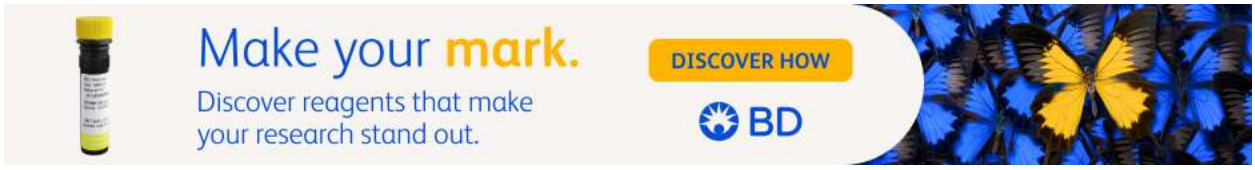

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J Immunol 2004; 173:2353-2361; ;
doi: 10.4049/jimmunol.173.4.2353
http://www.jimmunol.org/content/173/4/2353

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Initiation of Immune Responses in Brain Is Promoted by Local Dendritic Cells[1]

Jozsef Karman,* Changying Ling,† Matyas Sandor,*† and Zsuzsanna Fabry[2]*†

The contribution of dendritic cells (DCs) to initiating T cell-mediated immune response in and T cell homing into the CNS has not yet been clarified. In this study we show by confocal microscopy and flow cytometry that cells expressing CD11c, CD205, and MHC class II molecules and containing fluorescently labeled, processed Ag accumulate at the site of intracerebral Ag injection. These cells follow a specific pattern upon migrating out of the brain. To track their pathway out of the CNS, we differentiated DCs from bone marrow of GFP-transgenic mice and injected them directly into brains of naive C57BL/6 mice. We demonstrate that DCs migrate from brain to cervical lymph nodes, a process that can be blocked by fixation or pertussis toxin treatment of the DCs. Injection of OVA-loaded DCs into brain initiates a SIINFEKL (a dominant OVA epitope)-specific T cell response in lymph nodes and spleen, as measured by specific tetramer and LFA-1 activation marker staining. Additionally, a fraction of activated SIINFEKL-specific T cells home to the CNS. Specific T cell homing to the CNS, however, cannot be induced by i.v. injection of OVA-loaded DCs alone. These data suggest that brain-emigrant DCs are sufficient to support activated T cells to home to the tissue of DC origination. Thus, initiation of immune reactivity against CNS Ags involves the migration of APCs from nervous tissue to peripheral lymphoid tissues, similarly to that in other organs. ***The Journal of Immunology,*** **2004, 173: 2353–2361.**

The CNS is an immunoprivileged site based on the presence of the blood-brain barrier (BBB),[3] graft acceptance, lack of conventional lymphatics, low T cell trafficking, and low MHC class II expression (1). However, it is clear that brain-derived Ags can induce strong systemic immune responses that either protect against cerebral infections or cause inflammatory brain diseases (reviewed in Refs. 1 and 2). Until recently, it was thought that brain Ags reach the peripheral lymphoid system through three major routes: draining through the nasal lymphatics to the cervical lymph nodes (LNs), transported through the arachnoid villi and venous sinus to the periphery, or draining through the ventricular system via choroid plexus vascularizing blood vessels (reviewed in Refs. 2 and 3). It was shown that fluorescently labeled Ag can reach cervical LNs in a very short time, in minutes to hours (4–6), and this quick drainage could be decisive in the initiation of CNS immune responses (4, 6).

However, it has recently been reported that the rapid access of tissue-derived Ag to LNs is not sufficient to induce an optimal T cell response, and local APCs that arrive later from the tissue to the same LN are also required (7). This suggests that a similar mechanism could play an important role in the initiation of immune responses against brain Ags and would provide an explanation for why, despite the presence of nervous tissue Ags in secondary lymphoid organs (8), immune reactivity against CNS Ags only occurs at low frequency. However, the nature of brain APCs and CNS DCs is not well understood and is rather controversial. On the one hand, DCs are reported to be present in the choroid plexus and meninges in the inflamed brain, and their number dramatically increases in brain autoimmune diseases (9–13). We have shown that after intracerebral microinjection of Ag, APCs accumulate around the injection site (5). On the other hand, it was also shown that DCs isolated from the brain of mice with experimental autoimmune encephalomyelitis (EAE) were unable to prime naive T cells, suggesting that they limit, rather than stimulate, local immune reactivity in brain (14). As one of the characteristics of differentiated DCs is mobility, it is possible that brain-derived DCs can reach LNs and stimulate the activation of naive T cells. In our study we use GFP-labeled DCs (GFP-DCs) to answer these questions.

We show that microinjection of protein Ag into brain leads to accumulation of DCs in the CNS. Furthermore, Ag-pulsed CD205-, CD11c-, and MHC class II-expressing GFP-DCs traffic from brain to draining cervical LNs and induce a preferential recruitment of Ag-specific T cells to brain. GFP-DC homing to cervical LNs and the induced T cell recruitment to brain require the GFP-DCs to be alive and capable of signaling through G proteins. Under the conditions of T cell activation by brain-derived Ag, a characteristic distribution of GFP-DCs in the external capsule is observed. These studies indicate that T cell-mediated immune responses against brain Ags are initiated similarly to other tissues. Our findings, focusing on the nature of brain APCs, can lead to new therapeutic treatments of brain inflammatory diseases.

Materials and Methods

Mice

C57BL/6 and CD11cDTRtg mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The GFP-transgenic mouse strain on the C57BL/6 strain background was a gift from Dr. P. Marrack (National Jewish Medical and Research Center, Denver, CO; Ref. 15). The animals were housed according to the guidelines of the National Institutes of Health and University of Wisconsin-Madison Research Animals Resource Center.

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Received for publication February 23, 2004. Accepted for publication June 15, 2004.

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[1] This work was supported by National Institutes of Health Grant RO1NS37570-01A2 (to Z.F.).

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[3] Abbreviations used in this paper: BBB, blood-brain barrier; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; GFP-DC, GFP-expressing DC; LN, lymph node; PTX, pertussis toxin.

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Abs and MHC class I tetramers

Anti-LFA-1 (clone M17/4), anti-CD4 (clone RM4-5), anti-CD8 (clone 53.6-7), anti-B7-1 (clone GL1), anti-I-A^b (clone AF6-120.1), anti-I-A (clone 34-5-3), and anti-HLA-DR (clone L243, isotype control) Abs and streptavidin-CyChrome and streptavidin-allophycocyanin conjugate were purchased from BD Pharmingen (San Diego, CA). Streptavidin-Alexa568 conjugate was purchased from Molecular Probes (Eugene, OR). Anti-CD205, anti-CD11b, anti-CD11c, and anti-Vβ2 TCR (isotype control) Abs were purified from the supernatant of clones NLDC-145, M1/70, N418, and B20.6, respectively (American Type Culture Collection, Manassas, VA), and were used in an FITC- (Sigma-Aldrich, St. Louis, MO), biotin- (Sigma-Aldrich), or Cy5 fluorochrome- (Amersham, Piscataway, NJ) labeled format. Mouse CCR7-specific Ab raised in goats was purchased from Research Diagnostics (Flanders, NJ). Anti-goat IgG-Cy5 conjugate was obtained from Chemicon International (Temecula, CA), and anti-goat IgG-FITC conjugate was purchased from The Jackson Laboratory. H-$2K^b$-SIINFEKL MHC class I tetramer was purchased from Beckman Coulter (Fullerton, CA).

Dendritic cells (DCs)

DCs were differentiated from mouse bone marrow as previously described (16). Briefly, bone marrow cell suspensions were cultured for 7 days in the presence of 20 ng/ml GM-CSF (Sigma-Aldrich) or titrated 10% supernatant from the X63 cell line transfected with a construct encoding mouse GM-CSF (gift from Dr. A. Erdei, Eotvos University, Budapest, Hungary). On day 7, the nonadherent and loosely adherent cells were cultured overnight in the absence of GM-CSF, and nonadherent cells were collected.

The DCs were pulsed with OVA as previously described (17). OVA preparations were tested for endotoxin content and showed no significant B cell-activating capacity for naive C57BL/6 cells (data not shown). For some experiments DCs were incubated in the presence of 200 ng/ml pertussis toxin (List Biological Laboratories, Sacramento, CA) during Ag pulsing (18). After Ag pulsing, cell surface marker expression (MHC class II, CD205, CD11c, and B7-1) was measured by flow cytometry, and functional activity was measured by culturing the cells with OT-1 splenocytes. The average purity of the cell suspension obtained after Ag pulsing varied from 60–75%, with a mean of 70%, on the basis of forward scatter-side scatter distribution. Before injection, cells were washed thoroughly and finally resuspended in RPMI 1640 without serum. Fifty thousand to 250,000 DCs were injected intracerebrally or i.v. into 6- to 8-wk-old female C57BL/6 mice. For some experiments, Ag-loaded cells were fixed in ice-cold 1% paraformaldehyde in PBS for 10 min (19) and washed extensively before injection. Thirty microliters of DQ-OVA (Molecular Probes) or OVA (Sigma-Aldrich) at 2 μg/μl was injected intracerebrally as described previously (5). Three and 7 days after injection, brains were processed for sectioning or measurement of cell populations by flow cytometry.

Cell preparation and flow cytometry

All experimental animals were perfused with at least 50 ml of heparinized PBS, and lymphocyte suspensions were prepared from brains, cervical LNs, and spleens as described previously (5). Blood contamination was excluded in brain cell isolations using proflavin (5). Spleen cells (10^6) were processed for flow cytometric analysis. When brain tissue was analyzed, the typical yield of cells was 10^5 to 5×10^5, and all these cells were used for single-cell staining. Cell suspensions were incubated on ice with saturating concentrations of Abs supplemented with unlabeled 0.04 mg/ml FcγRII- and FcγRIII-specific Ab (clone 2.4G2) to block FcR-mediated binding. H-$2K^b$ SIINFEKL-tetramer staining was conducted for 30 min at room temperature. Cell surface staining on 10,000–50,000 events was acquired on a four-color FACSCalibur flow cytometer and analyzed with CellQuest software version 3.1 (BD Immunocytometry Systems, San Jose, CA) and FlowJo (TreeStar, San Carlos, CA) software version 4.5. Absolute numbers were calculated based on the percentage of specific T cells or GFP^+ from the total cell population acquired and the weight of the tissue.

Confocal microscopy and immunohistochemistry

For confocal microscopy of frozen sections and free-floating sections, mice were perfused with 50 ml of heparinized PBS, followed by perfusion with 50 ml of 3% paraformaldehyde in PBS. In the case of frozen sections, brain, cervical LNs, and spleens were removed and postfixed in 3% paraformadehyde/25% sucrose solution in PBS before freezing in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) on dry ice. Sections were stored at −80°C until further use. Sections (5 μm) were cut from all organs. Brain sections included the injection site from the brains. For free-floating sections, 50-μm sections were cut on a Vibratome (Pelco 101, Series 1000; St. Louis, MO) and kept in ice-cold PBS until staining.

Frozen sections were thawed for 10 min at room temperature and blocked with 1% BSA solution in PBS for 15 min. Some sections were stained with anti-CD205-Cy5 conjugate or with anti-I-A-biotin or anti-CD11c-biotin conjugate for 1 h at room temperature (frozen sections) or for 1 h on ice (free-floating sections), followed by extensive washes with PBS and, in the case of biotinylated Abs, 1-h incubation with streptavidin-allophycocyanin or streptavidin-Alexa568 conjugate. The sections were examined on a Diaphot 200 microscope (Nikon, Melville, NY) equipped with an MRC 1000 laser scanning confocal head (Bio-Rad, Hercules, CA), and the acquired digital images were processed and analyzed using Photoshop 7.0 software (Adobe Systems, San Jose, CA).

Results

CD205- (DEC-205), CD11c-, and MHC class II-expressing APCs accumulate in brain parenchyma at the site of intracerebral injection of Ag

To study the accumulation of DCs in the CNS as a response to neoantigen injection, we intracerebrally injected OVA or chemically modified DQ-OVA. In DQ-OVA, the protein OVA is conjugated with BodipyFL fluorescent dye that self-quenches its fluorescence in the intact molecule and emits green and red fluorescence upon proteolytic cleavage. Previously we have shown that DQ-OVA injected into brain parenchyma is processed in situ by CD11b-expressing cells and is distributed along the external capsule 7 days after intracerebral injection (5). In this report we extend these studies, showing that CD205- and CD11c-expressing cells can be isolated from normal mouse brain and that the number of these cells significantly increases after intracerebral injection of OVA protein Ag (Fig. 1, *A* and *B*). Flow cytometric analysis of CD11c-expressing cell populations from the brain revealed three subpopulations based on CD11c and CD205 expression, which also differed in MHC class II expression (Fig. 1*A*). $CD11c^+$/$CD205^+$ cells constituted ~1% of the total mononuclear cell population prepared from brain (Fig. 1*A*). There was only a slight change in the percentage of $CD11c^+$/$CD205^+$ cells after protein Ag injection (1.00 ± 0.16%). In contrast, the absolute number of $CD11c^+$/$CD205^+$ cells per gram of tissue significantly increased. This indicates that although more cells could be isolated from the brain of injected animals, the percentage of $CD11c^+$/$CD205^+$ cells stayed about the same. Our analysis showed that MHC class II molecule expression decreased on CD11c- and CD205-expressing cells 7 days after Ag injection, indicating maturation of these cells (Fig. 1, *A* and *B*). Maturing DCs have been shown to upregulate CCR7, allowing the homing of these cells to regional LNs (20). CCR7 was expressed on the $CD205^+$, $CD11c^+$, and MHC class II^+ populations before intracerebral injection (Fig. 1*C*). The $CD205^+$, $CD11c^+$, and MHC class II^+ cells may represent a sentinel DC population in the brain. Our data indicate that these cells accumulate at the site of injection and process Ags (Fig. 1, *D* and *E*). CD205- and MHC class II-expressing cells that colocalized with DQ-OVA appeared both 3 and 7 days postinjection in the injection site and the external capsule (only data from 7 days postinjection data are shown; Fig. 1, *D* and *E*) and were likely to represent ingested Ag. Not all CD205-expressing cells contained ingested DQ-OVA, but all processed DQ-OVA was associated with CD205-expressing cells. The CD205-expressing cells without DQ-OVA colocalization may be brain endothelial cells (21). Processed OVA was found in both lysosomes and cytoplasm of CD205-expressing cells (Fig. 1, *D* and *E*, *insets*), indicating Ag uptake and processing of this protein. Staining adjacent sections from the same brains revealed that all processed DQ-OVA was contained within cells expressing MHC class II (Fig. 1*E*).

Collectively, these data indicate that cells expressing DC markers accumulate in the brain after intracerebral Ag delivery. It is not

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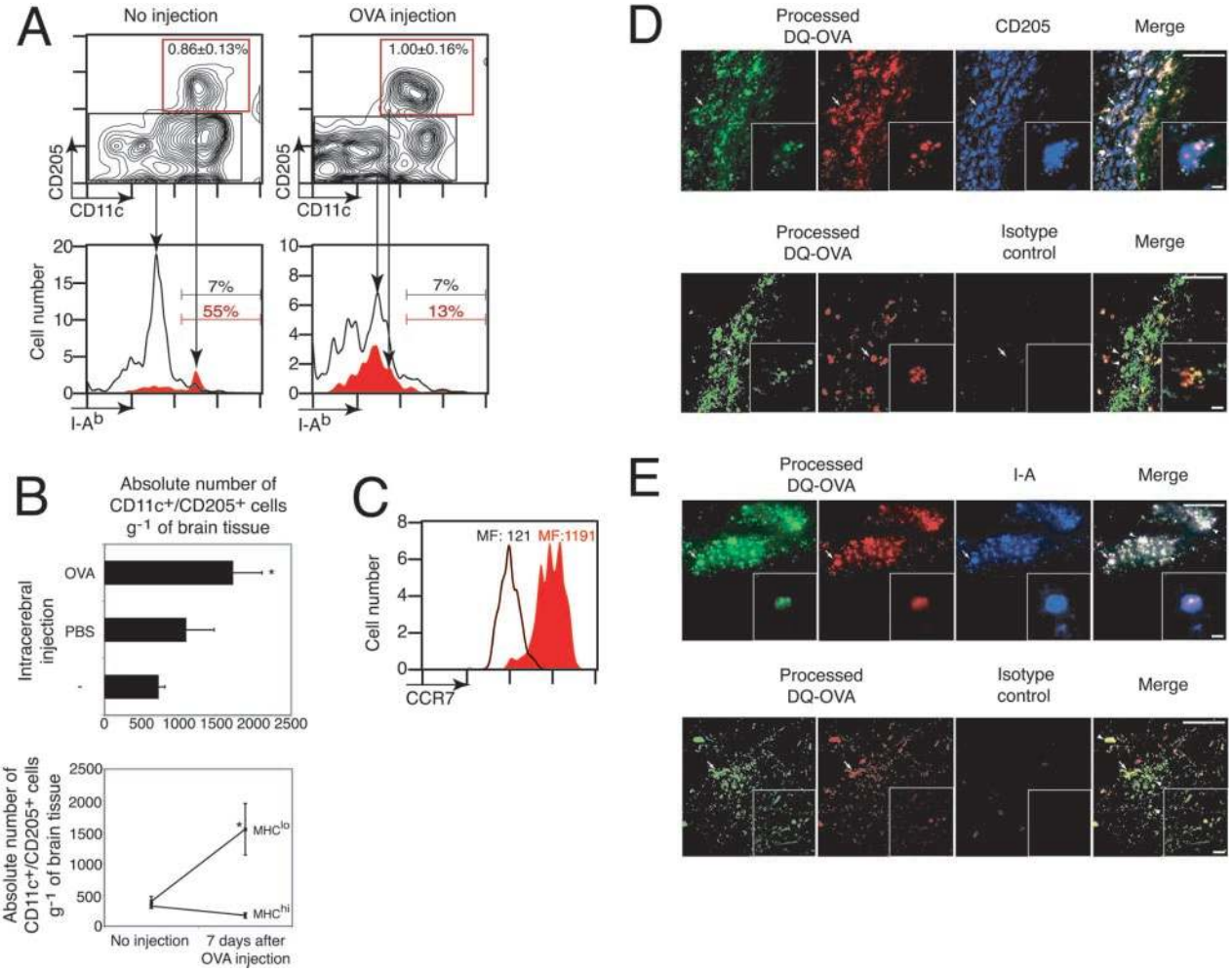

FIGURE 1. Dendritic-like cells are present in the naive brain and accumulate in higher numbers in response to Ag injection. *A*, Expression of CD11c and CD205 in leukocyte suspensions prepared from brain of naive C57BL/6 mice (*left column*, *upper plot*) and from OVA-injected C57BL/6 mice 7 days postinjection (*right column*, *upper plot*), gated on the forward scatter^{high}/side scatter^{medium} population. The *lower graph* shows the relative expression of I-A^b on the surface of the indicated cell populations. One representative experiment is shown of three performed with similar results. The numbers in the red boxes indicate the average percentage of CD11c$^+$/CD205$^+$ cells from the total mononuclear cell population prepared from brain ± SEM. $n \geq 5$ in both groups. *B*, Absolute numbers of CD11c$^+$/CD205$^+$ cells in the brain of differently treated C57BL/6 mice 7 days postinjection (*upper graph*). The mice were either naive (−) or injected with 30 μl of PBS intracerebrally (PBS) or 60 μg of OVA in 30 μl of PBS solution intracerebrally (OVA). The *lower graph* shows the absolute number of I-A$^{b(high)}$ and I-A$^{b(low)}$ populations from the CD11c$^+$/CD205$^+$ population in naive mice and in mice injected with 60 μg of OVA in 30 μl of PBS solution intracerebrally 7 days postinjection. Error bars represent the SEM. $n \geq 5$ in all groups. *C*, CCR7 expression on the surface of the CD11c$^+$/CD205$^+$ population in naive C57BL/6 mice. Black line, anti-goat-IgG-FITC secondary Ab control; solid red area, goat anti-mouse CCR7 Ab plus anti-goat-IgG-FITC. *D* and *E*, DCs appear in the brain upon OVA injection and take up and process OVA. Naive C57BL/6 mice were intracerebrally injected with 60 μg of DQ-OVA. On day 7 after injection, free-floating sections were prepared from brain tissue, and immunohistochemistry was performed. The sections were stained with anti-CD205 or its isotype control (rat IgG2a, κ; *D*) and anti-MHC class II or its isotype control (mouse IgG2a, κ; *E*). The white triangles and arrows indicate CD205-expressing (*D*) or MHC class II-expressing (*E*) cells that ingested DQ-OVA. The *insets* show high magnification of the cells indicated with the white arrows. Scale bars = 50 μm. Scale bars in *insets* = 5 μm.

known whether some of these Ag-processing cells traffic to cervical LNs. The fluorescent protein could drain to cervical LNs (5) and be processed by LN APCs, making it difficult to distinguish between brain-derived and local LN APCs. To determine whether brain DCs are capable of migrating from CNS to peripheral LNs, we injected GFP-expressing, Ag-loaded DCs into the brain and tracked the migration of these cells.

DCs injected into brain parenchyma migrate to cervical LNs along the external capsule in brain

To address the migration of DCs from the brain, we differentiated DCs from bone marrow precursors of GFP transgenic mice. These cells were pulsed with Ag (OVA) and injected intracerebrally. We measured the phenotype of these DCs before injection and found high expression of GFP, CD205, and I-A^b; moderate expression of CD11c molecules (Fig. 2*A*); and low expression of the CCR7 molecule (Fig. 2*B*). This phenotype resembles the characteristics of mature DCs described previously (16). OVA Ag-pulsed GFP-DCs also induced a robust T cell response in OT-1-transgenic mice, indicating the potent Ag-presenting properties of these cells before injection (data not shown).

To test the migratory abilities of GFP-DCs in the brain, we injected these cells directly into brain parenchyma and tracked their appearance in the CNS and peripheral lymphoid organs using GFP and CD205 expression as markers (Fig. 2, *C* and *D*). We show that GFP-DCs primarily migrated to cervical LNs, which is the draining site for CNS-derived Ags (2–4, 22). GFP-DCs could be detected in brain parenchyma both 3 days (data not shown) and

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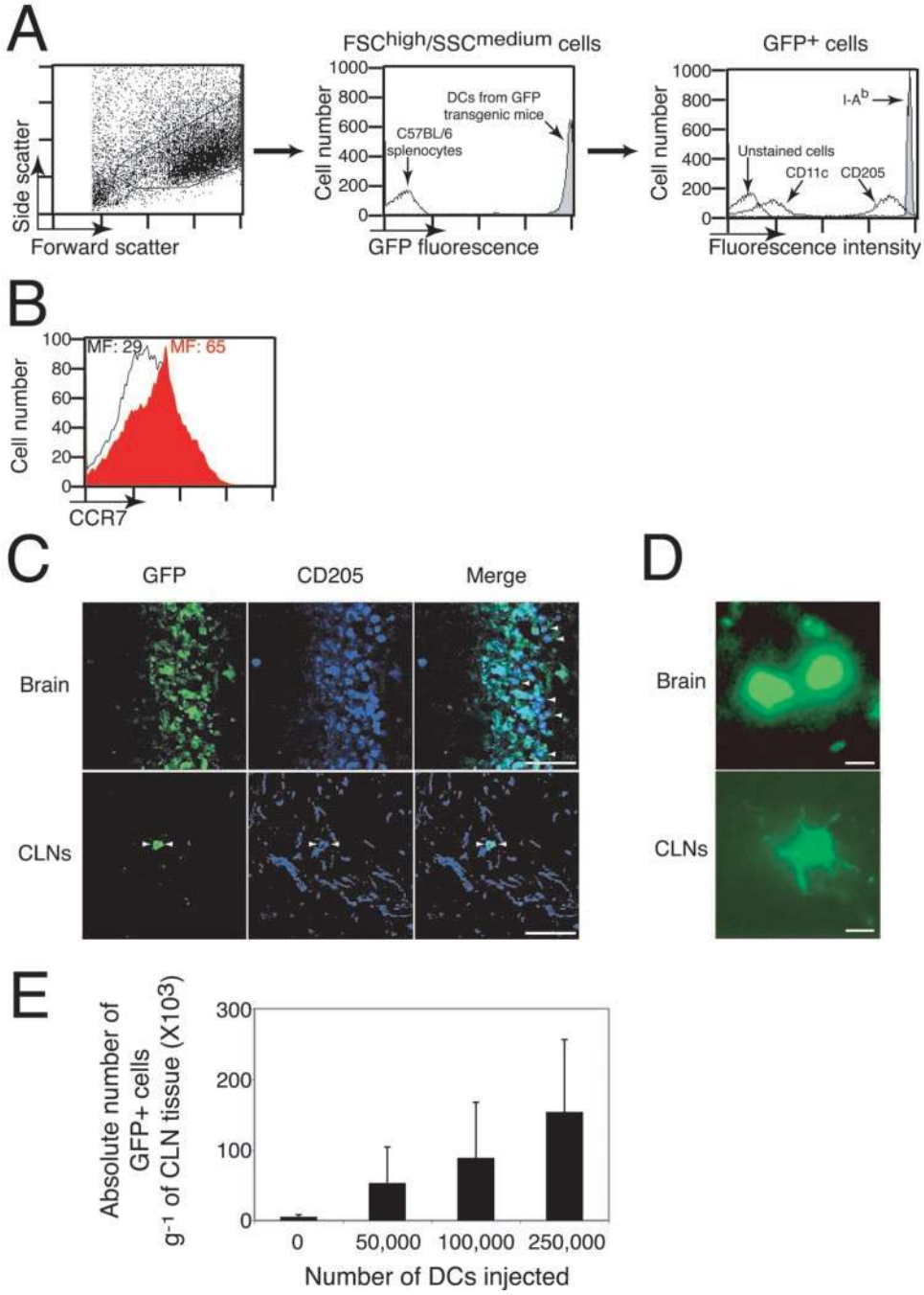

FIGURE 2. GFP-expressing DCs migrate to cervical LNs when injected intracerebrally. *A*, Characterization of GFP and surface marker expression of DCs. Bone marrow cells from GFP-transgenic mice were differentiated into GFP-DCs and characterized for forward and side scatter, GFP expression, and surface levels of CD11c, CD205, and MHC class II. The graphs are representative of >15 separate experiments. *B*, Expression of CCR7 on the surface of in vitro differentiated GFP-DCs before injection. Black line, anti-goat-IgG-Cy5 secondary Ab control; solid red area, goat anti-mouse CCR7 Ab plus anti-goat-IgG-Cy5. *C*, CD205 immunostaining of GFP-positive cells along the external capsule in brain and cervical LNs. White arrowheads indicate $GFP^+/CD205^+$ cells. Scale bar = 50 μm. *D*, Higher magnification images indicate that the DCs in brain and cervical LNs are intact. Scale bar = 2 μm. *E*, The absolute number of GFP^+ cells per gram of tissue in cervical LNs 7 days after the injection of varying amounts of GFP-DCs. Error bars represent the SEM. $n \geq 5$ in all groups.

7 days (Fig. 2*C*, *upper row*) after injection. Intact GFP^+ cells appeared at lower frequency in cervical LNs (Fig. 2*C*, *lower row*), indicating that intact injected GFP-DCs can reach cervical LNs. The trafficking of injected GFP-DCs was also indicated by the dose-dependent increase in the absolute numbers of these cells in cervical LNs 7 days after injection (Fig. 2*E*). Phenotypically, the GFP-DCs appeared intact in the brain and cervical LNs using confocal microscopy at high magnification (Fig. 2*D*). Intact GFP-expressing cells should show uniform cytoplasmic GFP fluorescence, whereas ingestion of the apoptotic injected cells would present a punctate fluorescence due to cellular granularity. GFP^+ cells with uniform cytoplasmic fluorescence could only be found in the injected hemisphere (Fig. 2, *C* and *D*, *upper rows*), and no cell with either uniform or punctate fluorescence could be found in the uninjected hemisphere (not shown). Together, these results indicate that intact DCs can migrate out of the brain to cervical LNs.

To further eliminate the possibility that injected GFP-DCs leak Ag and GFP protein that is picked up and carried to cervical LNs by a brain-resident APC, we tested the effect of pertussis toxin (PTX) or fixation on the migratory capability of injected GFP-DCs in the brain. PTX is a G protein inhibitor that blocks chemokine receptor signaling and prevents DC migration from the skin and monocyte migration into uninflamed tissues (7, 23, 24). We injected intact, paraformaldehyde-fixed, or PTX-treated, OVA-loaded GFP-DCs into the brain and followed their migration in the CNS. Fig. 3*A* shows the orientation of the injection site and the external capsule in the pictures shown in Fig. 3*B* and *D*. Intact GFP-expressing cells were present at the injection site in the brain and exhibited a migration pattern similar to the drainage pattern of injected DQ-OVA described previously (5) (Fig. 3*B*, *right panel*). Fixation or PTX treatment blocked the migration of GFP-DCs from brain detected by confocal microscopy (Fig. 3*B*, *center* and

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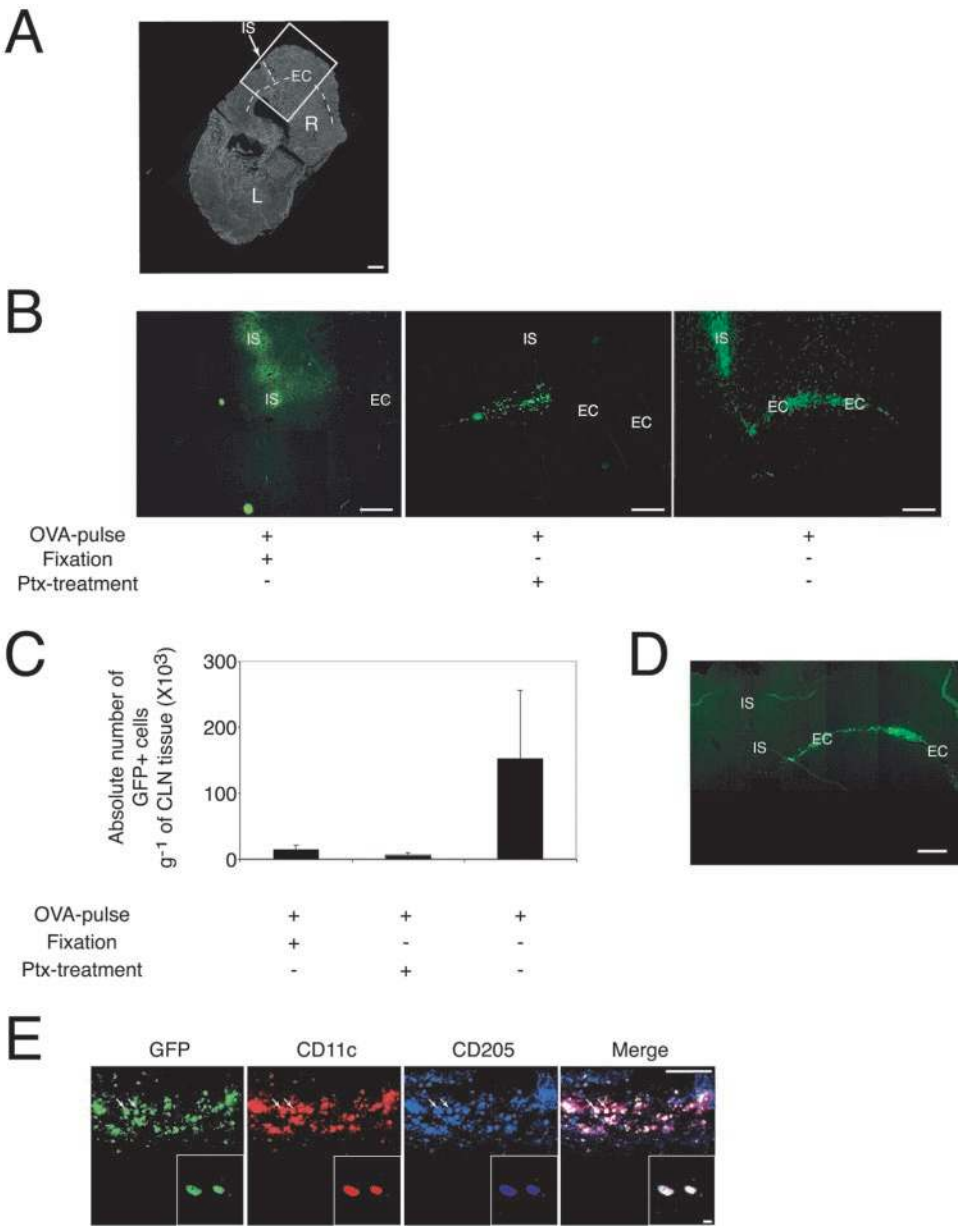

FIGURE 3. GFP-DC migration can be blocked by fixation or PTX treatment of GFP-DCs before intracerebral injection. *A*, Orientation of the injection site and external capsule on the sections shown in *B* and *D*. The injection site and external capsule are depicted by the dashed lines. The white box indicates the area of brain shown in *B* and *D*. L, left hemisphere; R, right hemisphere; IS, injection site; EC, external capsule. Scale bar = 500 μm. *B*, The injected OVA-pulsed GFP-DCs migrate along the external capsule (*right panel*), and this migration can be blocked by fixation (*left panel*) or PTX treatment (*middle panel*). Fluorescent microscopy images were taken 7 days after DC injection. Scale bar = 250 μm. *C*, The absolute numbers of GFP$^+$ cells per gram of tissue in cervical LNs 7 days after injection of GFP-DCs following the different treatments. Error bars represent the SEM. $n \geq 5$ in all groups. *D*, GFP$^+$ cells appear at the injection site in CD11cDTRtg mice and also migrate along the external capsule. CD11cDTRtg mice were injected with 60 μg of OVA in 30 μl of PBS solution intracerebrally. Seven days postinjection, frozen sections were prepared from the brain, stained, and examined by fluorescent microscopy. Scale bar = 250 μm. *E*, GFP$^+$ cells that accumulate and migrate along the external capsule in CD11cDTRtg mice in response to OVA injection are also CD11c$^+$ and CD205$^+$. CD11cDTRtg mice were injected with 60 μg of OVA in 30 μl of PBS solution intracerebrally. Seven days postinjection, frozen sections were prepared from the brain, stained with anti-CD11c and anti-CD205 Ab, and examined by fluorescent microscopy. The *insets* show the cells indicated with the white arrows at high magnification. Scale bars = 50 μm. Scale bars in *insets* = 5 μm.

left panels) and reduced the absolute number of GFP$^+$ cells in cervical LNs as measured by flow cytometry (Fig. 3*C*). Approximately 0.5–1% of injected GFP-DCs were found in cervical LNs at this time point, which corresponds to data published recently (25) quantifying the amount of injected DCs migrating out of the skin.

To address the concern that injected DCs may differ from endogenous brain DCs, we used a mouse strain in which only CD11c-expressing cells are labeled with GFP (26). In the CD11cDTRtg mouse strain, the CD11c promoter drives the expression of a chimeric transgene consisting of the human diphtheria toxin receptor and GFP separated by an internal ribosome entry site (26). This results in the expression of diphtheria toxin receptor and GFP only in CD11c-positive cells, enabling selective depletion and identification of these cells. We intracerebrally injected OVA into CD11cDTRtg mice and examined their brains 7 days postinjection. In response to the injection, endogenous GFP- and CD11c-expressing cells accumulated in brain parenchyma in the injected hemisphere, which were also CD205$^+$ (Fig. 3, *D* and *E*). The majority of these cells were distributed along the external capsule, which corresponds to our previous results (5) and the data shown in Fig. 3*B*. Together, these data show that CD11c$^+$ cells accumulate in the brain upon exogenous Ag injection and, regardless of whether they are endogenous or exogenous cells, migrate along the same pathway in the brain.

Ag-specific T cell response to Ag-loaded DCs in brain

The potential relevance of Ag-loaded DC migration from the brain to the periphery was assessed by measuring the activation of naive, Ag-specific CD8$^+$ T cells using MHC class I tetramers presenting the immunodominant SIINFEKL peptide from OVA. OVA-specific CD8$^+$LFA-1^{high} cells accumulated in a dose-dependent manner in the spleen in mice injected with OVA-loaded GFP-DCs 7 days after injection (Fig. 4*A*), but not 3 days postinjection (data not shown). In cervical LNs, between 1 and 2% of the total CD8$^+$

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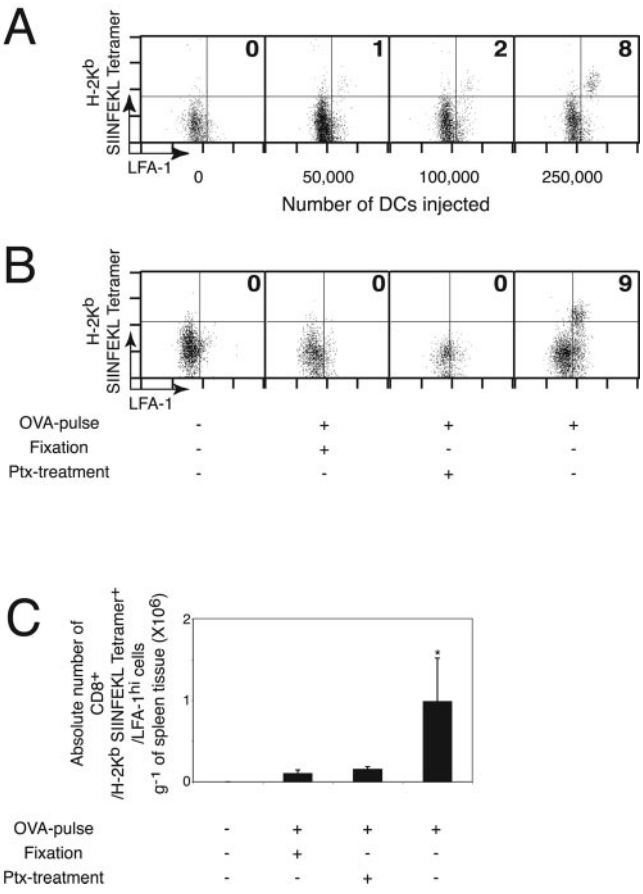

FIGURE 4. Intracerebral injection of OVA-loaded GFP-DCs leads to activation of specific $CD8^+$ cells in secondary lymphoid organs, which requires the presentation of OVA by GFP-DCs as well as their migration from the brain. *A*, Naive C57BL/6 mice were injected intracerebrally with the indicated number of OVA-loaded GFP-DCs. Seven days later, cell suspensions from spleens were prepared and stained. The dot plots show expression of LFA-1 and specific TCR on the gated $CD8^+$ lymphocyte population. Numbers in each quadrant indicate the percentage of H-$2K^b$-SIINFEKL $tetramer^+$/LFA-1^{high} cells in the quadrant from the gated population. The plots show one representative experiment of four performed. *B*, Comparison of the T cell-activating capabilities of intact, fixed, or PTX-treated GFP-DCs and unpulsed GFP-DCs after intracerebral injection into brain. GFP-DCs (250,000) were intracerebrally injected, and 7 days after injection, single-cell suspensions were prepared from spleens for flow cytometric analysis. The dot plots show the expression of LFA-1 and specific TCR on the gated $CD8^+$ lymphocyte population. Numbers in each quadrant indicate the percentage of H-$2K^b$-SIINFEKL $tetramer^+$/LFA-1^{high} cells in the quadrant from the gated population. The plots show one representative experiment of three performed. *C*, Quantification of the absolute number of activated Ag-specific $CD8^+$ T cell population in spleen of mice injected with differently treated GFP-DCs. $*$, $p < 0.05$ compared with the other groups combined. Error bars represent the SEM. $n \geq 5$ in all groups.

population was H-$2K^b$-SIINFEKL $tetramer^+$/LFA-1^{high} 7 days after intracerebral injection, possibly due to the exit of activated effector $CD8^+$ cells from LNs (not shown). These data support the conclusion that Ag-loaded GFP-DCs injected into brain parenchyma migrate to regional LNs and induce the activation of specific T cells that accumulate in secondary lymphoid organs.

We addressed the possibility that the OVA content of injected GFP-DCs could be cross-presented by endogenous APCs from the CNS before reaching the cervical LNs. We analyzed Ag-specific T cell activation in the periphery in response to fixed or PTX-treated GFP-DC injection into brain. Fixation or PTX treatment of the GFP-DCs prevented their migration out of the CNS (Fig. 3, *A* and *B*). Fig. 4*B* demonstrates that injection of fixed or PTX-treated GFP-DCs failed to induce specific $CD8^+$ T cell activation and accumulation in the spleen. The lack of specific T cell infiltration in these experiments was not due to the lack of presentation of OVA by GFP-DCs, because PTX-treated GFP-DCs activated naive OT-1 splenocytes equally well as did the untreated live OVA-pulsed GFP-DCs (data not shown). The data in Fig. 4*B* also show that the activation and infiltration of $CD8^+$ cells were Ag specific, because there was significantly less specific T cell activation when non-OVA-pulsed/live cells were injected (Fig. 4*B*). Quantification of the absolute numbers of activated Ag-specific $CD8^+$ T cells in the spleen revealed a significant enrichment of the Ag-specific T cell population in animals injected with the live, but not fixed, OVA-pulsed cells (Fig. 4*C*). Taken together, these results show that peripheral activation of Ag-specific T cells as a response to Ag-loaded GFP-DC in the CNS requires uptake, processing, and presentation of Ag and migration of GFP-DCs to peripheral lymphoid organs.

Brain-derived DCs induce preferential homing of Ag-specific T cells into brain

To determine the role of Ag-loaded DCs in inducing T cell homing into the CNS, we analyzed the frequency and absolute number of Ag-specific T cells in the CNS after Ag-loaded GFP-DC injection into brain. The injection of increasing numbers of OVA-pulsed, live GFP-DCs led to a dose-dependent increase in both the percentage and the absolute number of Ag-specific, activated $CD8^+$ T cells in brain (Fig. 5, *A* and *B*). The preferential homing of Ag-specific T cells into brain was dependent on live GFP-DCs capable of migrating out of brain tissue (Fig. 5, *C* and *D*). There was a significant decrease in the absolute number of Ag-specific T cells when fixed or PTX-treated GFP-DCs were injected into brain (Fig. 5*D*). Activated $CD4^+$ cells also accumulated in brain in response to DC injection, and the ratio of $CD4^+$ to $CD8^+$ T cells did not differ significantly from the 2:1 ratio detected in peripheral lymphoid organs (data not shown). Together, these data suggest that brain-emigrating GFP-DCs are necessary and sufficient to induce the accumulation of activated Ag-specific T cells in the brain.

Peyer's patch-derived DCs preferentially induce the accumulation of activated T cells in Peyer's patches (27). To test whether GFP-DCs need to derive from the brain to promote accumulation of T cells in the brain, we compared the effect of intracerebral and i.v. injection of OVA-loaded GFP-DCs. To exclude the possibility that accumulation of specific T cells in brain is an artifact of intracerebral injection, the animals receiving i.v. GFP-DC injection were also intracerebrally injected with medium (RPMI 1640 without serum). Intravenously injected GFP-DCs failed to induce accumulation of Ag-specific T cells in brain, leading to an ~10-fold decrease in absolute T cell number in the CNS (Fig. 5*E*). Thus, preferential homing of Ag-specific T cells into the CNS is induced by brain-derived GFP-DC migration to the periphery.

Discussion

Previous studies of DC appearance in the CNS have raised two main questions. Can these cells function in the CNS as in other organs and can these cells migrate from CNS tissue to peripheral lymphoid organs and participate in initiation of immunity against CNS Ags? Our data show that DCs accumulate in the CNS as a response to intracerebrally injected Ag and can migrate out of the CNS and induce systemic immune responses in the secondary lymphoid organs. One consequence of this migration is that Ag-specific T cells home to the brain. Thus, brain DCs behave similarly to skin-originated (7, 28) and Peyer's patch-originated (27) DCs in that they migrate from Ag injection sites to LNs and induce a

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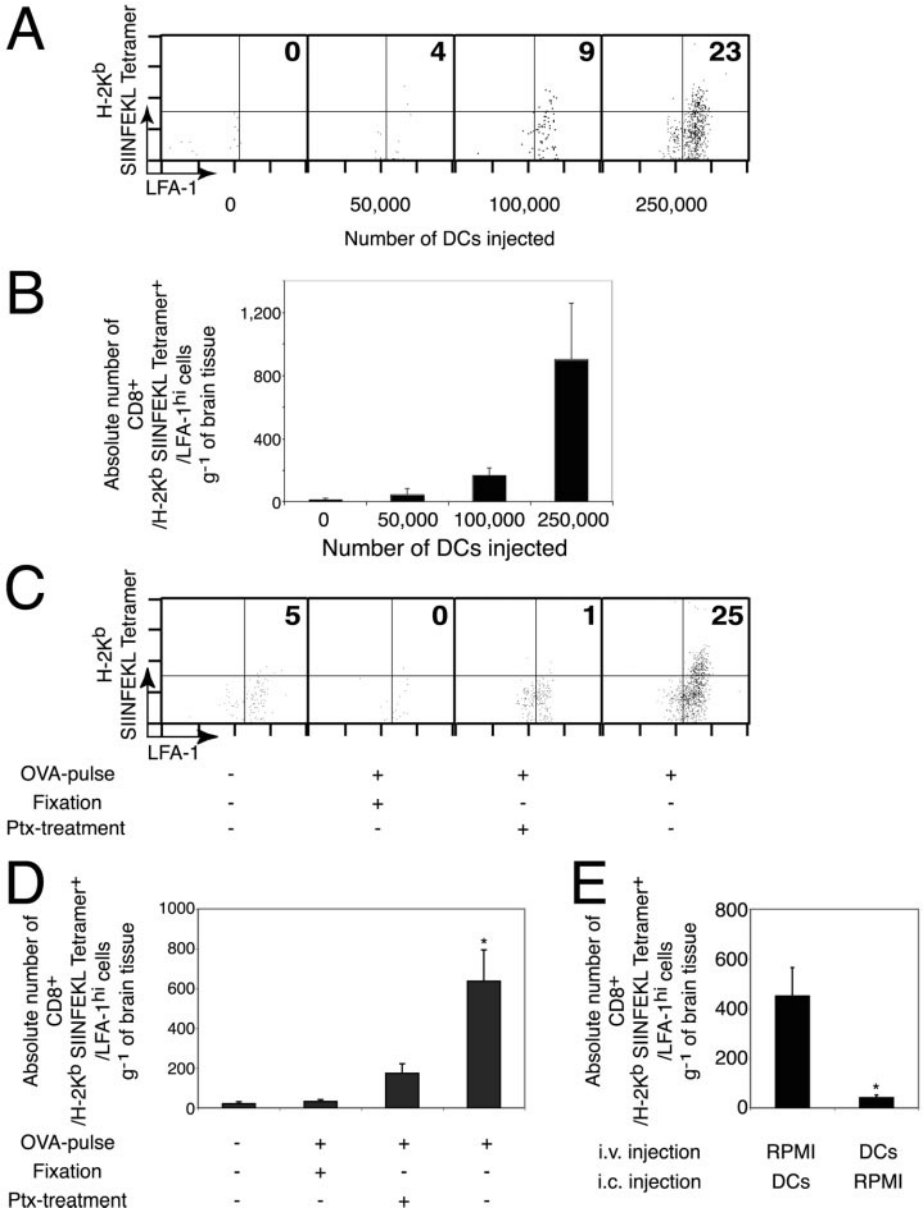

FIGURE 5. The brain-emigrant GFP-DCs induce the accumulation of specific T cells in the brain in an Ag-specific and migration-dependent manner. *A*, Naive C57BL/6 mice were injected intracerebrally with the indicated number of OVA-loaded GFP-DCs. Seven days later, cell suspensions from brains were prepared and stained. Dot plots show the expression of LFA-1 and specific TCR on the gated $CD8^+$ lymphocyte population. Numbers in each quadrant indicate the percentage of H-$2K^b$-SIINFEKL $tetramer^+$/LFA-1^{high} cells in the quadrant from the gated population. The plots show one representative experiment of four performed. *B*, Quantification of the absolute number of activated Ag-specific T cells after injection of different amounts of GFP-DCs. Error bars represent the SEM. $n = 5$ in all groups. *C*, Comparison of the T cell-activating capabilities of intact, fixed, or PTX-treated GFP-DCs and unpulsed GFP-DCs after intracerebral injection into naive mice. GFP-DCs (250,000) prepared from bone marrow of GFP-transgenic mice were injected intracerebrally into naive mice after the indicated treatment. Seven days after injection, single-cell suspensions were prepared from brains for flow cytometry. The dot plots show the expression of LFA-1 and specific TCR on the gated $CD8^+$ lymphocyte population. Numbers in each quadrant indicate the percentage of H-$2K^b$ SIINFEKL $tetramer^+$/LFA-1^{high} cells in the quadrant from the gated population. The plots show one representative experiment of four performed. *D*, Quantification of the absolute number of SIINFEKL-specific activated $CD8^+$ T cells in the brain in response to different DC injections. The graph shows the absolute number of $CD8^+$/H-$2K^b$-SIINFEKL $tetramer^+$/LFA-1^{high} cells per gram of brain tissue. $n \geq 5$ in all groups. $*$, $p < 0.02$ compared with the other groups combined. Error bars represent the SEM. *E*, Intravenously injected GFP-DCs fail to induce the accumulation of specific T cells in brain. The graph depicts quantification of the absolute number of SIINFEKL-specific activated $CD8^+$ T cells in the brain in response to different treatments. Live, OVA-pulsed GFP-DCs (250,000) were injected either intracerebrally (i.c.) or i.v. as indicated. On the same day, mice receiving i.v. DC injection received intracerebral RPMI 1640 medium (without serum; RPMI) injection and vice versa, as indicated. Seven days postinjection, mononuclear cell suspensions were prepared and analyzed by flow cytometry. The graph shows the absolute number of $CD8^+$/H-$2K^b$-SIINFEKL $tetramer^+$/LFA-1^{high} cells per gram of brain tissue. Intracerebral DC injection with i.v. medium injection, $n = 11$; i.v. DC injection with intracerebral medium injection, $n = 5$. $*$, $p < 0.01$. Error bars represent the SEM.

second wave of Ag presentation necessary for induction of immunity against tissue-localized Ag (7). Our data indicate that despite the dampened immune response at this immunologically privileged site, when an immune response is initiated, it follows the pattern previously described for Ags derived from other organs. This conclusion is based on the observation that intracerebrally injected, Ag-loaded GFP-expressing DCs accumulate in cervical LNs and induce the appearance of Ag-specific (H-$2K^b$-SIINFEKL

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tetramer$^+$), activated CD8$^+$ T cells in brain. Reverse transmigration of DCs through the endothelial cell monolayer has been demonstrated (29); however, reverse migration of DCs through the BBB has not been proven. These data correspond to previous results showing that intrathecally injected DCs migrate to cervical LNs, and CNS-derived Ags can be found in DCs in cervical LNs of monkeys with EAE (30, 31). T cell activation results from a primary response to Ag-presenting DCs and not from the cross-presentation of Ags by endogenous APCs. Thus, brain-originated DCs are important in the initiation of immunity against brain Ags.

The CNS is an immunologically privileged organ (1); however, immune responses against both pathogens and self-Ags in autoimmune demyelinating diseases point toward the presence of antigenic surveillance and inducible immune responses in this tissue. There must also be a delicate balance between induction of protective immune responses and that of autoimmune responses due to the sensitivity of irreplaceable neurons. A series of recent studies showed that DCs play an important role in the regulation of the T cell-mediated immune response against CNS-derived Ags, similarly to other tissues (32); in various diseases they accumulate in the CNS; and DCs prepared from the brain are able to induce specific T cell activation (33) or tolerance (14) in vitro.

A recent report showed that in an inflammatory skin model to achieve full activation of T cells against a skin Ag, the presentation of the Ag happens in two waves: direct drainage within a short time and DC-mediated transport thereafter. The latter type of Ag presentation accomplished by DCs emigrating from the skin is necessary to induce full T cell activation (7). However, it is not known whether presentation by only the tissue-emigrant DCs is necessary and sufficient to mediate full T cell activation. This is a fundamental question in the initiation of primary immune responses as well as for the reactivation of Ag-experienced T cells in LNs. Earlier studies from our laboratory showed that T cell accumulation in the brain peaks 7 days after intracerebral OVA injection, although direct drainage of OVA occurs within hours after Ag delivery (5). This suggests a requirement for APC migration to mediate Ag presentation at later time points. Importantly, the present study proves that brain-emigrant DC-mediated Ag presentation is necessary and sufficient to induce infiltration of the LFA-1^{high} T cell population into the brain.

DCs are present under steady state conditions in the absence of inflammation in the choroid plexus and meninges. Their number increases, and they also become detectable in brain parenchyma when inflammation is induced by pathogens or autoimmune attack (4, 11, 12, 33). More studies are needed to address whether cells with a DC phenotype that accumulate around the Ag injection site in brain are recruited from systemic sources or derived from CNS-resident cells. Mononuclear cells, probably of microglial origin, that can be isolated from brain parenchyma and cultured in the presence of GM-CSF obtain a DC-like phenotype and are able to efficiently prime naive T cells (9, 11). It is not known whether DCs present in the CNS under these conditions are able to migrate to draining LNs and function the same way as in other tissues. To our knowledge, there are no data on the ability of microglia to migrate out of the brain and present Ags to naive T cells. GM-CSF, which we used in this study to differentiate DC from bone marrow, is a major differentiation factor of DCs in brain and other organs (9, 34) and is produced in brain (35). In the absence of GM-CSF production, EAE fails to develop (36), indicating the importance of DCs in the initiation of CNS immunity.

The primary site for the interaction of DCs and T cells is the secondary lymphoid organs (37). There is limited information on how CNS DCs migrate out to LNs. Cserr and Knopf (3) demonstrated in rats that CNS Ags drain to cervical LNs via cerebrospinal fluid circulation, mostly through the cribriform plate, although the exact mechanism and types of cells transporting the Ags are not known. In this study we show a possible route of drainage to the periphery via the migration of injected DCs along the external capsule and analyze the specificity of CNS-infiltrating T cell populations. Interestingly, injected neural stem cells as well as stem cells in adult brain migrate along the external capsule in the brain (38); however, the reason for this migration pattern is currently unknown. It is possible, however, that this correlation is coincidental and not the cause of LN homing. Intracerebrally injected Ag is also distributed in this pattern, and the Ag is presented in cervical LNs (5). Our study shows that one of the possible cell types able to take up, process, and present brain Ags is the DC. Our results correspond to a previous study showing that intrathecally injected in vitro differentiated DCs accumulate in cervical LNs and induce the disproportionate recruitment of CD8$^+$ vs CD4$^+$ cells to the CNS (31). However, we did not find differences in the ratio of CD8$^+$ vs CD4$^+$ populations of T cells between the target organ and secondary lymphoid tissues (data not shown). We addressed Ag-specific CD8$^+$ T cell accumulation because MHC class I tetramers were readily available to us. We will also examine this process for CD4$^+$ T cells using moth cytochrome *c*/I-E^k tetramers that we have recently generated. Our preliminary data using this model indicate that the accumulation of Ag-specific, activated CD4$^+$ and CD8$^+$ T cells is regulated by similar mechanisms (our unpublished observations). Our previously published studies using a different model show that CD4$^+$ T cells accumulate in brain in response to intraventricular Ag delivery (39).

The injection of unloaded cells and cells fixed with 1% paraformaldehyde or treated with PTX demonstrated the specificity of the response as well as a requirement for migration of DCs to cervical LNs. Ag leaking out of fixed DCs is apparently insufficient to induce an immune response in a naive mouse. Our previous studies demonstrate that injection of OVA Ag into the brain induces immune responses in the periphery and T cell recruitment into the brain (5). The results of this study indicate that the cell type responsible for the induction of this T cell response is the DC. The timing and nature of the immune response in the present study argue that the presentation of Ag in cervical LNs is necessary for the recruitment and activation of specific CD8$^+$ T cells. DCs injected s.c. into mice accumulate in draining LNs and persist for time periods >7 days (40). Furthermore, DCs migrating out of the CNS induce T cell infiltration to the CNS (Figs. 4 and 5), showing that brain-emigrant DCs induce the accumulation of activated T cells in the CNS. Intravenous DC immunization together with intracerebral RPMI 1640 injection did not induce T cell infiltration to the CNS. This might indicate that the brain environment, the experimental injury induced by intracerebral injection, or both change the properties of DCs. Our results are in accordance with the recently described phenomenon that DCs induce the infiltration of T cells to the organ from which they originated (27). The lack of SIINFEKL-specific T cell activation in animals injected with fixed or PTX-pretreated DCs suggests that presentation of Ag by tissue-emigrating DCs is necessary, because possible cross-presentation of OVA due to local Ag processing or passive draining of OVA out of the brain failed to induce detectable SIINFEKL-specific T cell activation.

It is possible that DCs cells can migrate from brain to periphery through the bloodstream at the injury site induced by the injection. This represents one possible mechanism for our results. Indeed, DC migration from traumatic brain via the blood circulation to the periphery has very high relevance for brain Ag-induced immune responses after brain trauma. We are currently developing an in

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vivo brain traumatic injury model to explore this possibility. However, our data, presented in Fig. 3, indicate that an additional, new pathway can guide DC migration from brain to periphery. This pathway is along the external capsule in the brain. To understand the possible importance of guided DC migration from brain along the external capsule is the focus of a major effort in our laboratory. We believe that this migration pathway is important because we demonstrate that DCs need to originate from brain to induce preferential specific T cell accumulation in brain. Additionally, Fig. 5*E* demonstrates that OVA-pulsed DC injection i.v. together with RPMI 1640 medium injection into brain is not sufficient to induce preferential T cell accumulation in brain. These results do not exclude the direct route of DC exiting to blood, but show that the injury alone is not sufficient to induce T cell recruitment to the site of the injury.

In summary, Ag-loaded DCs injected into brain parenchyma are able to migrate to cervical LNs, transport Ags out of the CNS, induce a primary immune response in the periphery, and instruct Ag-specific T cells to home to the brain. It is not known whether DCs are local resident cells in the CNS or whether they infiltrate from the periphery into inflamed CNS. In either case, they are capable of migrating to secondary lymphoid tissues from the brain, which has not been proven previously. This process has significant implications for understanding the mechanism of presentation of CNS-derived Ags in immunity against foreign and self-Ags in the CNS. Immune responses are initiated in the immunoprivileged environment of the brain, much like in other organs of the body. This indicates that presentation of Ags by emigrant DCs is necessary and sufficient for peripheral Ag-specific T cell activation and consequent induction of preferential homing of these cells into the CNS.

Acknowledgments

We thank Dr. Laura Hogan, Dominic Co, and Emily Reinke for critical reading of the manuscript, and Sinarack and Khen Macvilay, Shin-Il Kim, and Tashi Kinoshita for excellent technical assistance.

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