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Immunopathology and Infectious Disease
Distinct Functions of Autoreactive Memory and Effector CD4+ T Cells in Experimental Autoimmune Encephalomyelitis

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The persistence of human autoimmune diseases is thought to be mediated predominantly by memory T cells. We investigated the phenotype and migration of memory versus effector T cells in vivo in experimental autoimmune encephalomyelitis (EAE). We found that memory CD4+ T cells up-regulated the activation marker CD44 as well as CXCR3 and ICOS, proliferated more and produced more interferon-γ and less interleukin-17 compared to effector T cells. Moreover, adoptive transfer of memory T cells into T cell receptor (TCR)αβ+ recipients induced more severe disease than did effector CD4+ T cells with marked central nervous system inflammation and axonal damage. The uniqueness of disease mediated by memory T cells was confirmed by the differential susceptibility to immunomodulatory therapies in vivo. CD28-B7 T cell costimulatory signal blockade by CTLA4Ig suppressed effector cell-mediated EAE but had minimal effects on disease induced by memory cells. In contrast, ICOS-B7h blockade exacerbated effector T cell-induced EAE but protected from disease induced by memory T cells. However, blockade of the OX40 (CD134) costimulatory pathway ameliorated disease mediated by both memory and effector T cells. Our data extend the understanding of the pathogenicity of autoreactive memory T cells and have important implications for the development of novel therapies for human autoimmune diseases.

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) white matter in which T cells and their mediators trigger injury of axons and their myelin sheaths through a complex sequence of events.1-5 T cell-mediated inflammation is driven by an autoimmune process, which in turn can trigger a degenerative phase that is independent of immune responses.6 Although the nature of the autoreactive T cells in MS patients is not yet fully understood, there is evidence that these T cells belong to the long-lived memory T cell pool.6-9 Memory CD4+ T cells express a pattern of surface markers different from that of naïve or effector cells, and their responses to immune stimuli are functionally different.10 Murine memory cells are high in CD44 and low in the expression of activation markers such as CD25. Memory cells may persist in the absence of antigenic stimulation as nondividing cells.10 Re-encounter with the same antigen can expand the population to a stable, higher level and generate a separate population of CD44 high effector T cells.11 Two subsets of memory T cells, called central-memory (Tcen) and effector-memory (Tcem) T cells, are described based on their anatomical location, expression of cell surface markers, and effector function.12 Tcem cells express molecules such as CD62L and CCR7, which allow efficient homing to lymph nodes, whereas Tcen cells lack expression of these lymph node homing receptors and are located in nonlymphoid tissues. However, both T cell subsets are present in the blood and spleen. Some studies have also shown that Tcen cells acquire effector functions, such as cytokine production and killing, more rapidly than do Tcem.13-15 The induction of immunological tolerance is an important clinical goal in autoimmunity. Strategies designed specifically to suppress the function of chronically activated memory T cells without impairing the function of naïve T cells therefore have value in the treatment of autoimmune diseases. Two signals are required for T cell activation: antigen recognition and costimulatory signals. Several costimulatory pathways important in the activation of T cells have been identified.16,17 Costimulatory molecules may deliver either a stimulatory (positive) or inhibitory (negative) signal for T cell activation.18 The delicate balance between positive and negative regulatory signals can determine the outcome of a specific immune response. It has been suggested that memory T cells are less dependent on positive costimulatory signals than naïve cells for activation on antigen encounter19,20 or may require different costimulatory signals. In humans, ex vivo activated myelin-reactive T cells from multiple sclerosis patients are independent of CD28-B7 costimulation.7,9 However, in vivo murine studies showed that activation of effector-memory CD4+ T cells may be abrogated by blocking the CD28-B7 pathway.29 Furthermore, it was recently shown that memory CD8+ T cells require CD28 costimulation to generate maximal secondary responses against pathogens.20 Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the central nervous system that mimics certain aspects of MS. Although no animal model can

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replicate MS completely, this model is valuable in addressing specific questions of pathogenesis and potential therapies. However, most studies have focused on targeting prevention of disease or therapy of early disease that may not be fully applicable to human disease, which is probably perpetuated by memory T cells. The understanding of CD4+ memory T cell function and regulation comes mostly from in vitro studies or limited in vivo studies, but there is no disease model mediated purely by memory T cells. Here we establish a new EAE disease model mediated by antigen-specific memory CD4+ T cells generated in vivo. Our data show that memory CD4+ T cells induce more severe disease than effector CD4+ T cells caused by a preferential differentiation of memory T cells into the Th1 phenotype and a differential expression of chemokine receptors and costimulatory molecules leading to severe CNS inflammation. Furthermore, our data using costimulatory blocking agents show the requirement of the Inducible costimulator-B7h and OX40 pathways in disease mediated by memory CD4+ T cells. Our findings have implications for the treatment of human autoimmune diseases and translating novel tolerance strategies to the clinic.

Materials and Methods

Mice and EAE Induction with Myelin Oligodendrocytes Glycoprotein (MOG)

Female wild-type (WT) and T cell receptor (TCR)αβ−/− C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). MOG-TCR mice (2D2) were provided by V. Kuchroo (Brigham and Women’s Hospital, Boston, Massachusetts). All animal experiments were conducted with the approval of the Harvard Medical Area Standing Committee on Animals. EAE was induced in C57BL/6 WT and TCRαβ−/− mice as described previously.22 MOG peptide 35-55 (M-E-V-G-W-Y-R-S-P-F-S-R-O-V-H-L-Y-R-N-G-K), corresponding to the mouse sequence, was synthesized by Quality Controlled Biochemicals Inc., a division of Biosource International (Hopkinton, Massachusetts) and purified to greater than 99% by high-performance liquid chromatography. C57BL/6 WT and TCRαβ−/− mice were immunized subcutaneously in the flank with 100 μg of myelin oligodendrocyte glycoprotein peptide of amino acids 35-55 (MOG35-55) peptide in 0.1 ml of PBS and 0.1 ml of complete Freund’s adjuvant (CFA) containing 0.4 mg of Mycobacterium tuberculosis (H37Ra; Difco Laboratories, Detroit, Michigan) and injected intraperitoneally with 200 ng of pertussis toxin (PT) (List Biological Laboratories Inc., Campbell, California) on the day of immunization and 2 days later. For clinical scoring, animals were kept for at least 1 month and EAE was scored as follows: grade 1, limp tail or isolated weakness of gait without limp tail; grade 2, partial hind and front leg paralysis; grade 3, total hind leg paralysis; grade 4, total hind leg and partial front leg paralysis; grade 5, moribund or dead animal.

Antibodies, Reagents, Enzyme-Linked Immunosorbent Spot (ELISPOT) Assay, and Enzyme-Linked Immunosorbent Assay (ELISA)

Murine CTLA4Ig was obtained from Bristol-Myers Squibb (Seattle, Washington). Anti-ICOS-L monoclonal antibody (clone HK5.3)24 and anti-OX40L (clone RM 134L)25 were produced by Bioexpress Cell Culture Inc. (Kaysville, Utah) and administered to mice intraperitoneally at 200 μg starting from the day of the immunization and every other day until day 10 postimmunization. Control IgG antibodies were given according to the same protocol. For ELISPOT assay, 96-well nitrocellulose plates (Millipore, Bedford, Massachusetts) were coated with 4 μg/ml purified mouse anti-interferon (IFN)-γ, anti-interleukin (IL)-10 (all from BD Biosciences, San Diego, California), and anti-IL-17 (ELISPOT kit; eBioscience, San Diego, California) overnight at 4°C. After blocking with 1% BSA, 2 to 5 × 105 cells were loaded in each well and incubated with 0 to 100 μg/ml antigen peptide for 36 hours at 37°C. After washing, corresponding plates were incubated with 2 μg/ml biotinylated anti-IFN-γ, anti-IL-10, or anti-IL-17 overnight at 4°C. The plates were washed, then incubated with alkaline phosphatase (Sigma, St. Louis, Missouri) at 1:10,000 for 2 hours at room temperature, and developed in BCIP/NBT (Sigma) solution. Spots were counted using a computer-assisted ELISPOT Image Analyzer (Cellular Technology Limited, Cleveland, Ohio). IFN-γ (ELISA kit; R&D Systems, Minneapolis, Minnesota) production was assessed in the culture supernatants by ELISA 2 days after ex vivo stimulation according to the manufacturer’s protocol.

Intracellular Cytokine Staining

Cells were isolated from peripheral tissues or from the CNS and were restimulated with phorbol 12-myristate 13-acetate (20 ng/ml), ionomycin (500 ng/ml), and 2 mmol/L monensin (GolgiStop; BD PharMingen, San Diego, California) for 4 hours at 37°C. Cells were washed and stained for surface markers by incubating at room temperature for 20 minutes. After two washes in PBS containing 2% FCS and 0.1% sodium azide, the cells were treated with 1% paraformaldehyde and fixed with buffer containing saponin according to the manufacturer’s recommendations (BD PharMingen). Following permeabilization, the phycoerythrin (PE)-conjugated anti-cytokine antibodies were added for 20 minutes at room temperature, and the cells were washed and analyzed by using a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, California). Cytokine Abs and their corresponding isotype controls from BD PharMingen, IL-4, IL-17, and IFN-γ, were used.

Confocal Microscopy

For immunohistochemistry, animals were sacrificed and perfused with 4% paraformaldehyde in PBS; then, brain and spinal cord tissues were harvested and postfixed, dehydrated with 30% sucrose, placed in optimal cutting temperature, and stored at −80°C until use. The following antibodies were purchased from BD PharMingen: rat anti-CD4 (1:100), rabbit anti-glial fibrillary acidic protein (GFAP) (1:100), rabbit anti-lectin B4 (1:100), mouse anti-SMI32 (1:100), rhodamine goat anti-rat (1:250), fluorescein isothiocyanate (FITC) goat anti-rabbit (1:500), and FITC goat anti-mouse (1:500). Thirty-μm-thick longitudinal sections of spinal cord tissues from mice exhibiting EAE, and control mice were washed with PBS, then blocked in PBS containing 8% horse serum, 3% BSA, and 0.3% Triton X-100. The sections were incubated with prima-
ry antibodies overnight at 4°C, washed, and incubated with the appropriate combination of secondary antibodies for 1 hour in blocking buffer. The sections were stained with the nuclear stain, TOTO-3 (Molecular Probes, Carlsbad, California), and mounted, and images were captured using confocal imaging and LSM510 software (Zeiss, Jena, Germany). Averaged numbers of CD4 and lectin B4-positive cells from at least three different levels of sectioning per spinal cord (two samples per group) were counted under X63 magnification.

T Cell Preparation, Phenotyping, and Adoptive Transfer

The memory CD4+ cell populations were characterized by flow-cytometric analysis using rat anti-CD44-FITC, CD62L-Allophycocyanin, ICOS-PE, CD28-PE, CTLA4-FITC, OX40-PE, (CXC chemokine receptor 3)-PE, and appropriate isotype antibody controls (all purchased from BD Biosciences). PE anti-mouse CCR7 and T-bet were from eBioscience (San Diego, California). For adoptive transfer studies, splenocytes from naïve, primed (immunized with MOG35-55 for 12 days), and long-term immunized mice (immunized with MOG for 100 days) were pooled separately and enriched for CD4+ T cells (negative selection) by magnetic cell separation (MACS) (Miltenyi Biotech, Auburn, California). The CD4+ cells were quantitated, stained with rat anti-mouse CD44-FITC, and sorted using a BD FACSAria cell sorter (BD Biosciences). Finally, 1 to 1.5 × 10^6 sorted naïve, effector, or memory CD4+ T cells were adoptively transferred by i.v. injection into TCRαβ-/- recipients. Mice were immunized in the flank with MOG/CFA and injected intraperitoneally with 200 ng of pertussis toxin on the day of immunization and 2 days later.

Activation-Induced Cell Death

Splenocytes were isolated from B6 mice immunized with MOG35-55/CFA for 10 days (effector cells) or for more than 120 days (memory cells). After activation with MOG peptide (0 to 25 μg/ml) in vitro for 4 days, cells were stained with 7-amino-actinomycin D (7-AAD) (BD Biosciences) and Annexin V (BD Biosciences), followed by fluorescence-activated cell sorting (FACS) analysis, and the percentage of apoptotic cells was quantititated.

Western Blot

The anti-Bcl-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California), and the immunoblot technique was performed according to standard protocols.

Expression Analysis by Real-Time PCR

Total RNA was extracted from specific cell populations using the Stratagene (La Jolla, California) RNA kit and transferred directly into the RT-PCR using the Applied Biosystems Taqman kit (Applied Biosystems, Foster City, California). Samples were subjected to real-time PCR analysis on an Applied Biosystems PRISM 7000 Sequencer Detection System under standard conditions. Genes analyzed were detected using commercially available assays (Applied Biosystems). Relative mRNA abundance was normalized against glyceraldehyde-3-phosphate dehydrogenase.

MHC II IAβ Tetramer Staining Based on Flow-Cytometric Analysis

The MHC II IAβ tetramer was provided by V. Kuchroo. Four days after the activation of lymphoid cells, cell suspensions were enriched for T cells by Ficoll-Hypaque density gradient centrifugation. As a control for the tetramer, another group of C57BL/6 mice was immunized for 14 or 100 days with ovalbumin (100 μg) in CFA + PT, and splenocytes were used for the tetramer staining. The cells from all groups were incubated with IAβ multimers (30 μg/ml) in Dulbecco’s modified Eagle’s medium supplemented with IL-2 (5 μmol/L) at 37°C for 3 hours. The cells were then washed and stained with anti-CD4 (CD4-Allophycocyanin) and 7-AAD (BD PharMingen). After incubating at room temperature for 20 minutes, the cells were washed and analyzed using a FACSort (BD Biosciences). The number of cells positive for tetramer-PE (MOG35-55) was then determined in live (7-AAD-negative), CD4-positive populations.

Statistical Analysis

The two-tailed Mann-Whitney and unpaired t-tests were used to compare disease, cell proliferation, and cytokine production. Fisher’s exact test was used to compare disease incidence.

Results

Characterization of Autoreactive Memory CD4+ T Cells

We isolated CD4+CD44hi T cells from C57BL/6 mice after recovery from acute disease, greater than 100 days after immunization with MOG35-55 in CFA and intraperitoneal injections of PT. Effector CD4+ T cells (CD4+CD44lo) were isolated directly from short-term MOG-immunized mice around the peak of the clinical disease (12 days). Both memory and effector CD4+ T cells express the activation molecule CD44, but memory T cells express significantly higher levels of CD44 compared to effector CD4+ T cells (P = 0.002 by paired t-test) and slight down-regulation of CD62L expression (see Supplementary Figure S1A, at http://ajp.amjpathol.org). One of the defining features of memory CD4+ T cells is their survival advantage mediated by up-regulation of anti-apoptotic molecules.26-28 We compared the survival of memory CD4+ T cells to that of effector CD4+ T cells isolated as described above and stimulated in vitro with MOG35-55 peptide (0, 1, and 10 μg/ml) at equal numbers (0.5 × 10^6/well) for 4 days in a 96-well plate. We found higher percentages of Annexin V binding cells in 7-AADlo/CD4+CD44hi in effector T cells compared to memory T cells. However, there was no difference in the 7-AADD+/CD4+CD44hi gate between the two populations (see Supplementary Figure S1B, at http://ajp.amjpathol.org). The ability of memory cells to resist activation-induced cell death is due, at least partially, to higher expression of the anti-apoptotic protein Bcl-2, as shown by Western blot, of freshly isolated memory CD4+ T cells from naïve, primed (immunized with MOG35-55 for 12 days), and long-term immunized mice (immunized with MOG for 100 days) were pooled separately and enriched for CD4+ T cells (negative selection) by magnetic cell separation (MACS) (Miltenyi Biotech, Auburn, California). The CD4+ cells were quantitated, stained with rat anti-mouse CD44-FITC, and sorted using a BD FACSAria cell sorter (BD Biosciences). Finally, 1 to 1.5 × 10^6 sorted naïve, effector, or memory CD4+ T cells were adoptively transferred by i.v. injection into TCRαβ-/- recipients. Mice were immunized in the flank with MOG/CFA and injected intraperitoneally with 200 ng of pertussis toxin on the day of immunization and 2 days later.

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ing shows that memory CD4+ T cells proliferate more than effector CD4+ T cells in response to MOG35-55 (10 μg/ml) stimulation as shown by quantification of CFSE dilution in the Annexin-V+/7-AAD+/CD4+CD44hi gate (P < 0.05) (see Supplementary Figure S1D, at http://ajp.amjpathol.org). Interestingly, although memory CD4+ T cells proliferated more than effector T cells only when exposed to the higher dose of MOG35-55 (10 μg/ml), they produced higher amounts of IFN-γ compared to effector T cells in response to 1 and 10 μg/ml of MOG35-55 peptide (see Supplementary Figure S1E, at http://ajp.amjpathol.org).

Memory and Effector Cells Display Differential Expression of Survival and Costimulatory Genes

To eliminate the possibility of contamination of the memory cell pool by effector cells from the antigen depot site, we “parked” the cells in naïve syngeneic animals. CD4+ T cells were isolated from spleens of MOG-TCR transgenic mice (2D2) immunized with MOG35-55/CFA 14 days earlier and parked in naïve B6 recipients for 100 days to generate memory T cells. CD4+ Vβ11+ Vα3.2+ T cells were sorted from the spleens of recipient mice with a recovery rate of 8 to 12 × 106 cells per recipient. Using quantitative RT-PCR, we found a higher level of the antiapoptotic molecule Bcl-2 (2.3-fold higher) and a lower level of the pro-apoptotic tumor necrosis factor receptor p55 (3.6-fold) and caspase-8 (2.4-fold) in the memory T cell population compared to effector CD4+ T cells. There was no difference in the mRNA expression of Bcl-xl (see Supplementary Figure S2A, at http://ajp.amjpathol.org). We then compared the mRNA expression of the costimulatory molecules CD28, ICOS, and OX40 in addition to CTLA4. In comparison to memory T cells, effector T cells expressed 2.5- and 2.8-fold higher levels of CD28 and CTLA4, respectively. In contrast, ICOS expression was higher in the memory T cell population (3.3-fold), suggesting different requirements for costimulation between the two populations. There was no significant difference in OX40 expression (see Supplementary Figure S2B, at http://ajp.amjpathol.org). Finally, we quantified the transcripts of chemokine receptors on MOG-specific effector and memory CD4+ T cells. We found that CXCR3 transcripts were at their highest level only in memory T cells (12.4-fold) compared to effector T cells. Similarly, higher levels of CCR5 were also detected on the memory T cell population (2.3-fold) compared to effector T cells. No significant differences in the levels of CXCR4 and CCR7 between the two populations were detected (see Supplementary Figure S2C, at http://ajp.amjpathol.org). Since CCR7 is an important receptor for central memory cell homing, we compared the protein levels of this chemokine receptor between memory subpopulations (Tcm and Tem) and effector cells. FACS analysis showed that although Tem (CD4+CD44hiCD62Llo) cells express higher levels of CCR7 as compared to short-term activated T cells (MFI: 785 ± 89.2 versus 456.5 ± 18.7; P < 0.05), there was no significant difference in CCR7 protein expression between Tem (CD4+CD44hiCD62Llo) and activated effector T cells (MFI: 479.3 ± 25.3 versus 456.5 ± 18.7).

Next, we measured the relative mRNA expression of the transcription factors T-box 21, RORyt, and GATA3. Interestingly, the T-box 21 message was very prominent in the memory T cell population (~15-fold increase) (see Supplementary Figure S2D, at http://ajp.amjpathol.org). We also measured the frequency of T-beta-positive cells by intracellular staining. The number of T-beta-positive cells was higher in the memory cell population compared to the effector T cells (22.3 ± 5.1 versus 12.6 ± 3.9, P < 0.01), although their mean fluorescence intensity levels were identical (data not shown).

Memory CD4+ T Cells Induce More Severe Disease Than Effector Cells

CD4+CD44hi memory T cells from long-term (100 days) immunized WT mice were sorted and transferred into lymphopenic TCRβ−/− mice by i.v. injection. Effector CD4+CD44hi and naïve CD4+CD62L+ cells isolated from immunized (12 days) and non-immunized WT mice, respectively, were used as controls. Following cell transfer, recipients were immunized with MOG35-55/CFA + PTan and observed for EAE development. Although EAE onset was similar between recipients of memory and effector cells, disease was significantly more severe in memoryCD4+ cell-induced disease compared to that induced by effector T cells (mean maximal score 3.0 ± 0.2 versus 2.2 ± 0.2, P = 0.006 by Mann-Whitney test). As expected, adoptive transfer of naïve T cells caused a mild and delayed EAE compared to disease induced by memory cells (mean maximal score 3.0 ± 0.2 versus 0.8 ± 0.4, P < 0.0001 by Mann-Whitney test) (Figure 1A; Table 1). The enhanced disease severity was not due to unequal frequency of MOG-specific cells at the time of transfer, since the precursor frequency of IAβ-MOG35-55tetramer. cells was actually in favor of the effector cell population (Figure 1B).

To analyze the peripheral immune responses of the transferred cells, we isolated draining lymph nodes and splenocytes from TCRβ−/− mice recipients of memory or effector CD4+ T cells 12 days after immunization. Cells from the memory T cell recipient group exhibited more proliferation than those from the effector T cell recipients after culture with 10 and 100 μg of MOG peptide in vitro (P < 0.001 and P < 0.01, respectively) (Figure 1C). The cytokine production of freshly isolated memory or effector cells showed that the frequency of IFN-γ-producing cells by intracellular staining was significantly higher in the memory cell population (15.6 ± 2.9 versus 7.6 ± 4.6, P < 0.05). We also observed that memory T cells produced slightly more IL-17 than effector cells (2.6 ± 0.9 compared to 1.1 ± 0.6) (Figure 1D). There was no difference in the frequency of cells producing IL-4 between the two groups (Figure 1D).

The heightened response in the memory recipients was not due to unequal homeostatic proliferation of the memory and effector T cells. We adoptively transferred CFSE-labeled MOG-specific effector or memory CD4+ T lymphocytes into naïve TCRβ−/− mice. Three, 6 and 10 days after adoptive transfer, spleen cells were isolated and the percentage of cells that underwent proliferation was calculated based on the CFSE dilutions as percentage of CFSEdim cells of alive CD4, 7-AADdim/Annexin-V−/−. We found that effector T cells expanded more than memory T cells at 3 and 6 days after transfer, but by day 10 the expansion was similar (see Supplementary Figure S3, at http://ajp.amjpathol.org). These data exclude a possible role of homeostatic mechanisms increasing the pool of memory T cells in the lymphopenic hosts.
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...Another possible confounding factor could be unequal expansion of CD4+CD25+ regulatory T cells after in vivo transfer of the effector versus memory T cells. At the time of transfer, the percentage of CD4+CD25+Foxp3+ cells was comparable between the memory and the effector populations (8.3 ± 0.7 versus 7.5 ± 0.6, P = 0.5) (data not shown). To explore the possibility of unequal expansion of regulatory T cell populations in vivo after immunization, splenocytes isolated from recipients of either effector or memory CD4+ T cells were cultured 10 days after immunization, and the percentage of regulatory T cells (representative of three independent experiments). C: Splenocytes from mice that received effector or memory T cells were cultured with MOG35-55 peptide at 1, 10, and 100 μg/ml, and cell proliferation was measured by [3H]thymidine incorporation. The proliferation rate was significantly higher in cultures from the memory (■) T cell group compared to the effector (▲) group. D: Intracellular FACS staining of splenocytes from MOG35-55-immunized B6 mice. Spleen cells were isolated and activated in vitro with anti-TCR and anti-CD28 antibody for 6 hours and stained with anti-CD4-peridinin-chlorophyll-protein complex, anti-CD44-FITC, and a variety of cytokine-PE antibodies. **P < 0.001; ***P < 0.0001.

In Vivo Dynamics of MOG-Specific Memory and Effector CD4+ T Cells

To study the disease dynamics induced by the transfer of antigen-specific memory CD4+ T cells, we generated two lines of 2D2 mice (MOG-TCR transgenic) that express Thy1.2 or the Thy1.1 congenic markers. First, we showed that the transfer of memory T cells from 2D2 mice into TCRαβ-/- mice induced more severe disease than did their effector counterparts (mean maximal score 3.8 ± 0.4 versus 2.8 ± 0.1, P = 0.004 by Mann-Whitney test) (Figure 2A). Second, we studied the expansion, phenotype, and trafficking of autoreactive memory and effector CD4+ T cells by transferring both cell types into one WT hybrid recipient. Using MOG TCR-transgenic mice that express Thy1.2 or the Thy1.1 congenic markers, we tracked the Thy1.1 (memory) and Thy1.2 (effector) CD4+ T cells in the periphery before and during disease onset, and in the CNS compartment during the peak of the disease. To do this, equal numbers of MOG TCR transgenic Thy1.1 memory and Thy1.2 effector CD4+CD44hi T cells were transferred i.v. into Thy1.1/Thy1.2 hybrid hosts and challenged with MOG35-55 peptide. At the time of transfer, there were phenotypic differences between memory and effector cell populations: as measured by flow cytometry, ICOS and...
memory transfer, there was a slight increase in the frequency of the memory cell population in the periphery and in the CNS of IFN-γ-producing cells was similar to our previous experiment into hybrid hosts and challenged with MOG35-55 specific memory (Thy1.1) and naïve (Thy1.2) were transferred into naïve 2D2 Thy1.2 cells activated in vivo. Therefore, MOG-specific memory (Thy1.1) and naïve (Thy1.2) were transferred into hybrid hosts and challenged with MOG35-55 peptide. The FACS analysis indicated that the distribution of IFN-γ-producing cells was similar to our previous experiments, with a higher frequency of IFN-γ-producing cells in the memory cell population in the periphery and in the CNS (Figure 2D). Unlike what we observed in the effector versus memory transfer, there was a slight increase in the frequency of memory IL-17-producing cells in the CNS (Figure 2D). 

Table 1. Induction of Disease by Transfer of WT Memory, Effector, or Naïve CD4+ T Cells in TCRββ−/− Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Mean day of onset ± SEM</th>
<th>Mean maximum score ± SEM</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Memory CD4+</td>
<td>16/16</td>
<td>12.0 ± 0.4</td>
<td>3.0 ± 0.2*</td>
<td>2/16</td>
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<tr>
<td>Naïve CD4+</td>
<td>4/8</td>
<td>16.0 ± 0.9</td>
<td>0.8 ± 0.4**</td>
<td>0/8</td>
</tr>
<tr>
<td>Effector CD4+</td>
<td>16/16</td>
<td>13.5 ± 0.6</td>
<td>2.2 ± 0.2</td>
<td>0/16</td>
</tr>
</tbody>
</table>

*p = 0.006; **p < 0.001

Increased CNS Infiltration of Memory CD4+ T Cell-Mediated Inflammation during Eae

Histological examination of the CNS of TCRαβ−/− mice sacrificed on day 12 and day 32 (Figure 3 and Table 2) postimmunization revealed significantly more T cell infiltrates in CD4+ memory T cell recipients (Figure 3A and Table 2) compared to CD4+ effector T cell recipients (Figure 3D and Table 2), and more microglia activation (Figure 3B versus 3E and Table 2). It should be noted that, unlike effector cell infiltration in the CNS, memory CD4+ T cell infiltrates and microglia activation were detected in the CNS through the late stages of the disease (day 32) (Figure 3 and Table 2). To analyze whether disease transfer affects axonal integrity, we used SM132 monoclonal antibody, which reacts with a nonphosphorylated epitope in neurofilament H in demyelinating axons, as a marker of axonal damage.29 We also used GFAP antibody, a marker of astrocytes that is induced during astrocytic activation.30 Our data showed that memory CD4+ T cells were associated with greater astrocytic activation (Figure 3H) and axonal damage (Figure 3N), as demonstrated by increased fluorescence staining of GFAP and SM132, respectively, compared to recipients of effector cells (Figure 3, K and Q). A potential explanation for the increase in CNS infiltration in recipients of memory CD4+ T cells may be that memory T cells migrate to the CNS more efficiently. This is in agreement with our present data of the mRNA and protein levels of the chemokine receptor expression on MOG-specific memory CD4+ T cells demonstrating that these cells express higher levels of CXC3 and CCR5 compared to effector CD4+ T cells, indicating that the severe disease induced by memory T cells is related, at least partially, to increased migration of these cells to the CNS.29

Costimulatory Requirements of Memory Versus Effector CD4+ T Cells in Vivo

Our group and others have shown previously that CD28–B7 blockade by CTLA4Ig protects animals from actively induced EAE.31,32 As shown above, CD28 expression is higher in MOG TCR-specific effector T cells as compared to the memory T cell population (Figure 2; see Supplementary Figure S2, at http://ajp.amjpathol.org). To investigate the costimulatory requirements of memory and effector CD4+ T cells in vivo, TCRββ−/− recipient mice that received either memory or effector CD4+ cells were treated with CTLA4Ig (200 μg) every 2 days from day 0 through day 10 after immunization. The control group received control Ig. Our data demonstrated that although CTLA4Ig administration dramatically suppressed the disease induced by transferring effector cells (mean maximal score 0.4 ± 0.1 versus 2 ± 0.2, P < 0.001; incidence 25% versus 100%, P < 0.001), there was only a slight decrease in the induction of clinical disease in the group of...
mice that received memory cells (mean maximal grade 2 ± 0.2 compared to 2.5 ± 0.1, P = 0.03) (Figure 4A), and overall disease incidence was similar to the control group (100%). We investigated the cytokine profile in treated mice. Spleen and lymph node cells were isolated from treated mice 12 days after immunization and exposed to MOG peptide (50 μg/ml), and the cytokine profile was characterized. The effects of in vivo CTLA4Ig administration on the frequency of IFN-γ-producing cells were evident in mice that received effector cells (P < 0.01) and to a lesser extent in the mice that received memory cells (P < 0.05) (Figure 4B). Interestingly, CTLA4Ig administration increased IL-10 production and inhibited IL-17 production in cells isolated from the effector T cell recipients (P < 0.05 compared to control Ig-treated mice), whereas no such effects were seen in the memory T cell recipients (Figure 4B).

Next, TCRβ−/− mice that received either memory or effector CD4+ cells were treated i.p. with anti-B7h (ICOS-L) (200 μg) every second day from day 0 through day 10 after immunization. Although anti-B7h antibody treatment worsened the disease induced by transferring effector cells (mean maximal grade 2 ± 0.2 versus 2.6 ± 0.1, P < 0.05), it provided significant protection in mice that received memory cells (mean maximal grade 2.9 ± 0.1 versus 1.8 ± 0.1, P < 0.005) (Figure 4C). In vitro, the cytokine profile of lymphoid cells isolated 12 days after immunization resembled in vivo disease, with decreased frequency of IFN-γ-producing cells (P < 0.05) in the memory cell recipients treated with anti-B7h compared to control Ig mice. There was no such decrease in effector cell recipients treated similarly (Figure 4D). Furthermore, lymphoid cells from memory T cell recipients produced significantly less IL-17 and more IL-10 after treatment with anti-B7h (P < 0.05 compared to control mice), whereas IL-17 production by the effector T cell recipients treated with anti-B7h was similar to that of recipients treated with control Ig (Figure 4D).

We tested the effect of OX40L-OX40 blockade in this model using a blocking antibody against OX40L. TCRβ−/− mice received either memory or effector CD4+ T cells in the presence of anti-OX40L (200 μg) every second day from day 0 through day 10 after immunization. Surprisingly, antibody treatment ameliorated disease in recipients of memory (mean maximal grade 2.9 ± 0.1 vs. 1.7 ± 0.4, P < 0.01) and effector CD4+ T cells (mean maximal grade 2 ± 0.2 versus 1.2 ± 0.2, P < 0.05) (Figure 4E). The frequency of IFN-γ-producing cells was decreased in the anti-OX40L-treated memory cell recipients (P < 0.05) as well as the effector cell recipients treated similarly (P < 0.01) (Figure 4F). The amelioration of the clinical score induced by OX40-OX40L pathway blockade was associated with enhanced IL-10 production and reduction of IFN-γ-producing lymphoid cells of memory and effector T cell recipients, whereas IL-17 production was not affected (Figure 4F).

**Discussion**

The success of experimental immunomodulatory and tolerance strategies has been largely restricted to naïve hosts that do not harbor significant numbers of memory T cells; for example, mice housed in a pathogen-free environment. These strategies induce immunological tolerance to a foreign antigen by exploiting the same principles that underlie tolerance to self-antigens: deletion, anergy, regulation, and immune deviation. Therefore, for such strategies to work in immunologically experienced hosts, such as humans, relevant experimental models must first be developed to address whether memory T cells can be “tolerized” in vivo. The role of...
of heterologous memory cells has been recognized as a barrier to transplantation tolerance.\textsuperscript{33} It is becoming clear that results from current experimental models of autoimmune disease may not predict outcomes in human studies; thus, it is critical to develop animal models that address the role of memory T cells in response to therapeutic strategies.\textsuperscript{<cb>}

To address this issue, we developed a new disease model of memory CD4\textsuperscript{+} T cells in recipients of memory or effector T cells by Immunohistology.

Table 2. Quantification of CNS-Infiltrating T Cells in Recipients of Memory or Effector T Cells by Immunohistology

<table>
<thead>
<tr>
<th>Group</th>
<th>CD4\textsuperscript{+} T Cells</th>
<th>Lectin B4\textsuperscript{+} Cells</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 12</td>
<td>68.8 ± 13.8</td>
<td>34.5 ± 7.9</td>
<td>(P &lt; 0.05)</td>
</tr>
<tr>
<td>Day 32</td>
<td>107 ± 21</td>
<td>76 ± 12</td>
<td>(P &lt; 0.005)</td>
</tr>
</tbody>
</table>

TCRαβ\textsuperscript{+} mice received either memory or effector CD4\textsuperscript{+} T cells on day 0 and were immunized on the same day with MOG35-55. Spinal cords from each group of animals were collected on day 12 and day 32 postimmunization, processed for frozen sectioning, and immunostained for CD4 and lectin B4. Statistical analysis was performed using an unpaired \(t\)-test. \(P\) values < 0.05 were considered significant.

abundance of memory T cells in the target organ occurred under non-lymphopenic conditions, suggesting that this persistence was not due to homeostatic proliferation-mediated accumulation of memory T cells. Moreover, in comparison to effector T cells, we demonstrated that memory T cells were less susceptible to activation-induced cell death and exhibited higher expression of the anti-apoptotic Bcl-2, whereas effector cells expressed higher levels of the death receptor tumor necrosis factor receptor p55 and caspase-8, which are known to interact physically to induce the pro-apoptotic protease cascade.\textsuperscript{35} Although the frequency of long-term memory MOG-specific T cells was lower than that of short-term effector cells, the higher proliferative capacity of memory cells could be linked to a lower susceptibility to AICD. Interestingly, we also showed that memory CD4\textsuperscript{+} T cells have increased expression in the Th1-associated chemokine receptor, CXCR3, and increased IFN-γ production compared to effector CD4\textsuperscript{+} T cells. A recent report showed that CXCR3 is required for recruitment of CD62L and CCR7-negative memory T cells to reactive lymph nodes of adjuvant inoculation.\textsuperscript{36} Thus, the memory T cells that we isolated from peripheral lymphoid organs of immunized mice are heterogeneous in the expression of these homing receptors since they are a mixture of T\textsuperscript{TH}, T\textsuperscript{EM}, Memory CD4\textsuperscript{+} T cells infiltrated more than effector T cells and persisted until the late stage of the disease. These data are consistent with previous reports showing that CXCR3 is expressed on memory CD4\textsuperscript{+} T cells and preferentially on activated Th1 cells\textsuperscript{37} and that a small-molecule compound targeting CXCR3 prevents acute and chronic allograft rejection in murine cardiac and islet transplant models.\textsuperscript{38} Other reports demonstrated that the role of CXCR3 is not limited to T cell migration and could play an important role in the differentiation of memory CD4\textsuperscript{+} T cells during inflammation in humans.\textsuperscript{39,40} In contrast to the implication of CXCR3 in migration, it recently was demonstrated that CXCR3\textsuperscript{−/−} mice exhibit exaggerated severity of EAE compared with wild-type (CXCR3\textsuperscript{+/+}) littermate mice.\textsuperscript{39} There were neither quantitative nor qualitative differences in CNS-infiltrating leukocytes between CXCR3\textsuperscript{−/−} and CXCR3\textsuperscript{−/−} mice with EAE.\textsuperscript{41} Although the discrepancies between the findings of the two groups highlight the need for further study, the expression of this chemokine receptor at high levels in memory CD4\textsuperscript{+} T cells suggests an important role in memory T cell migration and perhaps in memory T cell differentiation.

For the first time we compare the in vivo behavior of memory and effector T cells in the same recipient. We also show that in addition to the production of IFN-γ, memory CD4\textsuperscript{+} of heterologous memory cells has been recognized as a barrier to transplantation tolerance.\textsuperscript{33} It is becoming clear that results from current experimental models of autoimmune disease may not predict outcomes in human studies; thus, it is critical to develop animal models that address the role of memory T cells in response to therapeutic strategies.\textsuperscript{<cb>}

To address this issue, we developed a new disease model of memory CD4\textsuperscript{+} T cells. Spinal cord tissues from the recipients of memory CD4\textsuperscript{+} T cells (A-C, G-I, and M-O) or effector CD4\textsuperscript{+} T cells (D-F, J-L, and P-R) were harvested 32 days postimmunization, processed for frozen sectioning, and immunostained with rat anti-CD4 (A, D, G, I, M, and P; red), anti-lectin B4 (B and E; green), anti-GFAP (H and K; green), and mouse anti-SMI32 (N and Q; green). Sections were co-stained with the nuclear dye TOPO3 (blue). Merged pictures (C, F, I, L, O, and R) are shown of images captured using confocal imaging at ×63 magnification.
T cells produced IL-17, although this production was lower than that of effector cells. IL-17 expression has been linked to autoimmune diseases, although its regulation and function are still unclear. Two recent studies showed evidence that IL-17-producing effector CD4+ T cells develop via a lineage distinct from the Th1 and 2 lineages. This process is negatively regulated by T helper type 1 and type 2 cytokines. Interestingly, we observed reduced production of IL-17 associated with increased secretion of IFN-γ by memory T cells compared to their effector counterparts during EAE. This suggests that IFN-γ may down-regulate IL-17 in the memory population. One of the approaches to targeting autoimmune disease mediated by memory T cells is to block activation pathways unique to the recall of memory T cells. There is extensive evidence in vitro that human memory T cells do not require CD28 costimulation for full activation. On the other hand, there are no in vivo studies of the effect of CTLA4Ig on memory function in an autoimmune disease model. Recently, two reports about CD28-mediated costimulation in viral models of CD4+ and CD8+ memory T cells demonstrated that the CD28 costimulatory pathway blockade suppressed memory T cell activation. Previously, we and others showed the effectiveness of the costimulatory signal blocker CTLA4Ig in EAE, but we hypothesized that CTLA4Ig would not be sufficient to induce tolerance of memory T cells. A clinical trial of CTLA4Ig in MS is currently under way (S.J. Khoury and colleagues), so it is critical to understand the limitations of such an approach in treating human disease. In this report, we showed that the numbers of CD28 and CTLA4 transcripts were lower in MOG-specific memory T cells compared to effector and naïve T cells. Furthermore, blocking CD28-B7 costimulation with CTLA4Ig caused weak protection of EAE induced by memory cells and did not suppress the production of pro-inflammatory cytokines IFN-γ and IL-17, in contrast to the effective suppression of effector cell-induced disease that we usually obtain with the same regimen of CTLA4Ig.

Recent studies identified a novel costimulatory pathway involved in the activation of antigen-experienced but not naïve T cells. The pathway is activated by the interaction of ICOS on T cells with B7h (B7RP-1) on antigen-presenting cells. Costimulation through ICOS is crucial for the activation of previously primed T cell populations, which include memory T cells. Blocking this pathway in EAE during the effenter immune response (9 to 20 days after immunization) abrogated disease, whereas blockade during antigen priming (1 to 10 days after immunization) exacerbated disease. Our model has the advantage of unequivocally showing the differential effect of ICOS blockade on effector versus memory cells. Our present findings also demonstrate that the decrease in expression of mRNA and protein level of ICOS has functional relevance. It is noteworthy that Park et al. have shown that the ICOS-B7h pathway is required for the production of IL-17 in murine T cells. These data are consistent with our present findings that blockade of B7h was beneficial when EAE was induced by transfer of CD4+ memory cells and was associated with a decrease in IL-17 production, whereas it worsened the disease when induced by transfer of CD4+ effector cells. It is also striking that ICOS mRNA is expressed at a significantly higher level on memory T cells compared to effector cells, which is consistent with an important role of the ICOS-B7h pathway in memory T cell-induced EAE. It is commonly stated that activation of memory T cells is independent of costimulatory signals; however, our in vivo...
studies demonstrate that memory T cells are sensitive to co-stimulatory blockade but have different requirements from effector T cells.

The OX40-OX40L pathway appears to regulate multiple aspects of the T cell response. OX40 costimulation on effector T cells promotes cell survival, proliferation, and effector differentiation. Also, OX40 costimulation prevents T cell anergy and allows anergic T cells to acquire potent effector functions. Furthermore, OX40 is instrumental in the generation of the memory recall response. Studies using OX40-deficient mice clearly showed that the most profound impact of OX40 deficiency on the immune response is the impaired generation of memory T cells. 

Recently, in a mouse model of skin transplantation, memory T cells generated by homeostatic proliferation and resistant to CD28/CD154 blockade in transplant rejection are sensitive to OX40 blockade. This is in agreement with our present findings in which anti-OX40L therapy ameliorated EAE mediated by memory T cells and reduced IFN-γ-producing cells.

In our model, the percentage of CD4+CD25+FoxP3+ cells in the transferred population was comparable between the effector and memory populations. After immunization, the percentages of these CD4+CD25+FoxP3+ cells remained similar in the two groups. These data underscore that differential expansion of regulatory T cells is not the cause of disparity in disease severity between the effector and memory T cell recipients. Our findings also suggest that regulatory T cells may have less ability to control memory than effector T cells in vivo.

In conclusion, we provide a clinically relevant novel model of autoimmune disease mediated by memory T cells that is useful for the study of disease pathogenicity as well as for the investigation of immunological tolerance strategies.

Acknowledgments

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References


