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The Role of CD8⁺ T Cells and Their Local Interaction with CD4⁺ T Cells in Myelin Oligodendrocyte Glycoprotein35–55–Induced Experimental Autoimmune Encephalomyelitis

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T cells have an essential role in the induction of multiple sclerosis and its animal model experimental autoimmune encephalomyelitis (EAE). Although for CD4⁺ T cells it is well established that they contribute to the disease, less is known about the role of CD8⁺ T cells. Our aim was to determine the individual contribution of CD4⁺ and CD8⁺ T cells in myelin oligodendrocyte glycoprotein (MOG)35–55–induced EAE. We investigated MOG35–55–activated CD8⁺ T cells to clarify their potential to induce or attenuate EAE. We monitored the behavior of CD8⁺ T cells and their interaction with CD4⁺ T cells directly at the site of inflammation in the CNS using intravital imaging of the brainstem of EAE-affected living anesthetized mice. We found that mice without CD4⁺ T cells did not develop relevant clinical signs of disease, although CD8⁺ T cells were present in the CNS of these mice. These CD8⁺ T cells displayed reduced motility compared with those in the presence of CD4⁺ T cells. In mice that harbored CD4⁺ and CD8⁺ T cells, we saw a similar extent of clinical signs of EAE as in mice with only CD4⁺ T cells. Furthermore, the dynamic motility and viability of CD4⁺ T cells were not disturbed by CD8⁺ T cells in the lesions of these mice. Therefore, we conclude that in MOG35–55–induced EAE, CD8⁺ T cell accumulation in the CNS represents instead an epiphenomenon with no impact on clinical disease or on the effects of CD4⁺ T cells, the latter being the true inducers of the disease. The Journal of Immunology, 2013, 191: 4960–4968.

The online version of this article contains supplemental material.

Abbreviations used in this article: EAE, experimental autoimmune encephalomyelitis; EGFP, enhanced GFP; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; KIF, red fluorescent protein; TPLSM, two-photon laser–scanning microscopy; Treg, regulatory T.

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tory role of these Qa-1-restricted CD8+ cells in protecting against subsequent relapses has been confirmed in several studies (17–19), but the exact nature of the suppression is not yet clear. A recent study suggested a myelin oligodendrocyte glycoprotein (MOG)–specific MHC-I–dependent suppressor role of CD8+ T cells that were isolated from MOG35–55–immunized mice, restimulated, and transferred into ongoing EAE (20).

Therefore, as the role of different T cell subsets in EAE is still controversial, the aim of our work was to determine the contribution of CD8+ T cells in the presence and absence of CD4+ T cells in EAE. We analyzed the widely used active EAE in C57BL/6 mice with conventional MOG35–55 immunization, as this peptide has been repeatedly reported to be a reliable inducer of myelin-specific CD8+ T cells in EAE (21, 22). Whether CD8+ T cells really contribute to the disease or rather have a regulatory role, or both, is still unclear. In this study, we dissected the role of CD8+ T cells in MOG35–55–induced EAE by clinical disease monitoring, T cell phenotyping in different organs at different periods of the disease, and intravital two-photon laser–scanning microscopy (TPLSM) for motility and interactive analysis of CD8+ T cells in EAE lesions. We found that CD8+ T cells are present in the CNS of MOG35–55–induced EAE–affected mice. TPLSM revealed that there was neither interaction with nor disturbance of CD4+ T cells. Most importantly, in the absence of CD4+ T cells, the invading CD8+ T cells hardly induced any clinical disease signs, although there were CD8+ T cells accumulating in the CNS. These CD8+ T cells in the CNS did not show activation motility as seen in the presence of CD4+ T cells. We thus found neither a proinflammatory nor a suppressive effect of CD8+ T cells in conventional MOG35–55–induced active EAE.

Materials and Methods

Mice

Mice were bred under specifically pathogen-free conditions and kept in house for experiments in individually ventilated cages under specifically pathogen-free conditions. B6.Rag1−/− (B6.129S7-Rag1tm1Mom/J) mice were originally acquired from The Jackson Laboratory. B6.EGFP mice and C57BL/6 mice with omnipresent enhanced GFP (EGFP) expression under the β-actin promoter (originally acquired from The Jackson Laboratory); B6.td red fluorescent protein (RFP) have omnipresent expression of tandem-dimer RFP (23). All animal experiments were approved by local authorities and conducted according to the German Animal Protection Law. Active EAE in C57BL/6 mice was induced by s.c. immunization with 250 μg MOG35–55 peptide and 500 μg H37RA emulsified in CFA, followed by two i.p. doses of 400 ng pertussis toxin in PBS at the time of immunization and 48 h later. Induction of active EAE in Rag1−/− mice reconstituted with CD4+ and/or CD8+ T cells was induced by 100 μg MOG35–55 peptide and 400 μg H37RA emulsified in CFA, followed by two i.p. doses of 200 ng pertussis toxin in PBS at the time of immunization and 48 h later. After induction of EAE, mice were scored daily. Clinical signs of classical EAE were translated into clinical scores, as follows: 0 = no detectable signs of EAE; 1 = complete tail paralysis; 2 = partial hind limb paralysis; 3 = complete bilateral hind limb paralysis; 4 = total paralysis of forelimbs and hind limbs; 5 = death.

Cell isolation and analysis

CD4+ and CD8+ T cells, which were used for direct injection into lymphopenic hosts, were isolated from spleen and lymph node cells by negative magnetic sorting according to manufacturers’ protocols. CD8+ T cell isolation kit (CD4+ T cell isolation kit; Miltenyi Biotec). The purity of the isolated cell populations was checked by FACS surface stainings with fluorescently labeled CD4 and CD8 Abs and measured at a FACSCanto II. The purity of the negatively isolated CD8+ and CD4+ T cells was usually ~90%. In some experiments, we used FACS-sorted cells, which were usually of a purity of >90%. For lymphocyte isolation from the CNS, lethally anesthetized animals were transcardially perfused with ice-cold PBS. Brain and spinal cord were isolated, cut into small pieces, and diluted in IMDM (Life Technologies) substituted with 10 mg/ml collagenase/clostridiopeptidase (Sigma-Aldrich) and 200 U/ml DNAse (Roche). After incubation for 30 min at 37°C under continuous rotation, the CNS tissue was put through a mesh (70 μm) and mononuclear cells were separated by conventional 40/70 Percoll centrifugation. FACs analysis of surface markers was performed directly after isolation. Cytokine analysis was preceded by plate-bound anti-CD3/anti-CD28 stimulation for 4 h (brefeldin A was added after 2 h). Abs used for FACS surface and intracellular stainings are as follows: CD4–AlexaFluor 647 (Invitrogen); CD4–FITC, CD8–PE, CD8–biotin, CD62L–allophycocyanin, IFN-γ–PE, IFN-γ–V450, IL-10 allophycocyanin, IL-10–allophycocyanin, and IL-17 allophycocyanin (all from BD Biosciences); and Fox3–PE (eBioscience). MHC-Ipeptide constructs (H-2DbYRSPFSRVVHLRNYQallophycocyanin) were manufactured by Immudex and used for flow cytometry, as recommended by the manufacturer. Absolute numbers were calculated by the cell count as determined by FACS surface staining. For some experiments, several mice had to be pooled due to multiple analytic approaches. This was in particular the case for CD8–only mice, which yielded low cell numbers. Therefore, presentation of the results reflected this bias by correcting for the initial mouse numbers. Absolute numbers for cytokine-producing cells were derived from multiplication of percentage of cytokine producers with absolute count of the respective cell subset as defined by FACS surface stain analysis.

Microscopy setup and imaging

Operation procedures and TPLSM were performed, as previously described by Siffrin et al. (24, 25). Mice were anesthetized with 1.5% isoflurane in oxygen/nitrous oxide (2:1) using a facemask. The mice were then tracheotomized and continuously respirated with a Harvard Apparatus Advanced Safety Respirator. The anesthetized animal was transferred to a custom-built operation and microscopy table and fixed in a hanging position. The preparation of the imaging field was performed according to adapted protocols for cortical imaging (24). In brief, the brain stem was exposed by carefully removing musculature above the dorsal neck area and removing the dura mater between the first cervical vertebra and occipital skull bone. The head was inclined for access to deeper brain stem regions, and the brain stem was superfused with isotonic Ringer solution. A sterile agarose plate (0.5% in 0.9% NaCl solution) was installed on the now-exposed brain surface to reduce heartbeat and breathing artifacts. During surgery and microscopy, body temperature was maintained at 37°C. The depth of anesthesia was controlled by continuous CO2 measurements of exhaled gas and recorded with a CI-240 Microcanograph.

Imaging was performed using a specialized two-photon–scanning microscope (LaVision BioTec) previously described by Herz et al. (26), which allows for dual near infrared (700–1020 nm) and infrared (1050–1100 nm) excitation, that is, pulsed near infrared radiation is generated by an automatically tunable Ti:Sa laser, 10% of which is coupled into a scan head. Ninety percent of Ti:Sa laser power is coupled into a synchronously pumped optical parametric oscillator. The generated optical parametric oscillator beam first passes a system of spectral filters, enters the scan head, and overlaps the Ti:Sa beam. The colocalized beams are coupled into an upright microscope toward the objective lens (×20, NA 0.95). Fluorescence is collected by the same objective lens and directed to a spectrally resolving detection unit containing the respective dichroic mirrors, interference filters, and up to three nondescanned photomultiplier tubes for spectral separation of EGFP and RFP. XYZ stacks were typically acquired at 1-min intervals over a period of 1–2 h. Imaging depth was between 20 and 140 μm, with a usual stack covering 70 μm. The imaging field was 300 × 300 μm in the xy range.

Data analysis

Intravital images were postprocessed using acquisition software Imispector (LaVision Biotec). Three-dimensional presentation, quantitative cell-tracking analysis, and cell–cell contact determination were performed with the software Imaged (NIH), Velocity (Improvision), and Imaris (Bitplane). Statistical analysis was performed using Prism (GraphPad) software.

Cell–cell contact determination

Contacts between EGFP+ and RFP+ cells within the brain stem of imaged mice were analyzed, as described by Siffrin et al. (25). In short, double-positive EGFP/RFP voxels were highlighted by standard image analysis software. Using automated identification of double-positive voxels, a cell–cell contact determination was generated and the contact areas by white regions. To describe and compare the contact duration between EGFP+ and RFP+ cells, all individual contact areas were tracked over time. To quantitatively describe the interactivity of two differently labeled cell
populations, our group developed a means of contact quantification based on the law of mass action. By considering the volumes of the two different fluorescences as well as their contact volume and the total imaging volume, the contact index $k$ can be calculated, which is independent of the absolute number of cells. $k$ is a measure for the colocalization and therefore the interaction of two cell populations.

$$k = \frac{V_{\text{coloc}} \times V_{\text{total}}}{V_{\text{EGFP}} \times V_{\text{RFP}}}$$

Statistical analysis

Statistical analysis was carried out with GraphPad Prism 5 and SPSS 12.0. Data are usually presented as mean ± SEM. To compare two means, the unpaired $t$ test or Mann–Whitney $U$ test was used. To compare EAE curves, the nonparametric Kruskal–Wallis test was used. The $p$ values < 0.05 were considered significant.

Results

Important numbers of activated CD8$^+$ T cells are present in different phases of the disease in the CNS of mice with MOG$_{35-55}$-induced EAE

In a first step, to examine the role of CD8$^+$ T cells in EAE, the distribution and phenotype of CD8$^+$ T cells during the course of EAE in MOG$_{35-55}$-immunized C57BL/6-mice were analyzed. Immune cells from the lymph nodes, spleen, and CNS were isolated in the peak of disease and during remission. Quantification of isolated immune cells by flow cytometry revealed that ~10% of live-gated CNS-derived cells were CD8$^+$ T cells (Fig. 1A, 1B). This proportion was stable in the peak phase and the remission of EAE. Thus, CD8$^+$ T cells are about three to four times less abundant than CD4$^+$ T cells in the CNS, which could also be confirmed by quantification of absolute cell counts in the CNS of EAE-affected animals in the different phases of the disease (Fig. 1C). The phenotype analysis revealed that CD8$^+$ T cells isolated from the CNS contained large amounts of CD8$^+$ IFN-γ producers, reaching a maximum in the peak of the disease (Fig. 1D). Interestingly, no markers for regulatory T (Treg) cells, such as Foxp3 (Fig. 1E) or IL-10 (data not shown), were expressed in significant amounts by CD8$^+$ T cells in this model, neither in the periphery nor in the CNS, and neither in the peak nor remission phases. Furthermore, the proinflammatory cytokine IL-17 was also not detectable in CD8$^+$ T cells isolated from the EAE-affected CNS (Fig. 1F). Thus, important numbers of CD8$^+$ T cells are found in the CNS of MOG$_{35-55}$-induced EAE animals. We could confirm by staining with MOG-specific MHC-I/peptide multimers that there was an enrichment of MOG-specific CD8$^+$ T cells in the CNS (Supplemental Fig. 1C) in contrast to the spleen (Supplemental Fig. 1D). The percentage of Ag-specific cells was low, but within the range that has been reported for CD4$^+$ T cells in EAE (27–29). Our data are thus in agreement that the bulk of CNS-recruited T cells is not specific for the disease-inducing Ag.

Following the hypothesis that CNS-infiltrating CD8$^+$ T cells exert a function within the site of inflammation, cytotoxic activity of CD8$^+$ T cells could be directed against either the target tissue or the other inflammatory T cells, for example, CD4$^+$ T cells, and the potential outcomes would work in opposite directions. To distinguish explicitly between the influence of CD4$^+$ and CD8$^+$ T effector cells on the course of EAE, we used lymphocyte-deficient Rag1$^{-/-}$ mice, which were reconstituted with only CD4$^+$ T cells, only CD8$^+$ T cells, or a mixture of the two cell types, isolated by magnetic sorting from the spleens of wild-type C57BL/6 mice.

CD8$^+$ T cells do not efficiently induce EAE upon immunization with MOG$_{35-55}$/CFA

CD4$^+$ T cell–, CD8$^+$ T cell–, and CD4$^+$/CD8$^+$ T cell–reconstituted Rag1$^{-/-}$ mice were used after a period of 4 wk of homeostatic engraftment. This results in a stable naive/memory T cell pool, as described previously (30). To check whether the reconstitution of the mice with T cells was successful, a FACS staining for CD4 and CD8 was performed with cells isolated from peripheral blood of the reconstituted mice 4 wk after transfer of the T cells (Supplemental Fig. I). The reconstitution was successful, with 15–25% CD4$^+$/CD8$^+$ T cells of the total live cells in the blood. The T cell–replenished animals were actively immunized with the MOG$_{35-55}$ peptide in CFA. The disease course in the three different groups was monitored, and the phenotype of the T cells within the CNS of the mice in the three groups was analyzed. No significant difference in disease incidence, onset, and severity was observed between the groups that had received CD4$^+$ T cells alone or a mixture of CD4$^+$ and CD8$^+$ T cells (Fig. 2). However, mice that had received CD8$^+$ T cells alone showed a drastically reduced disease incidence and only a very mild disease course with delayed onset. In control experiments with highly purified FACS-sorted CD4$^+$ and CD8$^+$ T cells, CD8$^+$ T cell–transferred mice showed no signs of disease at all (Supplemental Fig. 2A). To exclude any artifacts that might result from using lymphocyte-

![Figure 1](http://www.jimmunol.org/) (A) Immune cells isolated from the CNS of C57BL/6 mice immunized with MOG$_{35-55}$ at the peak of disease ($n = 8$, day 13–15) and (B) in remission/chronic phase ($n = 13$, day 25–28). (C) CD8$^+$ T cells are present, but in lower numbers than CD4$^+$ T cells in the CNS of EAE-affected mice at the peak of disease and in remission. (D) Quantification of IFN-γ producers among CD8$^+$ T cells in the CNS is shown (absolute cell counts per CNS). (E) No CD8$^+$Foxp3$^+$ cells are detectable in the peak and remission phases compared with CD4$^+$Foxp3$^+$ cells. (F) CD8$^+$ T cells in the CNS produce no IL-17, in contrast to CD4$^+$ T cells. Pooled data from at least two independent experiments.
replenished lymphopenic mice, we performed T cell–depletion experiments with depleting mAbs in wild-type C57BL/6 mice. These induced CD4+ or CD8+ T cell–deficient animals showed similar disease phenotypes as the mice in the lymphopenic model (Supplemental Fig. 3a).

To investigate more closely the phenotype of the T cells within the three different groups of the lymphocyte-reconstituted Rag1<sup>−/−</sup> mice, lymphocytes were isolated from the CNS of the mice between days 26 and 36. Flow cytometric analysis of the isolated cells revealed that CD4+ and CD8+ T cells were present in the CNS, with high amounts of CD4+ T cells in the CD4-only group, high amounts of CD8+ T cells in the CD8-only group, and more CD4+ than CD8+ T cells in the group that had received both cell types (Fig. 3A, 3D), similarly to the findings for MOG<sub>35–55</sub>-immunized C57BL/6 mice (see Fig. 1). In mice that were replenished with MACS-sorted cells, the CD8-only group showed a dichotomy of strongly increased CD8+ T cells in the CNS of animals with EAE and only a mild increase of CD8+ T cells in the animals without clinical signs. This finding is analyzed further below. In animals that were replenished with FACS-sorted cells and in Ab-lymphocyte–depleted C57BL/6 mice, we did not see any signs of the disease. In these animals, a mild CD8+ T cell infiltration of the CNS (Supplemental Figs. 2b, 3c) and an increase in MOG-specific CD8+ T cells were observed relative to CFA-immunized controls (Supplemental Fig. 1c). CD4+ and CD8+ T cells isolated from the CNS produced large amounts of IFN-γ—the CD8+ T cells even more than the CD4+ T cells (Fig. 3B, 3E). A striking difference in clinical phenotype was the production of the proinflammatory cytokine IL-17 by CD4+ T cells compared with CD8+ T cells (Fig. 3C, 3F). The expression of this marker cytokine of the Th17 phenotype, which was shown to be a prerequisite for the induction of EAE (31), correlates with disease severity in this EAE model. Similar results were found in FACS-sorted lymphocyte-replenished Rag1<sup>−/−</sup> mice and Ab-mediated lymphocyte-depleted mice (Supplemental Figs. 2c, 3d). Only the groups with IL-17–expressing CD4+ T cells in the CNS showed clinical signs of disease, whereas the expression of IFN-γ by CD4+ and CD8+ T cells did not correlate with disease incidence. The question arose whether CD8+ T cells might affect induction of passive EAE, which is induced by transfer of myelin-specific CD4+ T cells. We therefore transferred in vitro differentiated encephalitogenic myelin-specific (2d2) Th17 cells into lymphocyte-replenished Rag1<sup>−/−</sup> mice and control Rag1<sup>−/−</sup> mice. In these experiments, we did not see any effect of the presence of CD8+ T cells on the clinical course of EAE (Supplemental Fig. 4a). Nevertheless, there was a strong infiltration of the CNS tissue with CD8+ T cells in this adoptive transfer EAE model in the CD8+ T cell–replenished Rag1<sup>−/−</sup> mice (Supplemental Fig. 4b). These CNS-isolated CD8+ T cells exhibited a strongly activated phenotype (Supplemental Fig. 4c). We did not detect IL-17–expressing CD8+ T cells in any of the investigated EAE models.

FIGURE 2. Disease course of Rag1<sup>−/−</sup> mice replenished with MACS-sorted CD4+ and/or CD8+ T cells and subsequently immunized with MOG<sub>35–55</sub> (day 0). Reconstitution with CD8+ T cells alone and subsequent immunization led to reduced EAE incidence and severity, with delayed disease onset. Data are shown as mean ± SEM, pooled from three independent experiments, and analyzed by Kruskal-Wallis test and Dunn’s multiple comparison posttest. A p value < 0.05 was considered significant. ***p < 0.001.

FIGURE 3. Flow cytometry data of CNS-derived immune cells isolated from MOG<sub>35–55</sub>-immunized Rag1<sup>−/−</sup> mice previously replenished with MACS-sorted CD4, CD8, or CD4+CD8 T cells. (A) Percentage of effector (CD62L<sup>−</sup>) CD4+ and CD8+ T cells in the CNS of mice from the three different groups. (B and C) Percentage of IFN-γ+ and IL-17+ cells of the CD4+ and CD8+ T cells in the CNS of mice from the three different groups. (D–F) Quantification of data as shown in (A)–(C). Data from five mice per group were pooled, shown as mean ± SEM and analyzed by Mann-Whitney U test. **p < 0.01, ***p < 0.001.
which restricted the signs of disease to CD4-harboring animals irrespective of the presence or absence of CD8\(^+\) T cells. Thus, the question of the role of CD8\(^+\) T cells in the CNS, which neither induced clinical signs nor dampened the disease, remains open. Proinflammatory and suppressor effects might balance each other out and thus not lead to a difference in signs of disease.

**CD8\(^+\) T cells do not disturb CD4\(^+\) T cell motility, nor is there any indication for contact-dependent CD8-mediated CD4 suppression in the CNS of EAE-affected mice**

To investigate the role of CD4\(^+\) and CD8\(^+\) T cells within the EAE lesions, in particular a potential CD8-mediated suppression of CD4\(^+\) T cells, we performed intravital imaging of EAE lesions. T cell dynamics can be very rapid and very interactive, which makes it difficult to track down the different steps in the cascade of damage. As the primary interaction partners of CD8\(^+\) T cells within the CNS during EAE are not known, the behavior of CD8\(^+\) T cells and their interactions with CD4\(^+\) T cells and axons were investigated using intravital TPLSM of the brain in anesthetized mice with EAE. The approach described above was adapted for use with TPLSM. Therefore, Rag1\(^{-/-}\) mice were replenished with green fluorescent (EGFP) CD4\(^+\) T cells and/or RFP CD8\(^+\) T cells, isolated from spleens of B6.EGFP and B6.RFP mice, respectively. After an engraftment period of 4 wk, mice were immunized with MOG\(_{35-55}\). Shortly after onset or at the peak of disease (between days 20 and 30), TPLSM of the upper brainstem, which is a major target site in EAE, was performed. This approach allowed for imaging the behavior of CD4\(^+\) and CD8\(^+\) T cells in living mice with EAE, independently of each other and also in combination.

In Rag1\(^{-/-}\) mice that were replenished with CD4\(^+\) and CD8\(^+\) T cells, the CD4\(^+\) T cells showed a vessel-associated behavior (Fig. 4A, left, Supplemental Video S1), whereas the CD8\(^+\) T cells did not show this confined movement. Instead, they moved rapidly through the parenchyma (Fig. 4A, right). These findings are in line with previous findings from our group using an ex vivo hippocampal slice model in which the same differential behavior of CD4\(^+\) and CD8\(^+\) T cells was shown (24). The movement of the CD8\(^+\) T cells through the parenchyma in this study did not seem to be random. The reason for this directed CD8 movement is, however, not clear, but matches the direction of axons frequently seen in experiments using mice with green fluorescent axons. It might, therefore, be due to this space restriction in the dense CNS tissue (Fig. 4B).

Analysis of the movement patterns of CD4\(^+\) T cells and CD8\(^+\) T cells in mice reconstituted with CD4\(^+\) and CD8\(^+\) T cells revealed that the mean velocity of both cell types was dependent on the disease stage, with cells at the onset of disease showing a significantly higher mean velocity than cells at the peak of disease (Fig. 4C). At the onset of disease, CD4\(^+\) T cells (0.085 ± 0.002 \(\mu\)m/s) moved faster than CD8\(^+\) T cells (0.076 ± 0.01 \(\mu\)m/s), whereas, at the peak of disease, no difference between CD4\(^+\) T cell velocity (0.059 ± 0.002 \(\mu\)m/s) and CD8\(^+\) T cell velocity (0.061 ± 0.002 \(\mu\)m/s) was observed (Fig. 4C, 4D). Similar behavior was observed for the displacement rate of the CD4\(^+\) and CD8\(^+\) T cells. The displacement rate is a measure of how fast an object moves away from its starting point, so, in comparison with velocity, it also includes a measure of the directionality of the movement. For cells moving in a directional way, the displacement rate is higher than for cells that show a spatially confined movement. In this study, displacement rates for CD4\(^+\) and CD8\(^+\) T cells were higher at the onset of disease than at the peak (Fig. 4D). At the onset of disease, the displacement rate of CD4\(^+\) T cells (0.047 ± 0.002 \(\mu\)m/s) was higher than that of the CD8\(^+\) T cells (0.036 ± 0.001 \(\mu\)m/s), whereas no difference between the two cell types was observed at

**FIGURE 4.** Analysis of CD8 and CD4 trajectories in Rag1\(^{-/-}\) mice reconstituted with CD4-EGFP and CD8-RFP cells and subsequently immunized with MOG\(_{35-55}\). (A) Tracks of CD4\(^+\) T cells recorded over time show a vessel-associated behavior (left). Vessels were stained by perfusion of the mouse with FITC-dextran. Tracks of CD8\(^+\) T cells show a directional movement of the CD8\(^+\) T cells in the parenchyma (right). One example of nine imaged mice is shown. (B) Axons visualized in a mouse with green fluorescent neurons (B6.Thy1.21.EGFP); same imaging technique as in (A). Velocity (C) and displacement rate (D) of CD4\(^+\) and CD8\(^+\) T cells were analyzed at the onset and at the peak of disease. Cell tracks of four movies per time point were pooled, shown as mean ± SEM, and analyzed by Mann–Whitney \(U\) test: \(p < 0.05\) was considered significant, \(*p < 0.01\), \(* * p < 0.001\). Analysis of CD4 movement in Rag1\(^{-/-}\) mice reconstituted with CD4-EGFP and CD8-RFP cells (CD4\(^+\)CD8\(^-\)) or CD4-EGFP cells only (CD4 only) and subsequently immunized with MOG\(_{35-55}\). Velocity (E) and displacement rate (F) of CD4\(^+\) T cells in the two groups were analyzed at the peak of disease. Cell tracks of four movies per time point were pooled, shown as mean ± SEM, and analyzed by Mann–Whitney \(U\) test. A \(p\) value < 0.05 was considered significant. (G) Visualization of contacts (white; some indicated by white arrows) between CD8-RFP cells (red) and CD4-EGFP cells (blue) in Rag1\(^{-/-}\) mice reconstituted with CD4\(^+\)CD8\(^-\) T cells and subsequently immunized with MOG\(_{35-55}\), imaged at the peak of disease.
the peak of disease (CD4, 0.029 ± 0.002 µm/s; CD8, 0.027 ± 0.001 µm/s).

To examine whether the presence of CD8+ T cells influenced the behavior of CD4+ T cells, cell movement of CD4+ T cells in Rag1−/− mice reconstituted with CD4+ and CD8+ T cells was compared with movement of CD4+ T cells in Rag1−/− mice reconstituted with CD4+ T cells alone. Mice from the two groups were imaged at the peak of disease. The mean velocity and displacement rate of the CD4+ T cells were comparable in both groups (Fig. 4E, 4F). In the mice that had been reconstituted with CD4+ and CD8+ T cells (CD4+CD8), the mean velocity of the CD4+ T cells was 0.59 ± 0.002 µm/s compared with a mean velocity of 0.063 ± 0.002 µm/s in the mice reconstituted with CD4+ T cells only (CD4 only) (Fig. 4E). The mean displacement rate of the CD4+ T cells in the CD4+CD8 group was 0.029 ± 0.002 µm/s compared with 0.026 ± 0.002 µm/s in the CD4-only group (Fig. 4F). The presence of CD8+ T cells therefore did not influence the behavior of the CD4+ T cells, indicating that they did not influence the activation status or viability of CD4+ T cells.

To explicitly study the interaction of CD8+ T cells with CD4+ T cells as potential target cells in the CNS, the recorded time-lapse image stacks were further analyzed for interactions between CD8-RFP T cells and CD4-EGFP T cells using a method developed in our group (25). Using this technique, the nonoverlapping spectra of EGFP and RFP are exploited to measure the extent of contact formation of green and red fluorescent cells, which can be visualized in a third-color channel, shown in this study in white (Fig. 4G, Supplemental Video 2). The area of contact formation (voxels positive for green and red fluorescence only in areas of close proximity) can subsequently be followed over time and then quantified. To describe the interactivity of two differently labeled cell populations, the contact index $k$ can be calculated as a means of contact quantification. In this study, from time-lapse imaging of four different mice, very low $k$ values between 0.273 ± 0.042 and 0.439 ± 0.045 were calculated, indicating only very few long-lasting specific contacts between CD4+ and CD8+ T cells at the peak of disease, which is very much lower than the $k$ values for CD4+ T cell–neuronal interactions as reported from our group ($k =$ 2–5) (25). Thus, a direct cytotoxic effect of CD8+ T cells on CD4+ T cells was not obvious in these EAE lesions.

CD8+ T cells in the CNS lack motility in the absence of CD4+ Th17 cells

The EAE incidence of CD8-harboring mice was very low. Only 3 of 13 mice showed classical signs of the disease. Furthermore, the clinical signs of disease were mild in CD8-replenished and MOG35–55/CFA–immunized Rag1−/− mice. Flow cytometry of CNS-derived lymphocytes revealed that CD8+ T cells were indeed present in the CNS of these mice. Large numbers of memory/effector CD8+ T cells could be isolated from CD8-only mice with clinical signs of disease (Fig. 5A). After stimulation, these CD8+ T cells produced large amounts of IFN-γ. Interestingly, the large number

**FIGURE 5.** Analysis of CD8 phenotype and behavior in CD8-only mice. (A) FACS analysis of CNS-derived lymphocytes of an EAE-affected CD8-only mouse (score 3). After stimulation with anti-CD3/anti-CD28, CD8-gated CNS-derived lymphocytes show strong IFN-γ production. (B) FACS analysis of CNS-derived lymphocytes of clinically unaffected CD8-only mouse (score 0). After stimulation with anti-CD3/anti-CD28, CD8-gated CNS-derived lymphocytes show strong IFN-γ production. (C) Comparison of total numbers of CD4+ IL-17–producing cells (black squares), total numbers of IFN-γ–producing CD8+ T cells (black triangles), and relative numbers of IL-17–producing cells of CD4+ T cells (red squares) in CD4-only, CD4+CD8, and CD8-only mice. The circles highlight data of individual mice according to the score of the respective mice. (D) Visualization of CD8-RFP cells (red) in B6.Rag1−/− mice reconstituted with CD8-RFP cells (CD8-only mice) and subsequently immunized with MOG35–55. CD8-only mice were imaged on day 28–30 after immunization (score between 0 and 1.25). (E) Velocity of CD8 T cells in CD8-only mice in comparison with the velocity of CD8 T cells in different phases of EAE in CD4+CD8 mice. Data from at least two mice per group were pooled, shown as mean ± SEM, and analyzed by the Mann–Whitney U test. ***$p < 0.001$. 

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of CD8-only mice without clinical signs also harbored similarly activated IFN-γ-producing CD8⁺ T cells as the affected mice, although in lower numbers (Fig. 5B, 5C). When we compared the profiles of CD8⁺ and CD4⁺ T cells, it became obvious that the CD8-only mice with clinical EAE signs displayed IL-17-producing CD4⁺ T cells in the CNS. The presence of CD4⁺ T cells did not only correlate with clinical signs of disease, but also with large-scale recruitment of memory/effector CD8⁺ T cells (Fig. 5C). Interestingly, these EAE-affected mice had in total very low absolute numbers of CD4⁺ IL-17⁺ T cells. In clinically normal CD8-only mice, similar amounts of CD4⁺ T cells were also found, which were, however, not producing important amounts of IL-17 (Fig. 5C). To study in more detail the behavior of CD8⁺ T cells in the CNS, mice that had been reconstituted with CD8⁺ T cells alone and subsequently immunized with MOG35–55 were imaged and analyzed. Mice were imaged using TPLSM between days 28 and 30 after immunization. Analogously to the experiments shown in Fig. 3, these mice did not show signs of EAE and CD8-RFP cells were present in the CNS (Fig. 5D). However, in contrast to the rapid and directed movement of the CD8⁺ T cells within the parenchyma in mice reconstituted with CD4⁺ and CD8⁺ T cells (see Fig. 4A), the CD8⁺ T cells in the mice reconstituted with CD8⁺ T cells only showed slow movements, that is, rather static behavior with strongly reduced velocity (Fig. 5D, 5E). The low velocity in all of the acquired time-lapse recordings indicated that the static behavior of the CD8⁺ T cells was probably due to an improper activation of the CD8⁺ T cells or a lack of local inflammatory milieu that might be present only if CD4⁺ T(h17) cells are available. In addition, low CD8⁺ T cell motility supports the lack of local reactivation in the absence of CD4⁺ T cells in the investigated active EAE model.

Discussion

MS has long been considered a prototypic CD4⁺ T-cell–mediated autoimmune disease, whereas the role of CD8⁺ T cells in MS and EAE remains controversial. The reason for this CD4 bias is the relatively strong association of MS susceptibility with MHC class II alleles (32) and the fact that CD4⁺ T cells are the main effector T cells in animal models of MS, such as EAE or Thielers’s murine encephalomyelitis virus–induced demyelinating disease. In general, CD8⁺ T cells, in contrast to CD4⁺ T cells, are equipped with the mechanisms to directly kill target cells via secretion of lytic granules containing perforin and granzymes. A pathogenic role for CD8⁺ T cells in MS has been proposed, as CD8⁺ T cells have been reported to be present in inflammatory brain lesions and demonstrate oligoclonal expansions in MS brain and CSF (2, 5, 33). A detrimental function of CD8⁺ T cells is further supported by the fact that MHC class I can be expressed under inflammatory conditions on potential target cells within the CNS, such as neurons and oligodendrocytes (3), and the ability of CD8⁺ T cells to kill oligodendrocytes and neuronal cells in vitro (34, 35). Direct evidence for CD8⁺ T cells as inducers of EAE in vivo or MS has not, however, been presented to date. Open questions in this context are as follows: Do CD8⁺ T cells induce the disease, contribute to the damage processes, or kill the self-reactive CD4⁺ T cells or APC, thereby regulating the disease? Or are they just an epiphenomenon like intrathecal Ab production is presumed to be?

We addressed these questions by investigating MOG35–55/CFA–induced EAE in lymphopenic mice that had been replenished with CD4⁺ and/or CD8⁺ T cells. We used the mice after a period of 4 wk for homeostatic proliferation, this period of time after engraftment having been shown to result in stable naïve/memory T cell pools (30). Due to immunization in CFA, this model is CD4-biased, but CD8⁺ T cells were also reported to be induced in this model (7, 21, 22). In line with these findings, we report in this study important numbers of activated, proinflammatory CD8⁺ T cells in the CNS of MOG35–55-immunized C57BL/6 and T cell–reconstituted lymphopenic mice. In fact, primarily CD8⁺-biased EAE models have rarely been described until now, which might be due to the more complex modes of activation, for example, by viruses or other infectious agents that abrogate tolerance in myelin-specific CD8⁺ T cells, which generally seems to be more challenging than breaking CD4 tolerance (36). A recent study showed that transgenic CD8⁺ T cells induced inflammatory CNS lesions. The disease course and pathology, however, differed from conventional CD4-mediated EAE. In this model, preactivated CD8⁺ T cells specific for an influenza hemagglutinin peptide, which were transferred into transgenic mice with hemagglutinin-expressing oligodendrocytes, induced mild clinical symptoms such as weight loss, but no pronounced paralysis or neurologic signs (9). This approach highlights the difficulty of mimicking MS-like disease in clinical terms in a CD8-restricted context. In another study, a humanized mouse model was generated in which the MS-associated MHC class I molecule HLA-A3 and a myelin-specific autoreactive TCR derived from a CD8⁺ T cell clone of a MS patient were expressed in mice (37). In this study, CD8⁺ T cells also induced a MS-like disease via infiltration of CD8⁺ T cells. However, CD4⁺ T cells were shown to be essential for progression of the disease and the development of more severe clinical symptoms, which underlines the necessity of CD4⁺ T cells for clinical disease in this model. With our approach, we showed that in CD8-only mice (Rag1⁻/⁻ replenished with CD8⁺ T cells) there was strongly reduced clinical EAE, although CNS infiltration of CD8⁺ memory/effector T cells was obvious. In fact, only if CD4⁺ Th17 cells were present, clinical signs of the disease could be seen in CD8-only mice. TPLSM of mice that had received only CD8⁺ T cells and showed no clinical signs of disease also showed low migratory capacity of these CD8⁺ T cells in the CNS. This contrasts with observations that highly activated CD8⁺ T cells have the capacity to move rapidly through CNS parenchyma (24, 38). Therefore, this indicates a lack of local reactivation or local inflammatory signals in the CD8-only mice. Similar low-motility patterns were shown for nonactivated T cells that did not find adequate migratory signals in CNS tissue (39). In contrast, when CD4⁺ T cells were present, the clinical disease course of CD4⁺CD8⁻ mice in comparison with CD4-only mice was strongly disabling, yet similar in both groups, which indicates that the CD4⁺ T cells are the inducers of the clinical disease. Interestingly, even very low numbers of CD4⁺ Th17 cells were sufficient to induce clinical signs of EAE in CD8-only mice with a massive recruitment of CD8⁺ T cells to the CNS. In the presence of CD4⁺ T cells, the CD8⁺ T cells showed the behavior of typical activated CD8⁺ effector T cells with rapid migration through the CNS parenchyma. The CD8⁺ T cells were present in the CNS in high numbers in these mice and showed rapid motility, similar to the CD4⁺ T cells. However, CD8⁺ T cells did not aggravate the CD4-mediated disease in this model.

Although the role of CD8⁺ T cells in EAE and MS is not fully understood, there are convincing data for the existence of regulatory CD8⁺ T cells that contribute to self-tolerance (1, 40). Different approaches for abolishing CD8 T cells showed rather a protective effect of CD8⁺ T cells (10, 11). Furthermore, a CD8⁺-mediated suppression of autoreactive CD4⁺ T cells in a Qu-1 (non-classical MHC-I molecule)–restricted way has been described (16–19), as well as direct suppression of MOG-specific CD4⁺ T cells by MOG-specific CD8⁺ T cells, which were conditioned by Ag-specific restimulation in vitro (20). In our work, a regulatory/suppressor role of CD8⁺ T cells, that is, a CD8 suppressor effect
on CD4+ T cells, was not observed in the CD4-plus-CD8 mice. In clinical terms, the group that received CD4+ and CD8+ T cells did not show an alleviated disease course compared with the group that received CD4+ T cells alone. The CD4-mediated disease course was so severe and nonremitting in the applied model that no remission phase could be observed. Importantly, the role of the CD8+ T cells in inducing the remission phase of the disease could also not be properly addressed by clinical observation in these two groups in this approach, because of the severity and nonremitting nature of the disease course in replenished lymphopenic mice, which might be intrinsic to the model. The most elusive information comes from analyzing the T cell populations of interest at the site of inflammation (41). It has previously been shown that T cell motility is highly correlated with their status of activation (42), as well as the suppression of T cells being correlated with changes in migration pattern (43, 44). We therefore used TPLSM to investigate whether CD4 motility changes or a contact-dependent CD8–CD4 interaction might be present subclinically. However, the presence of the CD8+ T cells had no effect on the behavior of the CD4+ T cells. In addition, there was no strong interaction of CD8+ T cells and CD4+ T cells as shown by TPLSM and contact evaluation. Using TPLSM, it has previously been shown that Treg cells influence the behavior of autoantigen-specific Th cells in the lymph node (43, 44). In these publications, the first indication of a regulatory effect of the Treg cells was the changed behavior, that is, increased speed, of the autoreactive effector T cells in the presence of Treg cells compared with their absence. Although the regulatory mechanism was not attributed to a direct interaction of Treg and effector T cells, the Treg compromised the Ag-dependent arrest of the effector T cells by reducing their interactions with dendritic cells. Concerning a CD8-mediated cytotoxicity on encephalitogenic CD4+ T cells, it has been described that peptide vaccination with 1-9NacMBP (normally protecting against subsequently induced EAE) as well as the unrelated peptide P277 (normally protecting against spontaneous development of type-I diabetes) induced cross-protection mediated by CD8+ T cells, as both vaccination strategies protected against subsequently induced EAE as well as type 1 diabetes independently of each other (45). In our experiments, neither changes of velocity in CD4+ T cell motility nor long-lasting contacts between CD4+ and CD8+ T cells were observed in the CNS of EAE-affected mice, which would be typical and necessary for cytotoxicity (46). However, we cannot definitively exclude mixed pro- and anti-inflammatory effects that lead to a neutral clinical effect, especially if they are effective outside the CNS. To date, only one publication has reported a regulatory as well as pathogenic role of CD8+ T cells in a single EAE model (47).

Taken together, we were able to show in this study that important numbers of CD8+ effector/memory cells are generated upon immunization with MOG₃₅₋₅₅ in C57BL/6 mice. CD8+ T cells can be found in the CNS of EAE-induced mice. However, clinical evaluation and analysis of T cell behavior within CNS inflammatory lesions supported neither a proinflammatory nor a suppressive role in this EAE model. Only in the presence of CD4+ T cells was full induction of EAE observed. Within the CNS, we found no effect of CD8+ T cells on CD4+ T cell motility, the latter cell subset being the relevant lymphocyte population for disease induction. Therefore, these findings suggest that the presence of CD8+ T cells within inflammatory CNS lesions might be an epiphenomenon rather than a disease-relevant feature. It remains open as to how far this is also applicable to the human situation, in which long-standing disease, regular challenges with viruses and bacteria from the environment, as well as interindividual differences in genetic susceptibility are important CD8-relevant factors. However, our findings prove that the presence of a certain immune cell subset is not necessarily associated with the pathophysiology of disease induction or damage processes.

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Disclosures

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References