, it has been attempted to also recapitulate the CD8$^+$ T cell component of the disease in several mouse models: MHC class I-restricted T cell receptor (TCR)-transgenic mice with specificity for myelin autoantigens have been developed (Friese et al., 2008; Huseby et al., 2001; Ji et al., 2010), and model antigens to which well-characterized TCR-transgenic mouse lines are available have been expressed in the CNS (Na et al., 2008; Saxena et al., 2008). In adoptive transfer experiments analogous to those previously performed with encephalitogenic CD4$^+$ T cells, however, it has been extremely difficult to induce clinically apparent disease. For example, transfer of very high numbers (3×10^7, corresponding to about 10% of the endogenous CD8$^+$ T cell population) of in vitro preactivated hemagglutinin (HA)-specific CTLs induces EAE in less than half of recipients expressing HA in ODCs (Saxena et al., 2008). In another model employing TCR-transgenic myelin basic protein (MBP)-specific CTLs, disease induction requires transfer of 2×10^7 preactivated cells, conditioning by irradiation, and treatment with interleukin-2 (IL-2) (Huseby et al., 2001). In contrast, as few as 10^6 and 10^5 MBP-specific cloned CD4$^+$ T cells suffice to induce EAE in rats (Ben-Nun et al., 1981) and mice (Zamvil et al., 1985), respectively, indicating that the pathogenic potency of activated autoreactive CD4$^+$ T cells exceeds that of their CTL counterparts by more than two orders of magnitude.

As a second option to providing overwhelming numbers of autoreactive CD8$^+$ T cells, TCR transgenic mouse lines have been generated in which all or most CD8$^+$ T cells are specific for a natural or artificial myelin antigen. Depending on central tolerance induction and a requirement for peripheral priming, disease is observed in some (Friese et al., 2008; Ji et al., 2010; Na et al., 2008) but not in other (Perchellet et al., 2004; Saxena

et al., 2008) settings. Thus, in spite of the evidence supporting a role for $CD8^+$ T cells in human MS, experimental induction of disease with autoreactive $CD8^+$ T cells in mice requires a $CD8^+$ T cell assault of a magnitude that can hardly be considered within the range of physiology.

In our own studies, we have generated mice that express ovalbumin (OVA) under the proximal MBP promoter (ODC-OVA mice) leading to sequestration of this model antigen in the cytosol of ODCs (Cao et al., 2006). When crossed to OT-I mice expressing a transgenic TCR recognizing the OVA-derived SIINFEKL peptide presented by H-$2K^b$, ODC-OVA mice spontaneously develop lethal EAE at 2 weeks of age; in contrast, OVA-specific TCR-transgenic $CD4^+$ T cells developing in ODC-OVA mice remain ignorant of the sequestered autoantigen (Cao et al., 2006; Na et al., 2008).

To study the effect of peripheral induction of ODC-specific autoimmunity in mice with a normal T cell repertoire, we have now infected ODC-OVA mice with OVA-expressing *Listeria monocytogenes* (Lm-OVA), which elicits a strong $CD8^+$ T cell response to the SIINFEKL peptide. Unexpectedly, we observed resistance to EAE induction that was mediated by clonal deletion of autoreactive $CD8^+$ T cells from the entire immune system. In contrast, infection of the CNS itself rendered the OVA-expressing ODCs susceptible to destruction by OVA-specific CTLs, indicating that the decision between effective immune surveillance versus tolerance induction is made by the perception of infection in the brain itself.

RESULTS

Lack of OVA-Specific $CD8^+$ T Cell Response in Lm-OVA-Infected ODC-OVA Mice

To induce a $CD8^+$ T cell-mediated autoimmune response against ODCs by peripheral immunization, ODC-OVA mice, in which ODCs express cytosolic OVA, were infected with Lm-OVA. However, EAE symptoms, T cell infiltration, or inflammation of the CNS were not observed either during primary or during secondary infection (data not shown).

In WT mice, Lm-OVA infection elicits massive expansion of SIINFEKL-specific $CD8^+$ T cells, which can make up more than 30% of the $CD8^+$ T cell compartment at the peak of the response (Figure 1A; Pope et al., 2001). This response was almost completely absent in Lm-OVA-infected ODC-OVA mice, indicating that a deletional mechanism acting either during T cell development or during the immune response itself protected the mice from autoimmune attack (Figure 1A).

ODC-OVA Mice Lack OVA-K^b-Reactive $CD8^+$ T Cells

To clarify whether OVA-reactive $CD8^+$ T cells were deleted before or after Lm-OVA infection, 2×10^8 WT or ODC-OVA lymph node (LN) cells were transferred into *Rag1*$^{-/-}$ mice, which were then infected with Lm-OVA. Mice that had received LN cells from ODC-OVA donors showed hardly any OVA-specific $CD8^+$ T cell response, whereas mice reconstituted with WT cells responded well (Figure 1B), indicating that OVA-reactive $CD8^+$ T cells were deleted in ODC-OVA mice already before Lm-OVA infection.

To confirm this without the need to induce a response to OVA, we crossed ODC-OVA mice with "Vβ5 mice" that express only the β chain of the OT-I TCR and therefore have a detectable population of K^b-OVA-reactive $CD8^+$ T cells generated by pairing of polyclonal TCRα chains with the transgenic β chain (Dillon et al., 1994). Vβ5 ODC-OVA mice remained healthy and without CNS infiltrates (not shown). When their $CD8^+$ T cell repertoire was compared to that of Vβ5 single transgenic mice, we observed only a small difference in the frequency of K^b-OVA-reactive $CD8^+$ T cells by using K^b-OVA-multimer staining, which detects high- and low-avidity cells (Figure 1C). However, comparison of the functional avidity of K^b-OVA-reactive $CD8^+$ T cells from single and double transgenic mice revealed that high-avidity OVA-reactive T cells had been completely deleted in ODC-OVA mice (Figure 1D).

Deletion of OVA-Reactive $CD8^+$ T Cells Is Not Mediated by Thymic or Peripheral Stroma Cells or by Hematopoietic Cells

Because purging of autoreactive $CD8^+$ T cells from the T cell repertoire can be mediated by radioresistant cells both during thymic maturation and in the periphery, we tested whether OVA-specific $CD8^+$ T cells developing in irradiated ODC-OVA mice would also be deleted. Adult mice, in which T cells are excluded from the CNS in the absence of immune activation, were chosen as recipients. Bone marrow cells from WT and OT-I Thy1.1 mice were transplanted at a 4:1 ratio into irradiated WT or ODC-OVA mice, and T cell reconstitution from both inocula was analyzed 6 weeks later. Representation of mature OT-I cells in the $CD8^+$ T cell repertoire was identical in WT and ODC-OVA recipients (Figure 1E), and their response (proliferation and interferon-γ [IFN-γ] secretion) to the SIINFEKL peptide was unaffected by the host (WT or ODC-OVA) in which they had matured (Figure 1F). In keeping with our previous biochemical results (Na et al., 2008), this indicates that the neo-self antigen OVA is expressed neither in the thymus nor in the periphery of ODC-OVA mice at an amount that would be detectable by the high-affinity TCR of OT-I cells.

To extend our survey to the hematopoietic system, we also repopulated WT mice with mixed bone marrow from OT-I and ODC-OVA mice (1:4 ratio). The presence of the ODC-OVA transgene in the bone marrow had no effect on the development of OT-I cells in the same host (Figure 1E). Thus in ODC-OVA mice, hematopoietic cells are also not responsible for the deletion of OVA-reactive cells.

Perinatal Access to the CNS Allows Deletion of Autoreactive $CD8^+$ T Cells

Next, we considered perinatal access to ODCs in the CNS as an opportunity for encounter with self-antigen leading to purging of the repertoire. Previously, we had observed OT-I ODC interactions in the brain of postnatal OT-I ODC-OVA double transgenic mice undergoing EAE (Na et al., 2008), demonstrating the occurrence of such interactions. When carboxy fluorescein diaceteate succinimidyl ester (CFSE)-labeled OT-I T cells were transferred into 10-day-old ODC-OVA mice, they were expanded on day 5 and then deleted from the cervical LN (Figure 2A) and spleen (not shown) on day 12 after transfer, indicating that they had contacted OVA-presenting cells. Indeed, OVA expression was readily observed in the developing brain as early as day 3 of life (Figure S1 available online). In contrast, OT-I T cells transferred into adult ODC-OVA mice were neither expanded nor

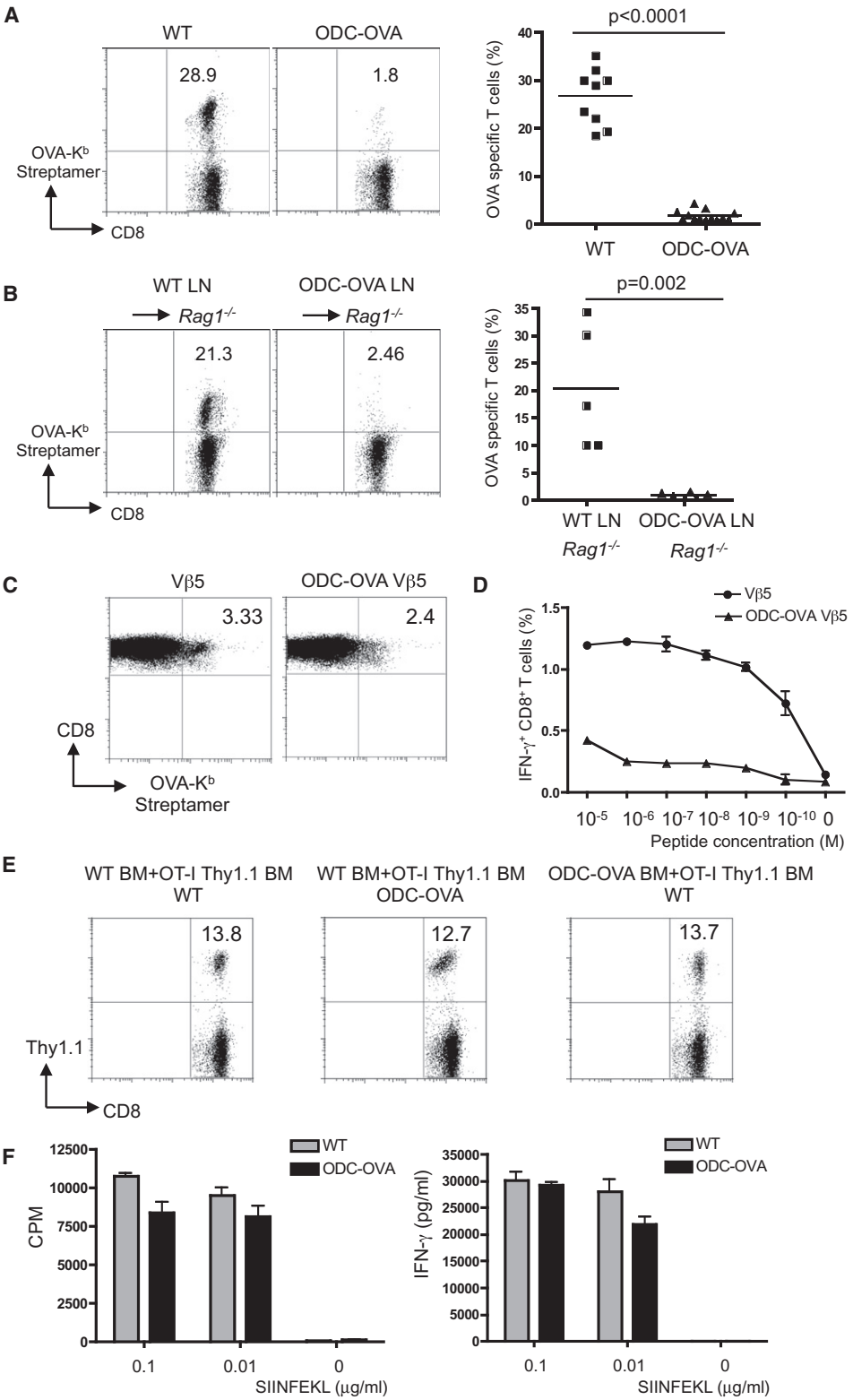

Figure 1. Defective OVA-Specific CD8+ T Cell Response in ODC-OVA Mice

(A) OVA-reactive T cells after Lm-OVA infection. WT or ODC-OVA tg mice were infected with 5,000 cfu Lm-OVA, challenged 3 weeks later with 10^5 cfu, and analyzed on day 6. OVA-K^b $CD8^+$ T cells were determined by streptamer staining.

(B) Lack of OVA-K^b-specific $CD8^+$ T cell precursors in LN cells from ODC-OVA mice. 2×10^8 total LN cells from WT or ODC-OVA mice were transferred into *Rag1*$^{-/-}$ mice. Spleens from recipient mice were analyzed 10 days after infection with 10^5 cfu Lm-OVA.

deleted (Figure 2B). These data indicate that before the blood brain barrier (BBB) closes, naive $CD8^+$ T cells are able to contact antigen-expressing ODCs in the CNS, leading to their activation and deletion. Once the BBB is established, however, naive $CD8^+$ T cells have no access to the CNS and hence remain ignorant of the autoantigen OVA.

Our finding that transfer of OT-I T cells into young ODC-OVA mice leads to their deletion rather than disease induction is seemingly at odds with the aggressive EAE observed in OT-I ODC-OVA double transgenic animals. Accordingly, we attempted to approach this extreme situation by increasing the number of transferred OT-I cells per 7-day-old mouse from the 10^7 presently used to 5×10^7. Surprisingly, after 18 days, only 1 in 8 recipient ODC-OVA mice developed EAE whereas the remaining mice remained healthy and effectively deleted the transferred cells (Figures S2A and S2B). Histologic analyses revealed extensive myelin damage in the diseased mouse whereas mice that had successfully removed the autoreactive $CD8^+$ T cells showed normal brain architecture (Figure S2C). Thus, overwhelming numbers of $CD8^+$ T cells (at least 5×10^7) specific for a cytosolic ODC antigen are required to cause serious damage even in a young mouse; below that threshold, they are deleted after initial clonal expansion.

Peripheral Activation of ODC-Reactive $CD8^+$ T Cells Provides Antigen Access in Adult Mice, Leading to Their Inactivation and Deletion

T cells specific for myelin antigens form part of the normal repertoire in rodents and humans (Martin et al., 1990; Ota et al., 1990; Pette et al., 1990; Schluesener and Wekerle, 1985). This situation is modeled by the persistence of transferred naive OT-I cells in the periphery of adult ODC-OVA mice. To investigate whether opening of the BBB by immunization under inflammatory conditions would provide access to the CNS and cause disease, we infected adult ODC-OVA and WT mice, which had received 10^7 OT-I cells 1 day earlier, with Lm-OVA. In both the spleen (not shown) and cervical LN (Figure 2C) of WT and ODC-OVA recipient mice, robust clonal expansion was observed on day 5 postinfection (p.i.). However, in ODC-OVA mice, this expanded population of OT-I cells showed impaired in vitro proliferation and IFN-γ secretion in response to the cognate peptide as compared to the OT-I cells reisolated from WT recipients (Figure S3). Furthermore, although the expanded population of OT-I cells persisted on day 10 p.i. in WT mice, it had been almost fully eliminated in mice expressing OVA in the CNS (Figures 2C and 3A). The disappearance of OT-I cells from the periphery was not due their sequestration as viable cells in the CNS (which did not exceed 1% of the inocula) (Figure S4), suggesting their clonal deletion as a result of antigen encounter.

In order to extend this observation to OVA-reactive $CD8^+$ T cells from a normal repertoire, WT LN cells were transferred into *Rag*-deficient mice with or without the ODC-OVA transgene and were then infected with Lm-OVA. As shown in Figure 2D, the robust SIINFEKL-K^b-specific $CD8^+$ T cell response seen in nontransgenic recipients was abolished by the ODC-OVA transgene, indicating purging of the normal OVA-specific repertoire during the response to Lm-OVA.

T cells may be enabled to cross the BBB either after specific activation or as a result of local or systemic inflammation. Because Lm-OVA infection leads to both systemic inflammation and specific activation of OVA-reactive $CD8^+$ T cells, we tried to separate these factors by infecting adult mice with a CFSE-labeled inocula of OT-I cells with either Lm or Lm-OVA. As expected, infection with Lm-OVA, but not with Lm, led to pronounced clonal expansion of OT-I cells in WT mice (Figure 3A). In ODC-OVA recipients, however, Lm infection sufficed to induce cell division followed by deletion (Figure 3A). Thus, inflammation-induced access to antigen resulted in proliferation of antigen-specific peripheral naive $CD8^+$ T cells, followed by their removal from the immune system.

To evaluate the effects of $CD8^+$ T cell activation itself on the ability to reach the target antigen in the CNS, OT-I cells were activated in vitro before transfer into adult recipients, and their presence in cervical LN was monitored. In ODC-OVA mice, the transferred activated OT-I cells were deleted, whereas they declined only moderately in WT recipients, as is expected during clonal contraction (Figure 3B). Note that as compared to the deletion of naive peripheral $CD8^+$ T cells stimulated by Lm-OVA (Figure 2C), deletion occurred faster, presumably because the phase of clonal expansion and sensitization to deletion had occurred already in vitro. These data suggest that activated $CD8^+$ T cells can cross the BBB and interact with antigens expressed by ODCs, leading to their removal from the peripheral repertoire.

Transplantation of OVA-Transgenic ODCs into the Cerebellum Suffices for Deletion of Peripheral OVA-Specific $CD8^+$ T Cells

To rigorously rule out a contribution of OVA expressed outside of the CNS to the observed deletion, in vitro cultured WT or ODC-OVA ODC precursors were stereotactically transplanted into the cerebellum of WT mice (Habisch et al., 2007). Three weeks later, OVA expression was readily detectable in the cerebellum of mice that had received transgenic, but not of those which had received WT, cells (Figure 4A). OT-I $CD8^+$ T cells were then transferred followed by Lm-OVA infection. In the periphery of mice transplanted with ODC-OVA ODCs, OT-I cells were strongly reduced compared to recipient mice with a WT ODC

Diagrams (A and B, right) show percentages of OVA-specific T cells of $CD8^+$ T cells. Aggregate data from two independent experiments are shown.
(C) Presence of K^b-SIINFEKL-reactive $CD8^+$ T cells in LN from Vβ5 or ODC-OVA Vβ5 mice as shown by streptamer staining.
(D) Absence of high-avidity K^b-SIINFEKL-reactive $CD8^+$ T cells in ODC-OVA Vβ5 mice. Polyclonally preactivated $CD8^+$ T cells were restimulated with titrated concentrations of SIINFEKL peptide and the response was measured by intracellular (i.c.) detection of IFN-γ.
(E) No deletion of developing OT-I cells by radioresistant thymic or hematopoeitic cells of ODC-OVA mice. As indicated, irradiated WT or ODC-OVA mice received mixed bone marrow cells from OT-I Thy1.1 mice together with WT or ODC-OVA mice (1:4). 6 weeks later, spleen cells were stained for reconstitution of T cells.
(F) $CD8^+$ T cells were prepared from LN of recipient mice and cultured for 3 days with SIINFEKL. Proliferation was measured by ^3H thymidine incorporation and IFN-γ secretion was detected by ELISA.
Data are representative of three independent experiments. See also Figure S5.

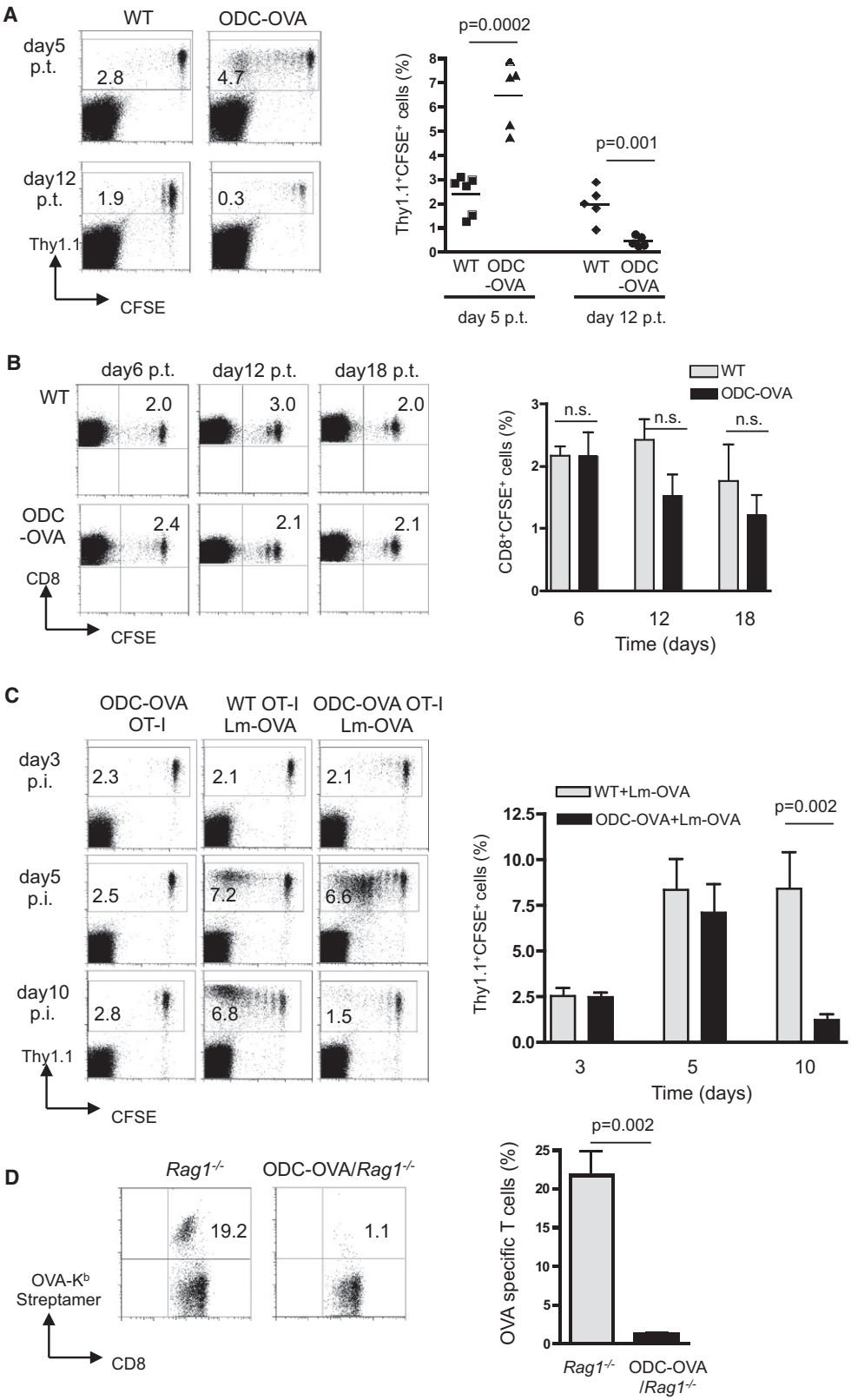

Figure 2. Access of OT-I Cells to OVA-Expressing ODCs Leads to Their Activation and Deletion

(A) Activation and deletion of postnatally transferred OT-I cells in ODC-OVA hosts. CFSE-labeled OT-I Thy1.1 CD8+ T cells were transferred into 10-day-old WT or ODC-OVA mice; cervical LNs were analyzed 5 and 12 days later. See also Figures S1 and S2.

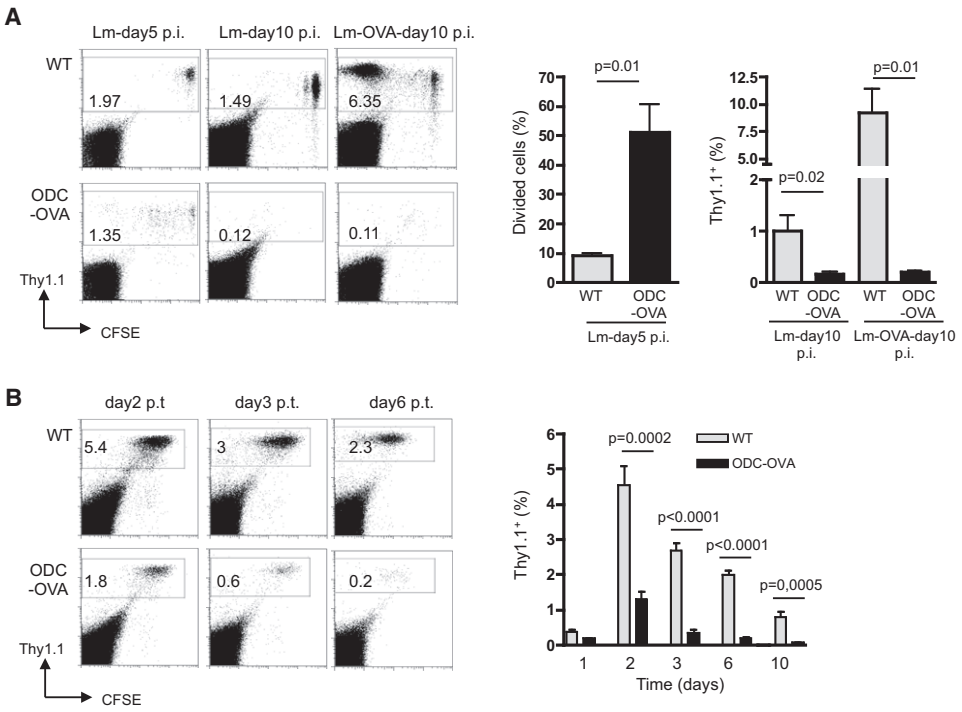

Figure 3. Both Systemic Inflammation and Cell-Intrinsic Activation Lead to Deletion of OT-I Cells in Adult ODC-OVA Mice

(A) Deletion by systemic inflammation. Adult WT or ODC-OVA mice adoptively transferred with CFSE-labeled OT-I Thy1.1 CD8+ T cells were infected with WT Lm or Lm-OVA; cervical LNs were analyzed 5 or 10 days after infection. Data are shown from two experiments with three to six animals, each with SD. See also Figure S4.

(B) Deletion of transferred in vitro activated OT-I cells. In vitro activated OT-I CD8+ T cells were transferred into adult WT or ODC-OVA mice and cervical LNs were analyzed at indicated time points. A CD8+ T cell gate was used in dot plots and graphs. Bar graph shows aggregate data from three experiments with two to three animals each with SD.

See also Figure S5.

transplant (Figures 4B and 4C). These data show that the presence of OVA in CNS-resident ODCs is sufficient for deletion of peripheral OVA-specific T cells after immune activation.

Deletion of ODC-Reactive CD8+ T Cells Is Blocked by VLA-4-Specific mAb

If OVA expression in the CNS suffices for deletion of peripherally activated OVA-reactive CD8+ T cells, these cells must migrate into the CNS via the BBB. Interaction of very late antigen-4 (VLA-4) with its ligand, vascular cell adhesion molecule-1 (VCAM-1), which is expressed on CNS endothelium, is required for T cell entry into the CNS (Yednock et al., 1992). Previous reports had shown that VLA-4 Ab treatment reverses clinical symptoms of EAE and results in the clearance of leukocytes from the CNS (Kent et al., 1995; Léger et al., 1997). We therefore transferred activated OT-I T cells into ODC-OVA mice undergoing treatment with VLA-4-specific Ab. VLA-4 Ab-treated ODC-OVA mice showed markedly reduced deletion of OT-I T cells compared to isotype control Ab-treated mice, in line with the notion that transgression of the BBB is required for the deletion of OVA-reactive CD8+ T cells (Figure 5A).

Deletion of Autoreactive CD8+ T Cells Is Probably due to Autoantigen Recognition on the ODCs Themselves

Recognition of OVA by OT-I cells could occur either directly on ODCs, which express low amounts of K^b-SIINFEKL complexes (about 90 per cell) (Na et al., 2009), or after antigen transfer to microglia or dendritic cells. First, we excluded expression of even small amounts of cell-intrinsic OVA by microglia and dendritic cells in ODC-OVA mice by using a sensitive polymerase chain reaction (PCR) and flow cytometry-sorted cells (Figure S5). We then tested for secondary presentation through the in vivo

(B) Antigenic ignorance of OT-I cells in adult ODC-OVA hosts. Naive OT-I CD8+ T cells were labeled with CFSE and transferred into adult WT or ODC-OVA mice. Cervical LNs were analyzed at indicated time points. Bar graph shows average percent cell of OT-I among CD8+ T cells from five to seven mice.

(C) Activation and deletion of formerly ignorant OT-I cells in adult ODC-OVA mice after Lm-OVA infection. ODC-OVA or WT mice received naive OT-I Thy1.1 CD8+ T cells followed by infection with Lm-OVA; cervical LNs were analyzed at indicated time points after infection. See also Figures S3 and S4.

(D) Deletion of OVA-reactive CD8+ T cells from the normal repertoire by transgenic expression of OVA in ODCs. 2×10^8 total LN cells from WT mice were transferred into *Rag1*$^{-/-}$ or ODC-OVA *Rag1*$^{-/-}$ mice. Spleens from recipient mice were analyzed 10 days after infection with 10^5 cfu Lm-OVA.

Data shown in diagrams (right panels) were obtained with a CD8+ T cell gate and are presented as mean ± standard deviation (SD) of two to three independent experiments. See also Figure S5.

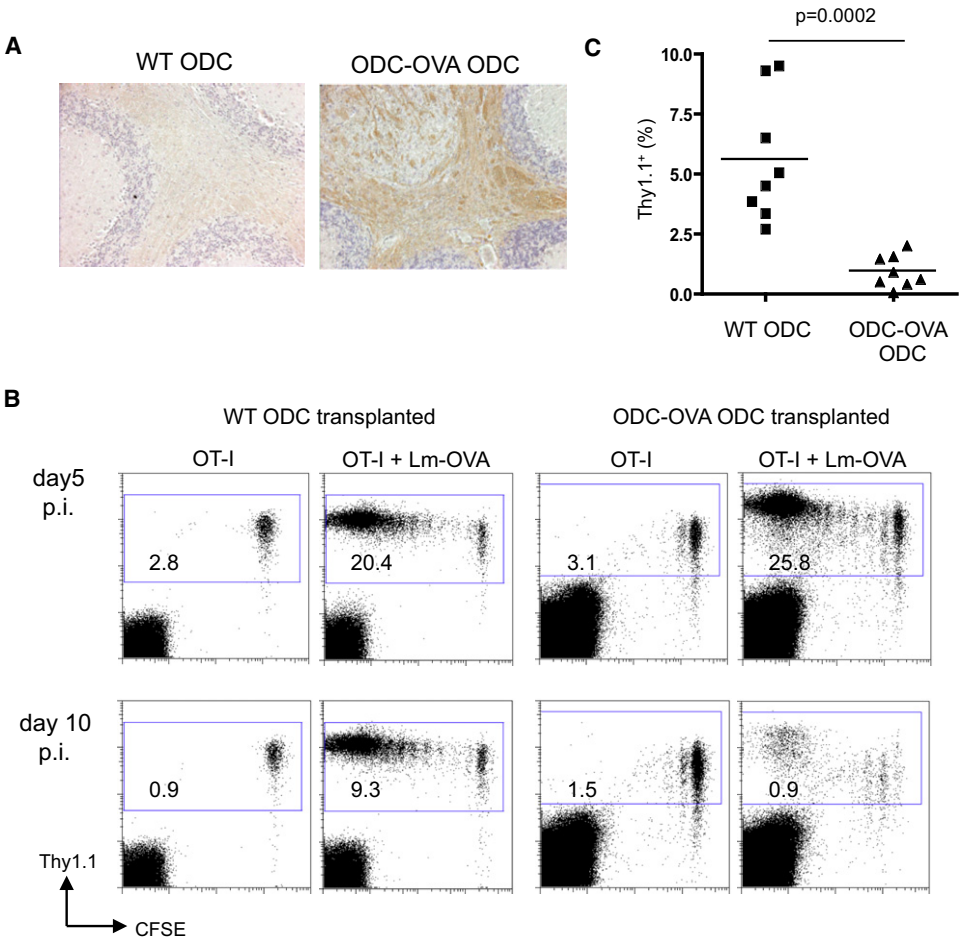

Figure 4. Transplantation of ODCs from ODC-OVA Mice Confers Deletion of OT-I Cells in Lm-OVA-Infected WT Mice

(A) Detection of OVA in transplanted ODCs. Cultured ODC precursors from WT or ODC-OVA mice were transplanted into the cerebellum of WT recipient mice 3 weeks before analysis by immune histology. OVA staining was seen throughout the whole cerebellar white matter only in mice transplanted with OVA-ODC. (B and C) Deletion of OT-I cells. 3 weeks posttransplantation, mice received CFSE-labeled OT-I $CD8^+$ T cells i.p. followed by Lm-OVA infection; OT-I cells recovered from cervical LN were analyzed on days 5 and 10 p.i. A $CD8^+$ T cell gate was used in dot plots and graphs. Aggregate data from two independent experiments are shown.

See also Figure S5.

activation of TCR transgenic $CD4^+$ T cells (OT-II cells). Previously, we had shown that if OVA is released from ODCs by an overwhelming number of $CD8^+$ T cells, as is the case in OT-I ODC-OVA double transgenic mice, activation of OT-II cells is readily observed in the cervical LN (Na et al., 2008). Accordingly, in vitro activated CFSE-labeled OT-I $CD8^+$ T cells were transferred to adult ODC-OVA mice together with naive CFSE-labeled OT-II cells as indicators for secondary presentation. As expected, transferred OT-I T cells were deleted in ODC-OVA but not in WT recipients, whereas the cotransferred OT-II cells remained quiescent in both WT and ODC-OVA mice (Figure 5B). Thus at least with regard to DCs, which also present antigens via the MHC class II endocytic pathway, secondary presentation of cytosolic OVA derived from ODCs does not seem to play a role in antigen recognition by and deletion of OT-I cells.

As a second test for the direct recognition of K^b-SIINFEKL on ODCs by OT-I cells, we used blockade with mAb 25D1.16, which selectively binds to this MHC-peptide complex (Porgador et al., 1997). In vivo, 25D1.16 is able to block activation of OT-I cells by OVA-transgenic ODCs, but not by exogenous OVA cross-presented by dendritic cells (Na et al., 2009). When in vitro preactivated OT-I cells were transferred into ODC-OVA mice, which had also received 25D1.16, deletion was fully prevented (Figure 5A). This result supports our hypothesis that deletion of OVA-reactive $CD8^+$ T cells is a consequence of antigen recognition at the surface of ODCs as opposed to cross-presentation by professional APCs.

Deletion of Autoreactive $CD8^+$ T Cells by Autoantigen-Expressing ODCs Involves Fas-Mediated Apoptosis

OVA recognition by OT-I cells on ODCs results in IFN-γ release in vivo (Na et al., 2008). Because IFN-γ induces FasL expression in ODCs (Pouly et al., 2000), triggering of apoptosis in antigen-activated $CD8^+$ T cells by the Fas pathway appeared as a possible mechanism for the observed deletion. To test this, we blocked FasL with a Fas-Fc fusion protein in

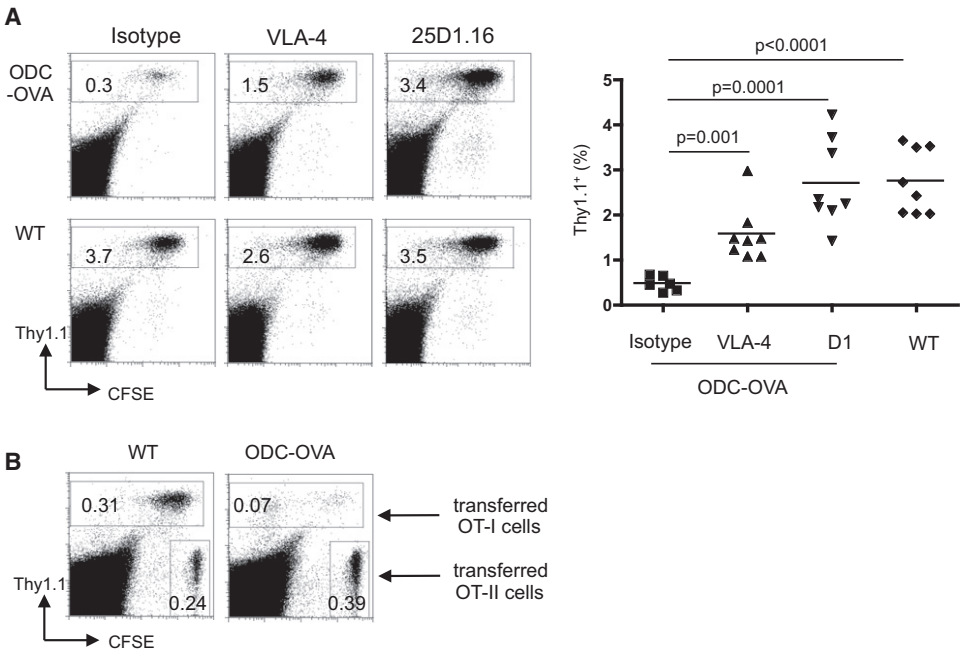

Figure 5. Deletion of OT-I Cells in ODC-OVA Mice Requires Migration and OVA Recognition on ODCs

(A) Blockade with antibodies. In vitro activated CFSE-labeled OT-I Thy1.1 cells were transferred into WT or ODC-OVA mice that received Abs to VLA-4 or to the K^b-SIINFEKL complex on days −1, +2, and +4 of cell transfer. On day 5, cervical LNs were analyzed with a $CD8^+$ T cell gate.

(B) Absence of OVA crosspresentation to OT-II cells in OT-I deleting ODC-OVA mice. Naive CFSE-labeled OT-II $CD4^+$ T cells were transferred into ODC-OVA mice together with in vitro activated CFSE-labeled OT-I Thy1.1 $CD8^+$ T cells; 5 days later cervical lymphocytes were stained for FACS analysis. The data are representative of three independent experiments.

See also Figure S5.

Lm-OVA-infected ODC-OVA mice and indeed observed substantial if not complete prevention of OT-I T cell deletion (Figure 6A).

Taken together, our data suggest that antigen recognition on ODCs by previously activated $CD8^+$ T cells scanning the brain induces their apoptosis in situ. As in other settings where high numbers of lymphocytes undergo apoptosis, such as thymic T cell selection, it was, however, difficult to "find the bodies" by immune histology or flow cytometry because of their very rapid removal by macrophages. We therefore devised a method that exploits transfer of the lipophilic dye CM-DiI to the phagocytes that eat the labeled cells. Indeed, a pilot experiment employing apoptosis induction in labeled thymocytes in the presence of peritoneal macrophages showed strong transfer of label from the thymocytes to the phagocytes (Figure 6B).

This method was then used to look for traces of dead OT-I cells in the CNS. Indeed, flow cytometry revealed a significant number of labeled microglia cells in ODC-OVA, but only a few in WT mice that had received labeled OT-I cells 3 days earlier (Figure 6C). Labeled microglia cells were also visible by immune histology of the cerebellum (Figure 6D). Taken together, our data indicate that removal of apoptotic ODC-reactive $CD8^+$ T cells occurs at least in part within the CNS itself.

CNS Infection Makes ODCs Susceptible to $CD8^+$ T Cell Attack

The ability of ODCs to delete even a large number of $CD8^+$ T cells from the repertoire begs the question how the immune system can successfully deal with intracellular pathogens infecting the brain. To address this issue, the site of infection with Lm-OVA was moved from the periphery to the CNS. Based on previous experience with mice expressing OVA in neurons (Sanchez-Ruiz et al., 2008), ODC-OVA and control mice received only 10^6 OT-I cells 1 day prior to an intracerebral infection with 10^3 Lm-OVA. Interestingly, starting at day 8 after infection, after complete elimination of the bacteria from the CNS, 100% of ODC-OVA mice developed limp tail weakness and hind leg paresis that persisted throughout the entire period of the study, i.e., 50 days after infection, whereas all WT mice (Figures 7A and 7B) and infected ODC-OVA mice without OT-I transfer (not shown) remained healthy. Neuropathological analysis identified a $CD8^+$ T cell-mediated autoimmune response directed against ODCs in brain and spinal cord. One day preceding neurologic symptoms, numerous $CD8^+$ T cells had homed to the brain of ODC-OVA mice, preferentially to the white matter where they formed inflammatory clusters (Figure 7C). In the spinal cord, $CD3^+$ T cells were present in the posterior column (Figure 7E). Inflammation progressed up to day 14 after infection, when demyelination in the brain and the spinal cord was prominent (Figure 7G). In contrast, only a few $CD3^+$ T cells patrolled the CNS in WT mice, and demyelination was absent (Figures 7F and 7H).

DISCUSSION

We have reported here the efficient deletion of autoreactive $CD8^+$ T cells from the immune system by ODCs expressing their

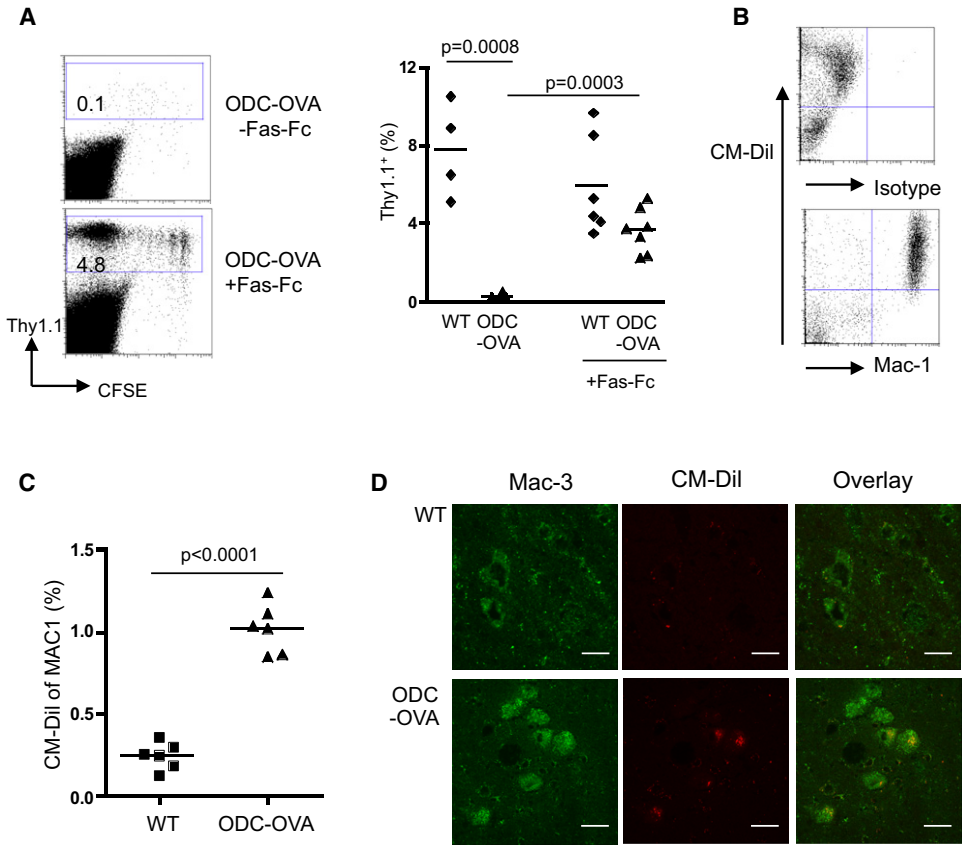

Figure 6. Deletion of OT-I T Cells in ODC-OVA Mice Involves Fas-Mediated Apoptosis

(A) Inhibition of deletion by blockade of FasL. ODC-OVA or WT mice were treated with 1 mg of Fas-Fc fusion protein every second day, starting 1 day before adoptive transfer of OT-I $CD8^+$ T cells. Next day, mice were infected with Lm-OVA; cervical LNs were analyzed on day 10 p.i. with a $CD8^+$ T cell gate.

(B) Use of lipophilic dye to detect macrophages having digested apoptotic cells. CM-DiI-labeled thymocytes were cultured overnight with peritoneal macrophages in the presence of dexamethason and analyzed by flow cytometry.

(C) Microglia cells dispose of apoptotic OT-I cells in ODC-OVA mice. In vitro activated CM-DiI-labeled OT-I cells were transferred into WT or ODC-OVA mice. 3 days later, leukocytes were prepared from brain and spinal cord and analyzed by flow cytometry for CM-DiI-labeled $Mac1^+$ cells.

(D) Detection of CM-DiI-labeled microglia cells in situ. Cryosections were stained for Mac-3 expression (green). Note scattered CM-DiI-labeled microglia cells in cerebellum of ODC-OVA mice 3 days after transfer of in vitro preactivated CM-DiI-labeled OT-I cells. Scale bars represent 20 μm. Aggregate data from two independent experiments are shown.

See also Figure S5.

cognate antigen in the uninfected brain, whereas demyelinating $CD8^+$ T cell-mediated disease ensues if the CNS itself is infected with an intracellular pathogen expressing the same ODC autoantigen. Deletion requires recirculation through the CNS and can occur either postnatally, when the BBB is not fully formed yet, or in the adult if $CD8^+$ T cells are enabled to enter the CNS. Enablement can be cell intrinsic, i.e., a result of their activation, or due to systemic inflammation. Both neonatal and adult purging of ODC-reactive $CD8^+$ T cells is preceded by a proliferative phase that is likely to be required for sensitizing the cells to activation-induced cell death (Plunkett et al., 2000) and during which the functional fitness of autoreactive $CD8^+$ T cells headed for deletion is already impaired. Deletion of ODC-reactive $CD8^+$ T cells in the perinatal period is seen both in mice with an unmanipulated repertoire and "Vβ5 mice" with a $CD8^+$ T cell repertoire enriched for OVA reactivity, the model antigen employed, and after transfer of high numbers (up to 5×10^7) of high-affinity OVA-reactive OT-I cells into 7-day-old ODC-OVA mice. Thus, ODCs readily cope with even an unphysiologically high representation of autoreactive $CD8^+$ T cells during the perinatal period.

Initially, we had suspected that perinatal deletion of K^b-SIINFEKL-reactive cells in ODC-OVA mice is due to intrathymic antigen expression. Medullary epithelial cells are potent negative selectors of the emerging T cell repertoire by ectopic expression of tissue antigens or fragments thereof (Kyewski and Klein, 2006). This has also been reported for MBP (Farhadi et al., 2003), the protein lending ODC specificity to OVA in ODC-OVA mice via its regulatory sequences, and for OVA itself expressed under the control of the rat insulin promotor (Kurts et al., 1996, 1997b). Our earlier search for thymic OVA by immune histology, immunoblotting, or RT-PCR had failed to provide such evidence in ODC-OVA mice (Cao et al., 2006), and in our current experiments, negative selection by thymic or extrathymic

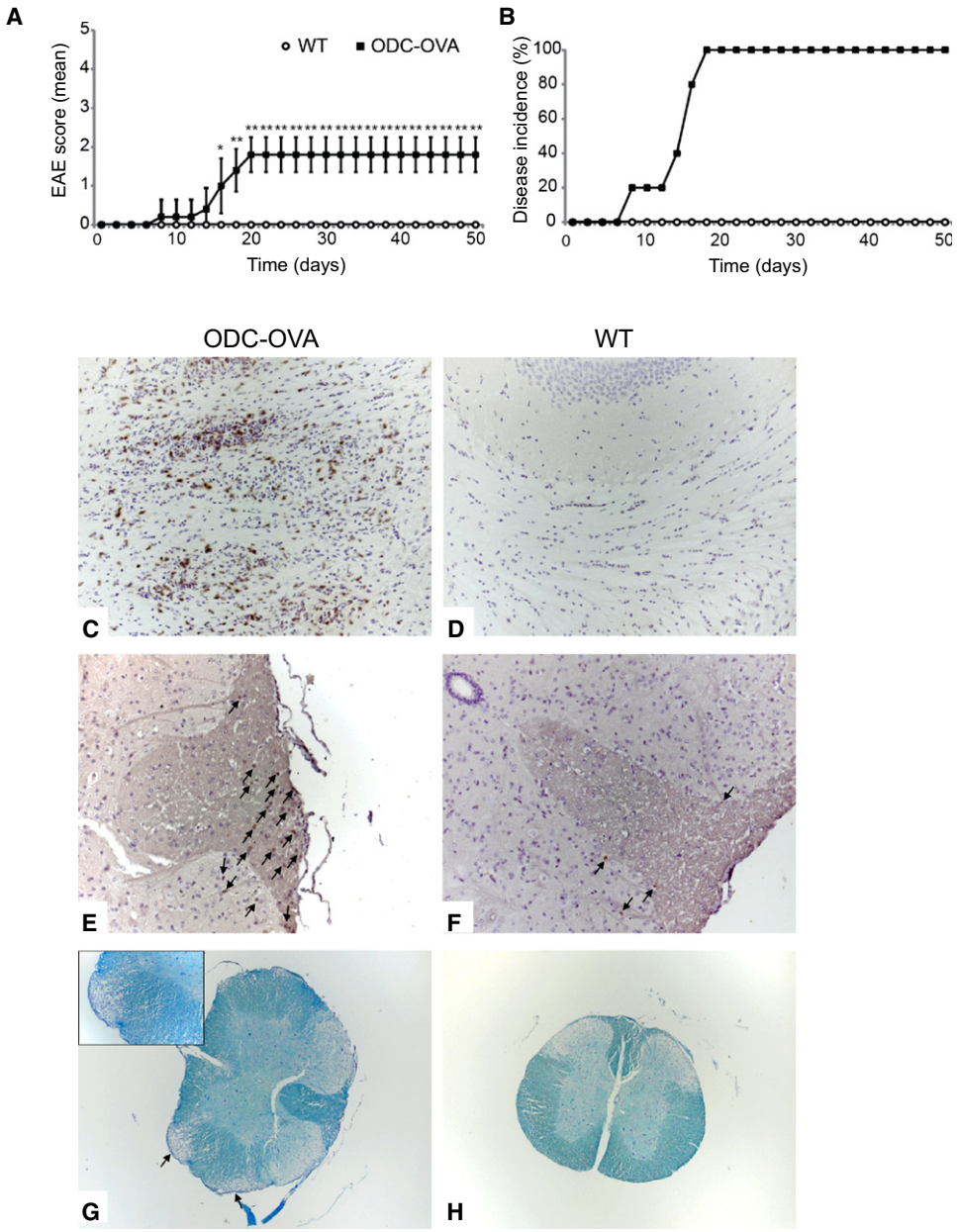

Figure 7. Intracerebral Infection of OT-I-Loaded ODC-OVA Mice Leads to Demyelinating Disease

(A and B) EAE score and incidence. ODC-OVA and WT mice received an i.v. injection of 10^6 OT-I CD8+ T cells 1 day prior to intracerebral infection with 10^3 Lm-OVA and were monitored for EAE score. Mean and SD are shown (Mann-Whitney U test; *$p < 0.05$; **$p < 0.025$).

(C–H) Neuropathological characteristics.

(C and D) CD8+ T cell infiltration in periventricular white matter 7 days p.i. in ODC-OVA (C) but not WT (D) mouse.

(E and F) Numerous CD3+ T cells in the posterior column of the spinal cord in an ODC-OVA mouse at day 7 after infection (arrows, E), whereas only single CD3+ T cells reside at the border of the gray matter to the white matter of the posterior column of the spinal cord in a C57BL/6 mouse (arrows, F). Immunohistochemistry with slight hemalum counterstaining, original magnification ×200.

(G and H) Widespread demyelination in the spinothalamic fascicle at day 14 after infection (arrows, G). Insert in (G) demonstrates vacuolar demyelination and myelin debris in the spinothalamic fascicle. Spinal cord of WT control mouse is normal (H).

See also Figure S5.

radioresistant or hematopoietic cells was ruled out by using adult bone marrow radiation chimeras.

Perinatal tolerance induction by a tissue-specific self antigen has previously been reported for CD8+ T cells recognizing transgenic K^b on keratinocytes (Alferink et al., 1998). Similar to our findings, access to antigen through trafficking in the neonate was required for tolerance induction, but the outcome was functional rather than deletional tolerance.

In spite of the remarkable efficiency of perinatal deletion currently observed, myelin-specific $CD8^+$ T cells are found in the periphery of both humans and rodents (Martin et al., 1990; Ota et al., 1990; Pette et al., 1990; Schluesener and Wekerle, 1985), presumably because of export from the thymus after formation of the BBB. By using adult ODC-OVA mice adoptively transferred with OT-I cells, we found that such cells can indeed persist in the immune system. However, provision of access to the CNS by systemic inflammation or by activation of the autoreactive $CD8^+$ T cells themselves was sufficient to induce deletion also in the adult setting. Accordingly, removal of autoreactive $CD8^+$ T cells from the repertoire by recirculation through the CNS appears to be a life-long series of events triggered by infections.

Several independent experimental approaches strongly suggest that deletion of the recirculating $CD8^+$ T cells is due to autoantigen expression by the ODCs themselves: PCR analysis showed its exclusive expression in ODCs and stereotactic transplantation mapped the effect to this cell type. In addition, interference of VLA-4 blockade with deletion provides further support for the need for $CD8^+$ T cells to cross the BBB (Yednock et al., 1992). A requirement for crosspresentation by professional APC appears unlikely for the following reasons. First, we have previously shown by using mixed ODC cultures from wild-type and ODC-OVA mice expressing $H\text{-}2^b$ or $H\text{-}2^d$ that $H\text{-}2^b$ ODC-OVA ODCs and not contaminating cross-presenting cell types directly activate OT-I cells (Na et al., 2008). Second, the failure to see activation of OT-II $CD4^+$ T cells cotransferred with OT-I T cells into ODC-OVA mice at least rules out the participation of DCs which re-present antigens to both $CD4^+$ and $CD8^+$ T cells. Finally, we have previously shown that recognition of exogenously applied OVA by OT-I cells cannot be blocked by the K^b-SIINFEKL-specific mAb 25D1.16 in vivo, whereas OT-I activation by OVA-expressing ODCs is readily blocked both in vitro and in vivo (Na et al., 2009). The ability of this mAb to fully interfere with OT-I deletion, therefore, strongly suggests that recognition leading to deletion occurred on the oligodrendocytes themselves, rather than by cross-presenting professional APCs. This is in contrast to the deletion of autoreactive OT-I $CD8^+$ T cells in the rat insulin promoter (RIP)-mOVA model, which depends on cross-presentation of pancreatic β cell-derived OVA by resting dendritic cells in the draining LN and reaches saturation of its protective capacity already at 10^6 transferred cells (Kurts et al., 1997a).

Together, our data indicate that antigen recognition on ODCs by $CD8^+$ T cells scanning the brain leads to their destruction. The observed participation of the Fas-FasL system in apoptotic removal of $CD8^+$ T cells is in keeping with the known ability of IFN-γ produced by the attacking $CD8^+$ T cells (Na et al., 2008) to upregulate FasL on ODCs (Pouly et al., 2000) but does not rule out the participation of FasL expressed by other cells.

Together, our results suggest a scenario where ODC-specific $CD8^+$ T cells gain access to the CNS as a result of specific activation or peripherally induced inflammation. Here, they acquire sensitivity to apoptosis induction by antigen recognition on ODCs while downregulating their effector functions. After further recirculation, through the periphery and the CNS, they finally receive a death signal from the ODCs that have upregulated FasL. Only recently, a distinct time-window of recirculation of OVA-specific $CD8^+$ T cells through the CNS has been described after Lm-OVA infection, in line with our proposal (Young et al., 2011).

Another nonlymphoid extrathymic cell type was recently reported to delete autoreactive $CD8^+$ T cells (Lee et al., 2007). These specialized LN stromal cells use the autoimmune regulator Aire to promiscuously express tissue-specific antigens to $CD4^+$ and $CD8^+$ T cells, much like thymic medullary epithelial cells. In contrast, ODCs do not express MHC class II glycoproteins (Turnley et al., 1991) and only low amounts of MHC class I, which are increased by IFN-γ exposure.

Death of $CD4^+$ T lymphocytes in the brain of mice undergoing EAE has been extensively described (Gold et al., 2006; Pender et al., 1991; Schmied et al., 1993). However, although these reports document T cell apoptosis in situ (Schmied et al., 1993), they do not describe purging of the peripheral immune system by filtration through the CNS. Myelin-reactive $CD8^+$ T cells may behave differently from $CD4^+$ T cells, because they can find their target antigen on ODCs themselves, not depending on their representation by microglia or DCs.

Importantly, we found one setting in which the ability of ODCs to purge the peripheral repertoire is turned into the opposite: if infection is in the CNS itself, the invading $CD8^+$ T cells are enabled to destroy ODCs expressing a pathogen-derived (and maybe also a crossreactive self-) antigen. Thus, presence or absence of local inflammation in the CNS decides whether $CD8^+$ T cells will destroy ODCs on which they detect an antigen, or will be destroyed themselves. Although this important decision-making process is clear from our present work, future research will clarify its precise cellular and molecular mechanisms. Whatever these may be, our studies may open the door for a better understanding of the role of antiviral $CD8^+$ T cell responses, in particular to EBV (Jilek et al., 2008), in the development of MS. Indeed, the reported crossreactivity of EBV-specific $CD8^+$ T cells with myelin antigens (Lang et al., 2002) and the presence of EBV in B cells of the CNS of MS patients (Serafini et al., 2007; Stowe et al., 2007) may provide a setting where local inflammation prevents ODCs from removing potentially harmful $CD8^+$ T cells.

EXPERIMENTAL PROCEDURES

Mice

ODC-OVA mice (Cao et al., 2006), OT-I TCR transgenic mice (Hogquist et al., 1993), Vβ5 TCRβ chain transgenic mice (Fink et al., 1992), and Thy1.1 congenic C57BL/6 mice (Jackson ImmunoResearch Laboratories) were kept in pathogen-free animal facilities. All in vivo studies were performed according to NIH guidelines under permits from the governments of Lower Frankonia (for Würzburg) and North-Rhine Westphalia (Cologne).

L. monocytogenes Infection

Mice were infected with 10^5 colony forming units (cfu) Lm-OVA (Pope et al., 2001) or WT Lm in PBS intravenously. To prime for secondary responses, mice were infected with 5,000 cfu 3 weeks earlier. Intracerebral infection of mice was performed and disease severity was scored on a 1 to 6 scale as described previously (Sanchez-Ruiz et al., 2008).

$CD8^+$ T Cell Isolation and In Vitro Activation

$CD8^+$ T cells were isolated from the LN of OT-I mice via MACS beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) and labeled with 10 μM CFSE (Invitrogen, Grand Island, NY). For stimulation, isolated $CD8^+$ T cells were cultured

with anti-CD3- plus anti-CD28-coated beads (Invitrogen) in the presence of recombinant human (rh) IL-2 (Novartis, Basel, CH) for 6 days and labeled with 10 μM CFSE. Cells were transferred i.p. into WT or ODC-OVA mice.

Determination of functional avidity of SIINFEKL-specific $CD8^+$ T cells followed the protocol by Zehn and Bevan (2006).

Bone Marrow Chimeras

Irradiated (11.5Gy) mice received 1.6×10^7 bone marrow cells from WT or ODC-OVA mice together with 4×10^6 bone marrow cells from OT-I Thy1.1 mice i.v. Analysis was 6 weeks later.

ODC Cultivation and Transplantation

ODC precursor cells were isolated and cultured as previously described (Na et al., 2008). 5×10^4 cultured ODCs were stereotactically implanted into left and right areas of the cerebellum. Coordinates were as follows: P, 6.5; L, ±1.7; V, 1.6 mm from bregma and dural surface with the toothbar set at 5, earbar at 10; injection rate, 0.25 μl/min; cannula left in place for 2 min. Mice were used for OT-I transfer experiments 3 weeks later.

In Vivo Application of Antibodies and FasL Blocking Fusion Protein

Mice were treated with 75 μg of VLA-4 Ab (Southern Biotechnology Inc., Cambridge, MA), 100 μg of isotype control, or 100 μg of 25D1.16 Abs (Porgador et al., 1997) i.p. on days −1, +2, and +4 of cell transfer. For FasL blocking, 1 mg Fas-Fc fusion protein (Apogenix, Heidelberg, Germany) was injected i.p. every second day after Lm-OVA infection for 10 days.

Flow Cytometry

Lymphocytes were stained with the following Abs (all from BD PharMingen): anti-CD8-FITC, anti-CD8-APC, anti-CD90.1-PerCP, and anti-TCR Va2-PE.

Streptamer staining was performed by Strep-Tactin kit (IBA, St. Louis, MO) as described by the manufacturer. Data were acquired on a FACSCalibur (BD Bioscience) and analyzed by FlowJo software (Tree Star Inc, Ashland, OR).

Histopathology

Brain and spinal cord were embedded in Paraffin or Tissue-Tek O.C.T. compound (Sakura Finetek). Cresyl vivolet-luxol fast blue staining and immunohistochemical staining was performed as described previously (Na et al., 2008; Sanchez-Ruiz et al., 2008).

Leukocyte Isolation from Spinal Cord

Leukocytes were isolated from spinal cords with percoll gradient as described previously (Na et al., 2008) and analyzed by flow cytometry.

Cell Labeling with CM-DiI

Thymocytes or LN cells were stained with 10 μM CM-DiI (Invitrogen) according to manufacturer's instructions. Labeled thymocytes were cocultured with peritoneal macrophages overnight in the presence of 10 nM dexamethasone (Sigma) and stained for flow cytometry analysis.

Statistical Analysis

Analysis was performed by GraphPad Prism 4.0 (GraphPad Software). Data are presented as mean ± SD; p values were determined by two-tailed Student's t tests.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.immuni.2012.04.009.

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