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Analysis of Autoreactive CD4 T Cells in Experimental Autoimmune Encephalomyelitis after Primary and Secondary Challenge Using MHC Class II Tetramers


Experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, is primarily mediated by CD4 T cells specific for Ags in the CNS. Using MHC class II tetramers, we assessed expansion and phenotypic differentiation of polyclonal self-reactive CD4 T cells during EAE after primary and secondary challenge with the specific Ag. After EAE induction in SJL mice with proteolipid protein 139–151, CNS-specific T cells up-regulated activation markers and expanded in the draining lymph nodes and in the spleen. Less than 20% of total autoreactive T cells entered the CNS simultaneously with Th cells of other specificities. Almost all tetramer-positive cells in the CNS were activated and phenotypically distinct from the large peripheral pool. When EAE was induced in Ag-experienced mice, disease symptoms developed earlier and persisted longer; autoreactive T cells were more rapidly activated and invaded the CNS earlier. In striking contrast to specific CTLs that respond after secondary viral challenge, the absolute numbers of autoreactive CD4 T cells were not increased, indicating that the accelerated autoreactivity in Ag-experienced mice is not related to higher frequencies of autoreactive CD4 T cells. The Journal of Immunology, 2004, 172: 2878–2884.
Materials and Methods

Mice, Ags, and Abs

SJL mice were purchased from Harlan Winkelmann (Borchten, Germany) and maintained in the animal facility at the Institute for Cell Biology, University of Tübingen. In all experiments, female 6- to 12-wk-old mice were used according to approved protocols. The peptides MBP84–96 (VHVFKKNIrTPRTPL, PLP139–151 (HLCGKWLGHPDKF), and myelin oligodendrocyte glycoprotein 92–106 (MOG92–106) (DEGGYTCF FRDHSYYQ) were synthesized using standard γ-fluorenylmethoxycarbonyl chemistry and purified by HPLC. All Abs were purchased from BD Biosciences (Hamburg, Germany).

Generation of T cell lines

Mice were immunized s.c. with 50 nmol of the respective Ag in CFA containing 50 µg Mycobacterium tuberculosis H37RA (Difco, Augsburg, Germany). Eight days later, single cell suspensions of DLN were prepared and cultured in vitro together with 10 µg/ml Ag in IMDM (Life Technologies, Invitrogen, Karlsruhe, Germany) containing 10% FBS (Life Technologies, Invitrogen). After 96 h of culture, cells were used for flow cytometric analysis.

Production of MHC II tetramers

Recombinant MHC class II tetramers were produced essentially as described (20, 21). The cDNA for the I-Aα- and β-chains was kindly provided by S. Miller (Northwestern University, Chicago, IL). The α-chain was elongated by overlapping PCR with sequences encoding for an acidic zipper sequence and a His6-tag and the β-chain with the complementary basic zipper sequence and the BirA-dependent biotinylation substrate sequence (22). The cDNAs encoding for the peptides MBP84–96 (VHVFKKNIrTPRTPL) and PLP139–151 (HLCGKWLGHPDKF) were attached to the 5′ end of the β-chain via a 6-aa linker. Corresponding α- and β-chains were cloned into the baculovirus transfer vector pAcDB3 (BD Biosciences) under control of individual p10 promoters. Recombinant baculoviruses were generated using the BaculoGold system (BD Biosciences). Recombinant monomers were purified under native conditions from the supernatant using Ni²⁺ chromatography (Ni-NTA; Qiagen, Hilden, Germany) containing 10% FBS (Life Technologies, Invitrogen). As a positive control for the tetramer signal, control stainings with the irrelevant tetramer were included in each analysis as a specificity control.

CFSE labeling and intracellular cytokine staining

Primary T cell cultures prepared from DLN 8 days after immunization with PLP139–151 were subjected to two rounds of in vitro restimulation with 10 µg/ml PLP139–151 peptide, labeled with 5 µM of CFSE (Molecular Probes, Mobitec, Göttingen, Germany), as indicated by the manufacturer. As a positive control for the detection of IFN-γ cells, cells were stained with the indicated tetramers overnight at 4°C, stained with anti-CD4 Ab (6 µg/ml; BD Biosciences) for 20 min, incubated with Cytofix/Cytoperm (BD Biosciences), and stained with PE-labeled anti-IFN-γ (BD Biosciences), as indicated by the manufacturer. CFSE-labeled cells were analyzed by flow cytometry.

Results

Detection of CNS-specific T cells

To identify autoreactive T cells, fluorescently labeled tetramers of recombinant peptide/MHC class II complexes (20, 21) were generated, in which the epitopes MBP84–96 or PLP139–151 are linked to the β-chain of the I-Aα MHC class II molecule. MHC
class II multimers have previously been used to detect CD4 T cells specific for viral Ags (27, 28) and self Ags (29), including Ags expressed in the CNS (30). The specific binding of the tetramers was first assessed by flow cytometry of polyclonal, in vitro restimulated lymph node cells isolated 8 days after immunization with either of the respective Ags MBP84–96 or PLP139–151, (31). The I-A\(^{a}\)-MBP84–96 tetramers stained T cells against MBP84–96, but not those specific for PLP139–151 (Fig. 1A), whereas I-A\(^{a}\)-PLP139–151 tetramers bound only to PLP139–151-specific T cells. These data demonstrate that tetramers bind specifically to T cells with their respective target Ag, as there was virtually no staining with T cells of other specificities. In all subsequent experiments, stainings were performed in parallel with I-A\(^{a}\)-PLP139–151 and I-A\(^{a}\)-MBP84–96 tetramers as specificity controls.

We then established the ability of the tetramers to identify specific T cells in mice with PLP139–151-induced EAE directly ex vivo. Cells derived from DLN and CNS of mice with PLP139–151-induced EAE stained with I-A\(^{a}\)-PLP139–151 tetramers, but not with I-A\(^{a}\)-MBP84–96 tetramers (Fig. 1B), while DLN cells of unimmunized mice were tetramer negative. In addition, there was no tetramer staining in DLN cells of mice that were immunized with CFA alone and received pertussis toxin i.v. (data not shown). I-A\(^{a}\)-PLP139–151 tetramers thus specifically detect polyclonal autoreactive T cells in actively induced EAE directly ex vivo.

FIGURE 2. Staining characteristics of tetramers. A, I-A\(^{a}\)-PLP tetramers bind specifically to blasting cells in polyclonal, PLP139–151-activated T cells in vitro. Polyclonal T cells were incubated with PLP139–151 peptide for 6 days and stained with anti-CD4 and I-A\(^{a}\)-PLP139–151 or I-A\(^{a}\)-MBP84–96, respectively. Histograms display tetramer binding to small (R2) and blasting (R1) CD4 T cells. B, I-A\(^{a}\)-PLP139–151 tetramers bind to T cells that proliferate in response to Ag-specific activation. Polyclonal, CFSE-labeled T cells were activated with PLP139–151 for 6 days and stained for CD4 and I-A\(^{a}\)-PLP139–151 or I-A\(^{a}\)-MBP84–96, as indicated. Density plots are gated on live CD4 T cells. C, Tetramers bind to T cells that produce IFN-\(\gamma\) after Ag-specific stimulation. T cells were prepared from DLN of PLP139–151 peptide-immunized mice and cultured in vitro with PLP139–151 for 6 days. Cells were stained with I-A\(^{a}\)-PLP139–151 tetramer and Abs against CD4 and intracellular IFN-\(\gamma\). Dot plots are gated on CD4:\(\text{H}1\)100 cells. D, A as a positive control, cells were activated for 4 h with 5 ng/ml PMA and 500 ng/ml ionomycin in the presence of 1 \(\mu\)M brefeldin A to block cytokine secretion. Negative controls were performed by staining PMA/ionomycin-activated cells with I-A\(^{a}\)-MBP84–96 tetramers and an isotype control Ab for anti-IFN-\(\gamma\) (data not shown). E, Tetramer staining triggers ultrastructural changes in Ag-specific T cells. A PLP139–151-specific T cell line was incubated with I-A\(^{a}\)-PLP139–151 tetramers (red) for 4 h at 4\(\degree\)C or 37\(\degree\)C, as indicated, and stained with anti-CD4 (green) for 20 min on ice. Incubation at 37\(\degree\)C, but not at 4\(\degree\)C, leads to internalization of the tetramer signal.

FIGURE 3. Detection of Ag-specific T cells in situ. Single cell suspensions of DLN (A) and life tissue sections of DLN (B) and CNS (C) of mice with PLP139–151-induced EAE were stained with CD4 and I-A\(^{a}\)-PLP139–151 and I-A\(^{a}\)-MBP84–96 tetramers, respectively. Thirty-two of 748 CD4:\(\text{H}1\)100 cells were I-A\(^{a}\)-PLP139–151:\(\text{H}1\)100. In control sections, none of 578 CD4:\(\text{H}1\)100 cells stained with I-A\(^{a}\)-MBP84–96 (data not shown).
To further characterize the sensitivity and specificity of the tetramer-staining reaction, tetramer binding was correlated to activation, proliferation, and cytokine production following Ag-specific stimulation in vitro. Polyclonal T cell lines were used in these assays that had undergone minimal in vitro restimulation and thus as closely as possible resemble the endogenous T cell repertoire. Six days after stimulation with PLP139–151 peptide, I-A<sup>-</sup>–PLP139–151 tetramers bound exclusively to activated, blasting cells and not to small resting cells, as determined by forward/side light scatter discrimination (Fig. 2A). Tetramer staining was then correlated to Ag-specific proliferation. To this end, CFSE-labeled T cells were incubated with APCs and PLP139–151 peptide for 6 days and stained with tetramers and anti-CD4. I-A<sup>-</sup>–PLP139–151 tetramers bound specifically to cells that had proliferated (i.e., were CFSE low) (Fig. 2B). A proportion of proliferating cells remained tetramer negative, indicating that tetramer staining does not detect all cells that proliferate in response to peptide-specific stimulation. We next combined the tetramer-staining reaction with staining for intracellular cytokines. DLN cells of peptide-immunized mice were activated in vitro with PLP139–151 peptide for 6 days and stained with I-A<sup>-</sup>–PLP139–151 tetramers and an Ab against intracellular IFN-γ. The majority of cells that produced IFN-γ were tetramer positive, and there was also a population of tetramer-positive cells that did not contain any detectable IFN-γ (Fig. 2C). To assess whether the tetramer-staining reaction induces morphological changes in specific T cells, we performed confocal imaging on T cells that were labeled with tetramers and anti-CD4 Abs (Fig. 2E). Incubation of specific T cells with I-A<sup>-</sup>–PLP139–151 tetramers at 37°C for 3 h resulted in internalization of the tetramers, whereas internalization was blocked at 4°C. Because TCR internalization is a hallmark of T cell activation, this indicates that incubation with the tetramers at 4°C does not induce significant T cell activation. In all subsequent experiments, tetramer staining was performed at 4°C.

Next, MHC class II tetramers were used to directly examine specific T cells in CNS sections of diseased animals in situ. For this purpose, we adapted a technique that recently had been developed for in situ detection of Ag-specific CD8<sup>+</sup> T cells (26). Single cell suspensions and life tissue sections of mice with PLP139–151-induced EAE were stained with mAbs against CD4 and I-A<sup>-</sup>–PLP139–151 or I-A<sup>-</sup>–MBP84–96 tetramers and examined by CLSM. Cells that stained for CD4 and I-A<sup>-</sup>–PLP139–151 tetramers were present in DLN and in the CNS of diseased animals (Fig. 3), while binding of the I-A<sup>-</sup>–MBP84–96 tetramers was not observed (data not shown). This method therefore provides the opportunity to directly examine autoreactive Th cells within their anatomical context.

**Autoimmunity after primary and secondary antigenic challenge**

The influence of an apparent antigenic challenge on the subsequent development of EAE was assessed. To this end, mice were primed with 25 nmol of PLP139–151. This first immunization was not sufficient to induce any clinical signs of autoimmunity. CNS sections of these mice were examined by conventional histology using H&E staining. No signs of inflammation could be detected (data not shown), indicating that this primary challenge did not induce any CNS inflammation. Four weeks later, EAE was induced and compared with EAE induced in previously unprimed and CFA-primed mice. EAE developed faster and persisted longer in mice after secondary challenge with the specific Ag (Fig. 4). We then compared expansion and phenotypic changes of autoreactive T cells after primary vs secondary challenge.

**Expansion of autoreactive T cells in PLP139–151-induced EAE**

After EAE induction in previously unprimed mice, the absolute numbers of CD4<sup>+</sup>/I-A<sup>-</sup>–PLP139–151<sup>+</sup> cells in the DLN increased until day 8 and subsequently declined (Fig. 5A). The numbers of PLP139–151-specific cells peaked in the spleen at day 10, and a
FIGURE 6. Activation phenotype of autoreactive T cells. Expression of CD25, CD44, and CD69 by CD4+ I-A\textsuperscript{s}-PLP139–151 and CD4+ I-A\textsuperscript{s}-PLP139–151 cells was determined by flow cytometry in mice in which EAE induction was a primary or secondary antigenic contact (n > 6 per time point; error bars indicate SD). Expression of CD25 and CD69 increased faster and peaked earlier after secondary as compared with the primary response (p = 0.031 for CD25 and p = 0.000026 for CD69, ANOVA).

Discussion

The immune system reacts faster and stronger upon re-encounter of the same Ag. This also holds true when the immune response is directed against self Ags, as demonstrated in the experiment shown in Fig. 4. The primary immunization did not induce any clinical or histological signs of CNS inflammation, and is thus representative of a clinically silent challenge with the Ag. Disease symptoms developed faster and persisted longer in mice that had encountered the Ag before. The maximal disease score was not increased in Ag-experienced mice most likely because primary EAE induction already causes very severe symptoms and a further increase in inflammation may thus not be reflected in a further increase in the disease score. A previous challenge with a self Ag thus enhances the subsequent development of autoimmunity. It is important to clearly distinguish these experiments from earlier studies that showed that i.p. injections of Ags in IFA or exposure to pertussis toxin or proteins from M. tuberculosis clearly distinguish these experiments from earlier studies that showed that i.p. injections of Ags in IFA or exposure to pertussis toxin or proteins from M. tuberculosis protect mice from EAE (36, 37). In the present study, mice were primed by s.c. immunization with peptide Ags emulsified in CFA, a regimen that induces strong Th1-type immune responses (24).

Because EAE is a prototypic CD4 T cell-mediated autoimmune disease (2), we sought to determine whether this accelerated self reactivity is related to differences in the expansion and phenotypic differentiation of autoreactive Th cells in vivo. Autoreactive Th cells in actively induced EAE had not yet been directly investigated, and we thus generated peptide/MHC class II tetramers that indicated that these cells had been recently (re)activated. These data collectively show that the majority of self-specific cells in the CNS express CD25, CD44, and CD69, whereas autoreactive cells in the spleen express CD44, but not CD25 and CD69. In vivo activated, polyclonal self-specific T cells are thus phenotypically heterogeneous, and this diversity is related to the anatomical environment.

When EAE was induced in Ag-experienced mice, the frequency of specific cells expressing CD25 in the DLN increased faster than after primary challenge and reached maximal levels at day 4, thus earlier than after primary challenge (p = 0.03; ANOVA). The accelerated kinetics of T cell activation during secondary expansion was even more evident when CD69 expression was observed, which peaked already after 24 h (p = 0.000026; ANOVA). This proportion declined subsequently more rapidly than after primary challenge, while the absolute numbers of tetramer-positive cells and the expression of CD25 and CD44 still increased.
the direct investigation of polyclonal, in vivo activated self-reactive Th cells within their anatomical context.

The staining characteristics of the tetramers were further defined using polyclonal short-term T cell lines that, in contrast to clones, had not undergone modifications and selection processes that occur during repetitive in vitro stimulation and thus display a range of TCR affinities that closely resemble the endogenous T cell repertoire. The tetramer staining was found to be very specific, as there was virtually no staining with T cells of other specificities and tetramers bound exclusively to cells that could be activated by the specific peptide in vitro. Approximately 50% of cells that proliferated in response to the target Ag in vitro remained tetramer negative (Fig. 1B). These low avidity T cells are not detected by tetramer staining and are thus not included in the further analysis. This underestimation of the absolute numbers of autoreactive T cells is likely to be more evident after primary than after secondary contact, because avidity maturation during the primary response results in the generation of a high avidity T cell repertoire (38–40). We next assessed the population dynamics of autoreactive T cells after EAE induction in unprimed mice. Consistent with earlier ELISPOT analysis (41), cells first expanded in the DLN and in the spleen, and only 5% of PLP139–151-specific T cells then migrated into the CNS. In absolute numbers, less than 15,000 tetramer-positive CD4 T cells were present in the CNS of heavily diseased animals. The analysis of the cell numbers recovered from the CNS indicates that CNS-specific and unspecific cells simultaneously migrate into the CNS and argues against the hypothesis that specific cells enter first and subsequently attract cells of other specificities. All cells in the CNS expressed high levels of CD44, indicating that peripheral activation is a prerequisite for entry into the CNS. Within the CNS, tetramer-positive cells rapidly re-expressed CD25 and CD69, indicating that they received local TCR triggering. The tetramer-positive cells in the CNS were phenotypically distinct (CD25^+/CD44^−/CD69^−) from the vast majority of cells that resided in the periphery (CD25^+/CD44^+/CD69^+).

Further characterization of the differences between autoreactive T cells within the CNS and those outside may provide new treatment options to selectively inactivate cells that enter the target organ. The majority of Th cells in the CNS were tetramer negative. These cells had been activated in the periphery, indicated by the expression of CD44. It is possible that they are specific for antigenic determinants present in the adjuvant that was used for immunization or may have been activated by bystander activation. Most of them did not re-express CD25 or CD69, and thus did not find an appropriate target within the CNS.

The in vivo expansion and phenotypic differentiation of autoreactive T cells in an unmanipulated T cell repertoire, as assessed in this study, clearly differ from the behavior of autoreactive T cells in adoptive transfer EAE. In adoptive transfer EAE, several millions of in vivo activated T cells are required to provoke disease symptoms (35). Immediately after transfer, autoreactive T cells lose expression of activation markers (42), and >90% migrate into the CNS (42). In actively induced and adoptively transferred EAE, CNS-specific T cells are reactivated within the CNS, indicating self Ag recognition in situ (42).

When we induced EAE in Ag-experienced mice, the kinetics of T cell migration into the CNS were 2–4 days accelerated as compared with primary EAE induction. In the DLN and in the spleen, the absolute numbers of tetramer-positive cells peaked and subsequently declined earlier after secondary challenge, indicating that expansion and migration in the periphery were also enhanced. A 3- to 4-day faster invasion of the CNS after secondary challenge is remarkably similar to the kinetic advantage of memory CTLs that expand after secondary influenza infection (17). In marked contrast to influenza-infected mice, however, the absolute numbers of specific Th cells were not increased, demonstrating that the enhanced autoreactivity in Ag-experienced mice is not related to increased numbers of self-specific CD4 T cells. This was surprising because it has been shown in several systems that the accelerated protective immunity after repetitive viral infections is at least partially due to an increased frequency of virus-specific CD8 T cells (16–18, 43). The severity of an autoimmune disease thus does not necessarily correlate with the number of autoreactive T cells.

Comparative analysis of phenotypic changes of self-reactive T cells clearly showed that Ag-experienced cells more rapidly express activation markers after in vivo activation. This was most prominent for CD69, which peaked within 24 h after secondary challenge. These data indicate that qualitative rather than quantitative changes in self-reactive CD4 T cells account for the enhanced autoimmunity in Ag-experienced mice. Therefore, when T cell reactivities in autoimmune diseases are analyzed, functional differences in autoreactive T cells are probably more important than cell numbers. Additional differences in Ag-experienced mice are likely to contribute to the enhanced autoreactivity. Among these are functional changes in autoreactive CD4 T cells or alterations in CTLs or B cells, which are present in the fully competent mice used in the current investigation.

The data presented in this work provide a clear picture on the population dynamics and phenotypic differentiation of polyclonal autoreactive T cells, from in vivo activation to development and remission of autoimmunity. They show that inapparent antigenic challenges influence the development of autoimmunity and further indicate that CD4 T cell memory is a matter of more efficient cells rather than of more cells. This is likely to be advantageous for host defense in a normal environment, in which individuals are constantly subject to successive infections by different pathogens, and the number of lymphocytes specific for a first Ag declines (44). Under these circumstances, qualitative properties of Ag-experienced lymphocytes would enable the immune system to maintain protective immunity (45), as productive immune responses can be generated from rare, but highly efficient cells. Although this property increases the chances of the individual to successfully fight infections, it favors the development of autoimmune diseases, such as multiple sclerosis.

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References


