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Adam P. Kohm; ... et. al

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Cutting Edge: CD4⁺CD25⁺ Regulatory T Cells Suppress Antigen-Specific Autoreactive Immune Responses and Central Nervous System Inflammation During Active Experimental Autoimmune Encephalomyelitis¹

Adam P. Kohm, Pamela A. Carpentier, Holly A. Anger, and Stephen D. Miller²

Autoreactive CD4⁺ T cells exist in normal individuals and retain the capacity to initiate autoimmune disease. The current study investigates the role of CD4⁺CD25⁺ T-regulatory (T_R) cells during autoimmune disease using the CD4⁺ T cell-dependent myelin oligodendrocyte glycoprotein (MOG)-specific experimental autoimmune encephalomyelitis model of multiple sclerosis. In vitro, T_R cells effectively inhibited both the proliferation of and cytokine production by MOG₃₅₋₅₅-specific Th1 cells. In vivo, adoptive transfer of T_R cells conferred significant protection from clinical experimental autoimmune encephalomyelitis which was associated with normal activation of autoreactive Th1 cells, but an increased frequency of MOG₃₅₋₅₅-specific Th2 cells and decreased CNS infiltration. Lastly, transferred T_R cells displayed an enhanced ability to traffic to the peripheral lymph nodes and expressed increased levels of the adhesion molecules ICAM-1 and P-selectin that may promote functional interactions with target T cells. Collectively, these findings suggest that T_R cells contribute notably to the endogenous mechanisms that regulate actively induced autoimmune disease. *The Journal of Immunology*, 2002, 169: 4712–4716.

An essential characteristic of intrathymic CD4⁺ T cell development is the generation of TCR diversity which permits T cells to respond to a huge number of foreign Ags. However, one consequence of TCR diversity is the inevitable emergence of autoreactive T cell populations that persist in normal individuals with the capacity of mediating various autoimmune diseases (1). To combat this, regulatory cell populations and other protective mechanisms, such as clonal deletion and activation-in-

duced energy, have evolved with the collective goal of inhibiting the generation and/or function of autoreactive CD4⁺ T cells.

CD4⁺ T-regulatory (T_R)³ cells display a mixed phenotype of naive and activated cell surface markers, e.g., CD4⁺CD25⁺CD62L^{high} (2). Importantly, the T_R cell population does not contain previously activated CD4⁺ T cells (3) and inhibits T cell proliferation in a TCR-dependent manner, possibly via direct T-T cell interactions. Although the exact mechanism by which T_R cells exert their inhibitory influence is still unknown, IL-10 production, surface CTLA-4 expression, IL-2 binding, costimulatory molecule blockade, and surface TGF-β expression are all proposed mechanisms by which T_R cells may down-regulate CD4⁺ T cell responses (2).

Consistent with their proposed role as active regulators of autoimmune responses, the depletion of T_R cells in neonatal animals results in the spontaneous induction of autoimmune gastritis in both the thymectomy and *nu/nu* model systems (4, 5). Importantly, T_R cells also block the gastritis resulting from the transfer of H/K ATPase-specific effector T cells (4, 5). Similarly, the transfer of CD4⁺CD25⁺ T_R cells in an adoptive model of diabetes conferred significant protection against the onset of spontaneous diabetes (6), and transfer of either CD4⁺CD25⁻ or CD4⁺CD25⁺ T_R cells has been reported to suppress spontaneous experimental autoimmune encephalomyelitis (EAE) mediated by naive myelin basic protein (MOG)-specific T cells in recombination-activating gene-1-deficient TCR-transgenic mice (7, 8). Collectively, these findings suggest that T_R cells may block both the initiation of autoimmune responses and inhibit the function of established autoreactive effector cells.

In this study, we investigate the role of T_R cells in regulating the progression of active EAE in conventional C57BL/6 mice. MOG₃₅₋₅₅-specific EAE (9), a mouse model of multiple sclerosis, is a CD4⁺ Th1-mediated autoimmune disease (10) in which autoreactive T cells specific for myelin components enter the CNS, initiating a cascade of inflammation and demyelination. We report here that CD4⁺CD25⁺ T_R cells inhibit both the proliferation of and IFN-γ production by a MOG₃₅₋₅₅-specific T cell line in vitro. In addition, supplementation of T_R cell numbers by adoptive transfer before active and adoptive EAE induction significantly reduced

Department of Microbiology-Immunology and Interdepartmental Immunobiology Center, Northwestern University Medical School, Chicago, IL 60611

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² Address correspondence and reprint requests to Dr. Stephen D. Miller, Department of Microbiology-Immunology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611. E-mail address: s-d-miller@northwestern.edu

³ Abbreviations used in this paper: T_R cell, CD4⁺CD25⁺ T-regulatory cell; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; LN, lymph node.

the severity of clinical disease potentially by promoting a disease-protective Th2 immune response and preventing CNS inflammation via a mechanism that may involve up-regulated expression of ICAM-1 and P-selectin. Together, these findings support a role for T_R cells in protection from the onset/progression of autoimmune demyelination in wild-type mice induced by active MOG₃₅₋₅₅/CFA immunization or adoptive transfer of differentiated autoreactive Th1 cells.

Materials and Methods

Mice

Female C57BL/6 mice, 5–6 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained on standard laboratory food and water ad libitum. Paralyzed animals were afforded easier access to food and water.

T_R cells

Peripheral lymph nodes (LNs) were harvested from 6- to 7-wk-old mice, mechanically dissociated, and depleted of APC populations before positive selection of CD25⁺ T_R cells using anti-CD25 Ab (7D4), anti-rat κ microbeads (Miltenyi Biotec, Auburn, CA), and an AutoMACs (Miltenyi Biotec). The resulting population consisted of between 85 and 95% CD4⁺CD25⁺CD62L^{high} T cells.

In vitro proliferation and ELISPOT assay

Draining LN cells or MOG₃₅₋₅₅-specific T cells (AG1) (11) were cultured with medium alone or different concentrations of MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK; Genemed Synthesis, San Francisco, CA) for 72 h and then pulsed with 1 μ Ci/well [³H]TdR for the final 24 h of culture. [³H]TdR uptake was detected using a Topcount Microplate Scintillation Counter, and results are expressed as the mean of triplicate cultures \pm SEM. ELISPOT assays were performed as previously described (11).

Induction and clinical evaluation of MOG₃₅₋₅₅-induced EAE

Female mice 6–7 wk old were immunized s.c. with 200 μ l of an emulsion containing 800 μ g of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI) and 200 μ g of MOG₃₅₋₅₅ distributed over three spots on the flank. Each mouse additionally received 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) in 200 μ l of PBS i.p. on days 0 and 2 postimmunization. For adoptive transfer, 5×10^6 MOG₃₅₋₅₅ blasts were coinjected i.v. with either 2.5×10^6 T_R (CD25⁺) or non- T_R (CD25⁻) cells into naive C56BL/6 mice (11). Individual animals were observed daily, and clinical scores were assessed in a blinded manner on a scale of 0–5 as follows: 0 = no abnormality; 1 = limp tail; 2 = limp tail and hind limb weakness; 3 = hind limb paralysis; 4 = hind limb paralysis and forelimb

weakness; and 5 = moribund. Data are reported as the mean daily clinical score. Mice were age and sex matched for all experiments.

Immunohistochemistry and immunofluorescence

CNS immunohistochemistry was performed as previously described (11). For immunofluorescence, single-cell suspensions were washed and incubated with fluorescently tagged Abs directed against a panel of cell surface markers (BD PharMingen, San Diego, CA). Fluorescent staining was analyzed using a FACSCalibur and CellQuest Pro (BD Biosciences, San Jose, CA).

Statistical analysis

Comparisons of clinical scores and ELISPOT frequencies between the various treatment groups were analyzed by unpaired Student's *t* test. Values of *p* < 0.01 were considered significant.

Results

CD4⁺CD25⁺ T_R cells suppress the proliferation of both CD4⁺ and CD8⁺ T cells; however, the exact mechanism by which these cells exert their effector function remains elusive. To elucidate the potential of T_R cells for regulating the induction and/or progression of EAE, we first determined whether T_R cells could suppress the in vitro proliferation of a T cell line (11) specific for an immunodominant epitope of MOG (MOG₃₅₋₅₅) known to initiate EAE in C57BL/6 mice. As seen in Fig. 1A, coculture of a fixed number of a MOG₃₅₋₅₅-specific Th1 line with an increasing number of T_R cells isolated from naive C57BL/6 mice inhibited the level of T cell proliferation, regardless of whether the T_R cells were isolated from either the LN or spleen. Overnight culture of LN T_R cells with recombinant IL-2, before coculture with target cells, also had no observed effects on suppressive function. In addition to effects on cellular proliferation, T_R cells (E:T ratio, 2:1) also reduced the level of IFN- γ secreted by the MOG₃₅₋₅₅-specific Th1 cell line (Fig. 1B), which is the predominant cytokine produced by this line as determined by gene array analysis (data not shown). As with cellular proliferation, T_R -mediated suppression of IFN- γ production was directly proportional to the E:T ratio (data not shown). Thus, T_R cells appear to be competent in suppressing MOG₃₅₋₅₅-specific T cell proliferation and IFN- γ production in vitro.

On the basis of the above findings, we next investigated the role of T_R cells in modulating EAE disease progression in vivo. First, T_R cells were isolated from LNs of naive C57BL/6 donors, and

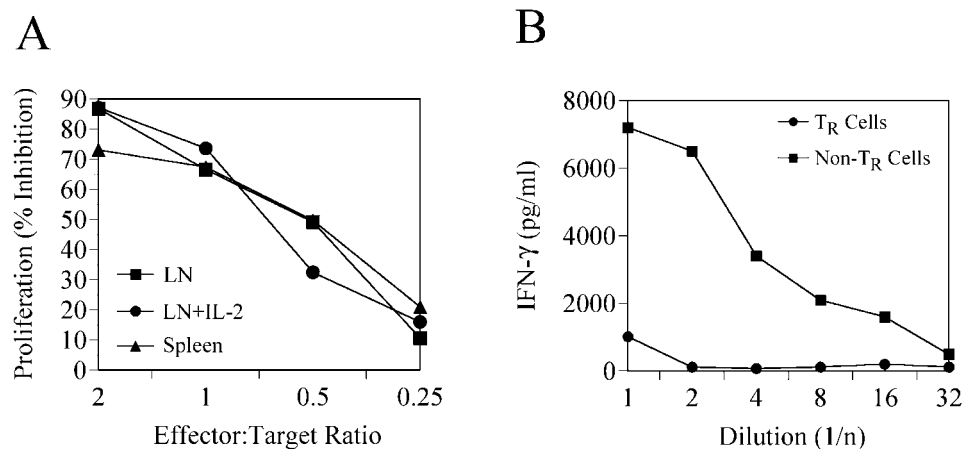


FIGURE 1. Effect of T_R cells on MOG₃₅₋₅₅-specific CD4⁺ Th1 function. *A*, T_R cells isolated from LN or spleen cells were cultured overnight in either the presence or absence of rIL-2. An increasing number of T_R cells (effector cells) were added to a fixed number (5×10^5 /well) of MOG₃₅₋₅₅-specific T cells (target cells), irradiated APC (5×10^6 /well), and MOG₃₅₋₅₅ (10 μ M). Data are presented as percent inhibition of proliferation in comparison with groups cocultured with equivalent numbers of CD4⁺CD25⁻ T cells. *B*, Effect of T_R cell coculture on Ag-specific IFN- γ production. Ninety-six hours after coculture of MOG₃₅₋₅₅-specific T cells with either T_R or non- T_R CD4⁺ cells (E:T ratio, 2:1), supernatant levels of IFN- γ were determined by ELISA. Data are presented as picograms per milliliter of IFN- γ after serial dilution of supernatants and are representative of two separate experiments.

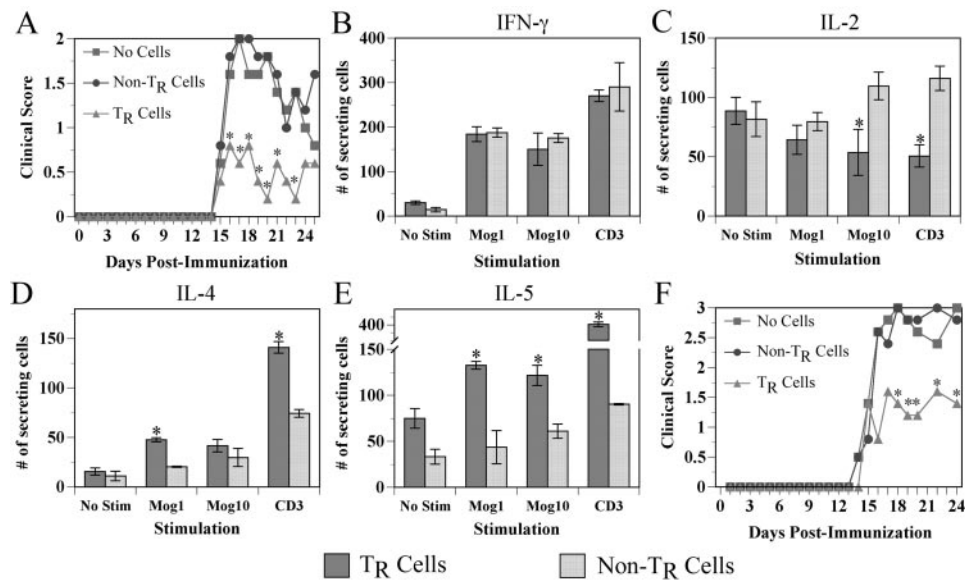


FIGURE 2. Active and passive EAE disease progression after supplementation of T_R cells. *A*, Two $\times 10^6$ T_R or non-T_R cells were isolated from LNs of naive donors and transferred i.v. into recipients 3 days before active EAE induction with MOG₃₅₋₅₅/CFA. Data are presented as the mean clinical score of five mice per group and are representative of three separate experiments. *B–E*, Effect of T_R cells on cytokine production by MOG₃₅₋₅₅-specific T cells during EAE. At the peak of EAE (15–20 days postimmunization), LN cells were harvested and cocultured with irradiated APC and stimulated (Stim) with 2 concentrations of MOG₃₅₋₅₅, 1 μ M (Mog1) and 10 μ M (Mog10), or anti-CD3 Ab (2C11, 0.5 μ g/10⁶ cells) for 24–36 h. Data are presented as the number of MOG₃₅₋₅₅-specific IFN- γ (*B*), IL-2 (*C*), IL-4 (*D*), and IL-5 (*E*)-secreting cells. *F*, Five $\times 10^6$ MOG₃₅₋₅₅ blasts were coinjected i.v. with either 2.5 $\times 10^6$ T_R (CD25⁺) or non-T_R (CD25⁻) cells into naive C56BL/6 mice. Data are presented as the mean clinical score of five mice per group and are representative of three separate experiments. *, Significant differences, $p < 0.01$.

2 $\times 10^6$ T_R cells were adoptively transferred into naive recipients 3 days before active induction of EAE. Supplementation of T_R cells conferred significant protection from the development of clinical EAE (Fig. 2*A*), in comparison with mice receiving either no cells or non-T_R CD4⁺ T cells. Surprisingly, the number of cells secreting disease-promoting Th1 cytokines, such as IFN- γ and TNF- α (12), were similar in the LNs (Fig. 2*B*) and spleens (data not shown) at the peak of disease in all groups of mice. Of potential importance, the number of MOG₃₅₋₅₅-specific IL-2-secreting cells was significantly diminished (Fig. 2*C*), and the number of IL-4 and IL-5-secreting cells was elevated in regulatory cell recipients (Fig. 2, *D* and *E*). Lastly, we examined the capacity of T_R cells to regulate the effector function of previously activated autoreactive T cells by coinjecting T_R or non-T_R cells with MOG₃₅₋₅₅-specific T cell blasts. Clinical symptoms in mice receiving T_R cells were reduced by $\sim 50\%$ in comparison with mice receiving non-T_R cells (Fig. 2*F*). Thus, supplementation of T_R cell numbers conferred protection against both active and passive EAE disease progression, in the absence of any observable effect on the frequency of T cells producing disease-promoting cytokines, whereas the number of cells secreting disease-protective Th2 cytokines was elevated.

As previously discussed, CNS inflammation is a characteristic of EAE disease progression. The degree of lymphocyte infiltration correlates with both the level of macrophage infiltration/activation and disease severity (13). In agreement with this, CD4⁺ T cell infiltration and F4/80⁺ APC activation/infiltration was substantially reduced in the spinal cords of T_R recipients (Fig. 3) despite the normal frequency of peripheral MOG₃₅₋₅₅-specific Th1 cells. These findings raised the possibility that T_R cells may enter the CNS to locally regulate immune cell activation and inflammation. To address this possibility, we examined the peripheral lymphoid organs and CNS tissues of individual mice for the presence of the transferred T_R cells. Although the number of non-T_R CD4⁺ T cells

was markedly reduced 6- to 12-fold within the CNS of T_R cell recipients (Fig. 4*A*), we failed to detect the transferred T_R cell population within either the brain or spinal cord during the peak of EAE. In contrast, there were 3.5-fold more donor cells in the LNs

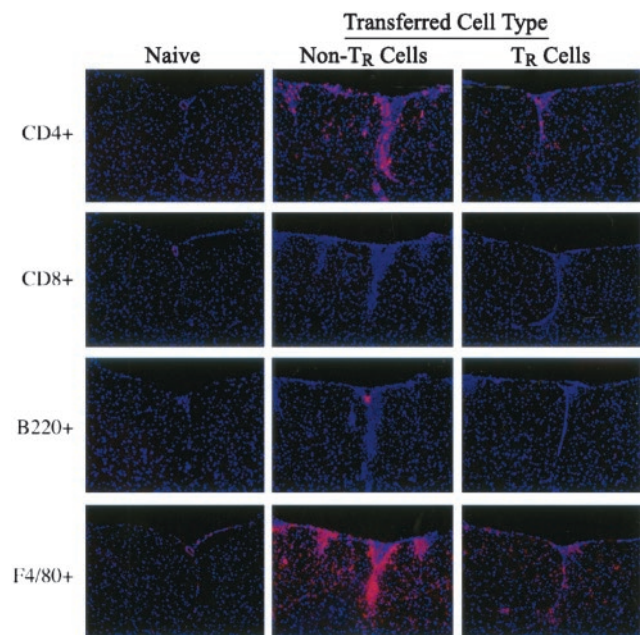


FIGURE 3. T_R cells diminish CNS inflammation during EAE. Spinal cord tissues from mice sacrificed days 15–20 postpriming were examined for the presence of CD4⁺, CD8⁺, B220⁺, and F4/80⁺ (APC) cells (red). Tissues were also counterstained with DAPI (blue). No positive staining was observed in isotype-matched controls (data not shown). $\times 100$ magnification.

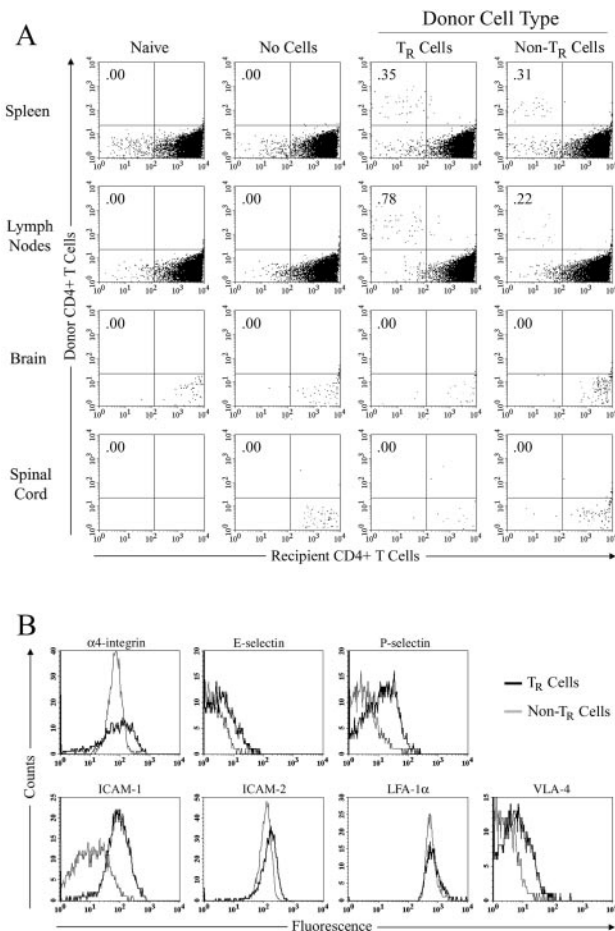


FIGURE 4. In vivo homing properties and adhesion molecule expression on T_R cells. *A*, Homing properties of T_R cells. A total of 2×10^6 T_R or non- T_R cells isolated from Thy1.2⁺ wild-type C57BL/6 mice were adoptively transferred into Thy1.1⁺-congenic mice 3 days before active EAE disease induction. At the peak of clinical disease, mice were perfused before harvest of spinal cords, brains, spleens, and peripheral LNs and adhesion molecule analysis. Data are presented from individual mice as recipient (Thy1.1) vs donor (Thy1.2) $CD4^+$ T cells. The percent of the $CD4^+$ T cell population originating from donor cells is indicated in each upper left quadrant. Data are representative of three separate animals in three separate experiments. *B*, Adhesion molecule expression on T_R and non- T_R $CD4^+$ T cells. LN cells isolated from naive mice were stained with Abs specific for CD4, CD25, CD62L, and a panel of adhesion molecules. Data are presented as the relative fluorescence of each adhesion molecule on both T_R and non- T_R cells and are representative of three separate experiments.

of T_R vs non- T_R recipients, suggesting differential trafficking of these populations. This finding may be explained by differential adhesion molecule expression on T_R cells, which expressed elevated levels of both surface P-selectin (CD62P) and ICAM-1 (Fig. 4*B*). Therefore, T_R cells appear to confer protection to mice against progression of EAE by a mechanism involving enhanced Th2 cytokine production and inhibition of CNS inflammation, which may be dependent on expression of specific adhesion molecules.

Discussion

It is currently believed that TCR ligation enables T_R cells to non-specifically inhibit $CD4^+$ T cell proliferation both in vitro and in vivo (3). However, there is a surprising deficiency in the literature concerning the effects of T_R cells on additional functional measures such as cytokine production and cellular differentiation. We

report that coculture of MOG_{35–55}-specific T cells with T_R cells inhibits the level of both Ag-specific proliferation and IFN- γ production. T_R cells appear not to prevent the initial activation of target $CD4^+$ T cells but subsequently induce cell cycle arrest (3). In light of these findings, it is not clear whether the measured reduction in IFN- γ production is the result of direct effects on cytokine production or whether it results from inhibition of Ag-specific cell expansion. Previous studies indicate that T_R cells must be activated via TCR ligation before exerting their immunosuppressive phenotype (14); however, the limiting frequency of MOG_{35–55}-specific T_R cells undoubtedly present in our in vitro culture system suggests that T_R cells are more efficient inhibitors of Ag-specific than mitogen-induced T cell responses and/or that these cells become activated as a result of the isolation process.

To investigate the role of T_R cells in regulating the progression of autoimmune diseases, we used a model system in which the T_R cell population in naive C57BL/6 mice was supplemented before both active and passive induction of EAE. Augmentation of T_R cell numbers by ~50–75% (2×10^6 T_R cells transferred to naive recipients normally containing an estimated $2.5–3 \times 10^6$ T_R cells) conferred significant protection against EAE induction/progression as measured by both disease score and the promotion of protective Th2 cytokines. In addition, we observed markedly less $CD4^+$ T cell infiltration into the CNS at the peak of disease in T_R cell recipients which corresponded with decreased levels of APC infiltration/activation within the CNS (Fig. 3). One explanation of these findings is that T_R cells inhibit the expansion of MOG-specific T cells in peripheral lymphoid organs. This possibility is supported by the decreased frequency of MOG_{35–55}-specific IL-2-producing cells in T_R cell recipients, but not by the normal numbers of Ag-specific IFN- γ ELISPOTs (Fig. 2). Alternatively, we detected an increased frequency of cells producing Th2-like cytokines in the LNs and spleens of T_R cell recipients after disease initiation. Therefore, it is possible that T_R cells differentially influence either the differentiation or effector function of Th1 and Th2 cells, with the normal frequency of Th1 cells better supporting the latter possibility. In addition, the elevated number of MOG_{35–55}-specific Th2 cells in T_R recipients may result from inhibition of the pathogenic Th1-like responses, subsequently allowing the progression of bystander Th2 responses. This possibility is further supported by our findings that mitogenic (anti-CD3 Ab) stimulation of LN and spleen cells isolated from T_R recipients induced a significant increase in the number of cells producing Th2-like cytokines in comparison with cells stimulated with the specific MOG_{35–55} peptide (Fig. 2). However, further study is necessary to gain a better understanding of the exact effector mechanisms of T_R cells during EAE disease progression.

Little is currently known about the homing patterns and site(s) of T_R cell function in vivo. During EAE, T_R cells may traffic to the CNS to inhibit the local activation of myelin-specific autoreactive T cells, a prerequisite for development of inflammatory demyelination (11). However, we failed to detect donor T_R within the CNS at a time corresponding with the peak of disease in non- T_R cell recipients (Fig. 4*A*), whereas T_R cell populations were detectable in recipient spleen and LNs. This finding supports the hypothesis that T_R cells may influence the activation of autoreactive T cells within peripheral lymphoid organs and/or the homing of activated lymphocytes to the CNS. This latter hypothesis gains further support from current findings (Fig. 2*F*) and those of others (4, 5) showing that T_R cells inhibit the in vivo function of previously activated T cells. It is possible that the numbers of regulatory cells within the CNS were below our detection limits or that the kinetics of T_R cell homing was such that these cells may be detected within the CNS either earlier or later than currently measured.

Chemokine gradients are an obvious mechanism that may regulate T_R cell trafficking in vivo (8, 15). In addition, T_R homing may be influenced by differential expression of adhesion molecules. Our findings support this possibility, because T_R cells expressed elevated levels of both ICAM-1 and P-selectin in comparison with non-T_R cells (Fig. 4B). The detection of P-selectin expression on T_R cells was surprising, because previous reports have indicated that expression is limited to activated platelets and endothelium (16). However, because P-selectin facilitates endothelium-T cell interactions (17), P-selectin expression may prove to be one mechanism promoting the direct interaction of T_R cells with target CD4⁺ T cells in vivo. This is an attractive hypothesis in light of previous findings suggesting that T_R cells must directly interact with target T cells to exert their suppressive phenotype and that P-selectin glycoprotein ligand-1 is expressed preferentially on Th1 cells (18). This is supported by our recent studies showing that T_R from P-selectin-deficient mice are functionally defective in vitro (data not shown). We are currently exploring the dependence of P-selectin/P-selectin glycoprotein ligand-1 interactions, as well as other adhesion molecules, in governing T_R function both in vitro and in vivo.

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