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Blocking OX-40/OX-40 Ligand Interaction In Vitro and In Vivo Leads to Decreased T Cell Function and Amelioration of Experimental Allergic Encephalomyelitis¹

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The OX-40R is a member of the TNF receptor family and is expressed primarily on activated CD4⁺ T cells. When the OX-40R is engaged by the OX-40 ligand (OX-40L), a potent costimulatory signal occurs. We have identified a population of CD11b⁺ cells, isolated from the central nervous system (CNS) of mice with actively induced experimental allergic encephalomyelitis (EAE), that expresses OX-40L. Moreover, the expression of OX-40L was found to be associated with paralytic episodes of EAE and was reduced or absent at disease recovery. These CD11b⁺ cells also coexpressed B7 and MHC class II. Therefore, to address the relative contributions of OX-40R/OX-40L and CD28/B7 to the costimulation of myelin-specific T cells, blocking studies were performed using soluble OX-40R and/or soluble CTLA-4. CD11b⁺ cells isolated from the CNS of mice with actively induced EAE were able to present Ag to proteolipid protein 139–151-specific T cell lines in vitro. The addition of soluble OX-40R:Ig to CD11b⁺ brain microglia/macrophages inhibited T cell proliferation by 50–70%. The addition of CTLA-4:Ig inhibited T cell proliferation by 20–30%, and the combination inhibited T cell proliferation by 95%. In vivo administration of soluble OX-40R at the onset of actively induced or adoptively transferred EAE reduced ongoing signs of disease, and the mice recovered more quickly from acute disease. The data imply that OX-40L, expressed by CNS-derived APC, acts to provide an important costimulatory signal to EAE effector T cells found within the inflammatory lesions. Furthermore, the data suggest that agents designed to inhibit the OX-40L/OX-40R complex may be useful for treating autoimmune disease. *The Journal of Immunology*, 1999, 162: 1818–1826.

The combination of two signals is necessary to activate CD4 and CD8 T cell responses (1). The first signal is delivered through the TCR by Ag bound to MHC class I or II expressed on an APC. The best characterized second signal is delivered to the T cell via the CD28 receptor by its ligand CD80 (B7.1) or CD86 (B7.2) expressed on APC (1). The interaction between CD80 and CD86 with CD28 results in increased IL-2 production and T cell proliferation. CTLA-4, which is expressed on activated T cells, also binds to CD80 and CD86, but this interaction appears to deliver a negative signal to the T cell (2). A soluble form of CTLA-4 (CTLA-4:Ig) binds to CD80 and CD86 and inhibits T cell proliferation generated by the CD28-mediated second signal (3–5).

The OX-40R is a membrane-bound protein that is expressed primarily on activated CD4⁺ T cells (6). It is part of a growing family of proteins in the TNF receptor superfamily, many of which are expressed by lymphocytes, for example, FAS, CD30, CD27, the TNF receptors, DR3, CD40, 4-1BB, and lymphotoxin- β receptor. All of these proteins have several cysteine

residues in the extracellular N-terminal domain, and the disulfide bonds formed within these proteins are thought to create a tertiary structure involved with ligand binding. These receptors appear to be involved in either cell growth and differentiation or cell death (apoptosis). The 4-1BB, CD30, and OX-40 receptors are all involved in T cell activation and have been shown to deliver costimulatory signals when engaged by their corresponding ligands or specific Abs (7, 8, 9–12).

Recently, a membrane-bound protein with homology to TNF was cloned and found to be the ligand for OX-40R (7, 8). The OX-40 ligand (OX-40L)⁴ is a type II membrane protein of ~34,000–40,000 m.w. (13) with homology to TNF, which is expressed on activated B cells (14) and activated endothelial cells (15) but not expressed on normal tissue. In addition to delivering a costimulatory signal, OX-40R/OX-40L interaction also mediates an adhesion event between endothelial cells and T cells (15).

The OX-40R is selectively expressed on T cells isolated from the site of inflammation in a number of disease states, including experimental allergic encephalomyelitis (EAE) (16, 17), rheumatoid arthritis (18), and graft-vs-host disease (GVHD) (19), and on tumor-infiltrating lymphocytes (20). OX-40⁺ T cells isolated from the inflammatory site in EAE (rat spinal cord) exhibited T cell receptor CDR3 binding motifs that are characteristic of those described for myelin basic protein (MBP) reactivity (17) and produced proinflammatory cytokines (IL-2 and IFN- γ) (21). Thus, Abs to the OX-40R T cells can be used to identify and isolate autoantigen-specific T cells from the site of inflammation without prior knowledge of the autoantigen. In addition, MBP-reactive CD4⁺ T cells are activated following costimulation with Abs specific for OX-40R, and this activation leads to the generation of

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⁴ Abbreviations used in this paper: L, ligand; EAE, experimental allergic encephalomyelitis; GVHD, graft-vs-host disease; MBP, myelin basic protein; CNS, central nervous system; R-EAE, relapsing EAE; PLP, proteolipid protein.

EAE effector T cells capable of transferring EAE into naive Lewis rats (9). These data imply that blocking OX-40R signaling *in vivo* should inhibit activation and/or proliferation of autoantigen-specific T cells at the site of inflammation and may dampen clinical signs of EAE.

In this manuscript we follow the time course of OX-40R expression on CD4⁺ T cells and OX-40L expression on CD11b⁺ cells isolated from the central nervous system (CNS) of SJL mice with relapsing EAE (R-EAE). Disease onset correlated with the appearance of OX-40R⁺ T cells, which peaked at disease onset, declined as the disease regressed, and increased during relapses. Similarly, OX-40L⁺ CD11b⁺ cells were present at disease onset, appeared to remain during the acute phase of disease, disappeared at disease recovery, and reappeared during relapses. These results suggested that OX-40R:Ig-Fc chimeric protein might be useful in blocking stimulation of OX-40R⁺ T cells by interfering with the ability of the OX-40L⁺ CD11b⁺ APC to costimulate via OX-40L/OX-40R interaction. We show that OX-40R:Ig was able to inhibit peptide-specific proliferation of proteolipid protein (PLP)-specific T cell lines when stimulated by either peptide-pulsed thymocytes or CNS-isolated CD11b⁺ cells. The combination of OX-40R:Ig and CTLA-4:Ig completely inhibited T cell proliferation *in vitro*. *In vivo* administration of OX-40R:Ig was able to dampen clinical signs of acute disease and inhibit relapses if administered during the clinical signs of the relapse. Our data are suggestive of a critical costimulatory role for OX-40R/OX-40L interaction in EAE. We hypothesize that the OX-40R is engaged by the OX-40L on CD11b⁺ cells during Ag-specific T cell priming in the CNS of animals with EAE and inhibition of this process leads to dampening of an ongoing autoimmune response.

Materials and Methods

Mice

Female SJL/J mice (5–20 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in the Animal Resource Facility at the Portland Veterans Affairs Medical Center under pathogen-free conditions according to institutional guidelines.

Antigens

Mouse spinal cord homogenates were prepared from SJL spinal cord as previously described (22). High-pressure liquid chromatography-purified PLP_{139–151} (amino acid sequence HCLGKWLGHDPKF) was purchased from the Beckman Center (Stanford, CA) or synthesized on an ABI Syn-gergy 432A peptide synthesizer (Perkin-Elmer, Foster City, CA).

Induction of R-EAE

To induce active EAE, female SJL/J mice were inoculated s.c. in the flanks at four sites with a total of 0.2 ml of emulsion of saline containing 150 µg of PLP_{139–151} and an equal volume of CFA containing 200 µg of *Mycobacterium tuberculosis* H37RA. Mice were examined daily by an investigator blinded to treatment for the development of neurological deficits. Degrees of hindlimb and forelimb weaknesses were assessed as previously outlined (22, 23).

Animals with minimal hindlimb weakness were easily flipped onto their backs with a twist of the tail but could easily right themselves. With mild hindlimb weakness, mice had difficulty righting themselves after being flipped onto their backs. Animals with moderate hindlimb weakness could walk with no difficulty but could not right themselves after being flipped onto their backs. Mice with moderately severe hindlimb weakness could walk upright only with difficulty. Mice exhibiting severe hindlimb weakness could not walk upright but were still able to move hindlimbs. Animals with hindlimb paraplegia displayed no volitional leg movement.

With mild forelimb weakness, mice walked with chests close to the ground. With moderate forelimb weakness, mice could not lift their chests off the ground but could move around the cages with difficulty. With severe forelimb weakness, mice could not place forelimbs under their chests and were unable to move around the cage.

Clinical severity of disease was graded as follows using a modification of a previously published grading scale (24): 0, normal; 1, minimal hind-

limb weakness; 2, mild hindlimb weakness; 3, moderate hindlimb weakness; 4, moderately severe hindlimb weakness; 5, severe hindlimb weakness; 6, hindlimb paraplegia; 7, hindlimb paraplegia with mild forelimb weakness; 8, hindlimb paraplegia with moderate forelimb weakness; and 9, hindlimb paraplegia with severe forelimb paralysis.

Gelatin cubes were placed in cages of animals with severe clinical disease. In addition to gelatin cubes, animals involved in treatment studies also received injections of sterile saline or human Ig in sterile saline (0.2 ml) as a diluent that also served to rehydrate animals.

Induction of adoptively transferred EAE

Adoptive transfer of EAE was accomplished by stimulating Ag-specific T cell lines for 3 days in culture with PLP_{139–151} peptide (5 µg/ml) and irradiated syngeneic thymocytes as APCs. Cells were then washed and resuspended in RPMI 1640 and transferred to naive recipient SJL/J mice by i.p. injection of 5×10^6 viable blasts.

Generation of PLP peptide-specific T cell lines

To generate Ag-specific T cell lines, mice were inoculated s.c. in the flanks at four sites on days 0 and 7 with a total of 0.3 ml of emulsion containing 1.0 mg of mouse spinal cord homogenate and an equal volume of CFA with 30 µg of *M. tuberculosis* H37RA (Difco, Detroit, MI). Draining inguinal lymph node cells (LNC) were obtained 14 days after immunization and stimulated with PLP_{139–151} to produce Ag-specific T cell lines as previously described (25). Briefly, LNC (7×10^6 /ml) were cultured in RPMI 1640 and 1% normal mouse serum (stimulation medium) for 3 days with Ag (50 µg/ml of PLP_{139–151}) and then expanded in RPMI 1640 and 10% FCS (growth medium) containing human rIL-2 (National Cancer Institute, Frederick, MD) for 4–10 days. The T cell lines (5×10^5 cells/ml) were restimulated with Ag (5 µg/ml of PLP_{139–151}) and a 10-fold excess of irradiated (2400 rad) syngeneic thymocytes as APCs and then alternately cycled between growth medium and stimulation medium.

Isolation of brain mononuclear cells

Mononuclear cells were isolated from SJL/J brains as previously described, (21, 26, 27). Mice were not perfused before removal of CNS tissue. Briefly, brains were removed from donor animals, and a single cell suspension was prepared by passage through a wire mesh. Cells were washed in RPMI 1640, resuspended in 7 ml of 80% Percoll (Pharmacia, Uppsala, Sweden), and then overlaid with 8 ml of 40% Percoll to form a discontinuous gradient in a 15-ml centrifuge tube. The gradient was centrifuged at $500 \times g$ for 35 min, and the 40–80% interface was harvested. The cells were washed three times with RPMI 1640 and stained for FACS analysis or used as APC for proliferation assays.

Enrichment of macrophage/microglia populations from the CNS

The CNS-isolated leukocytes were enriched for CD11b⁺ cells in a two-step process. Macrophages/microglia were stained with Mac-1 FITC for 20 min at 4°C and stained with a secondary anti-FITC-labeled microbead (Miltenyi Biotec, Auburn, CA). The CD11b⁺-labeled cells were then purified by positive selection using a magnetic bead separation column (Miltenyi Biotec). Following enrichment, the CD11b⁺ cells were analyzed by FACS analysis and found to be >80% pure.

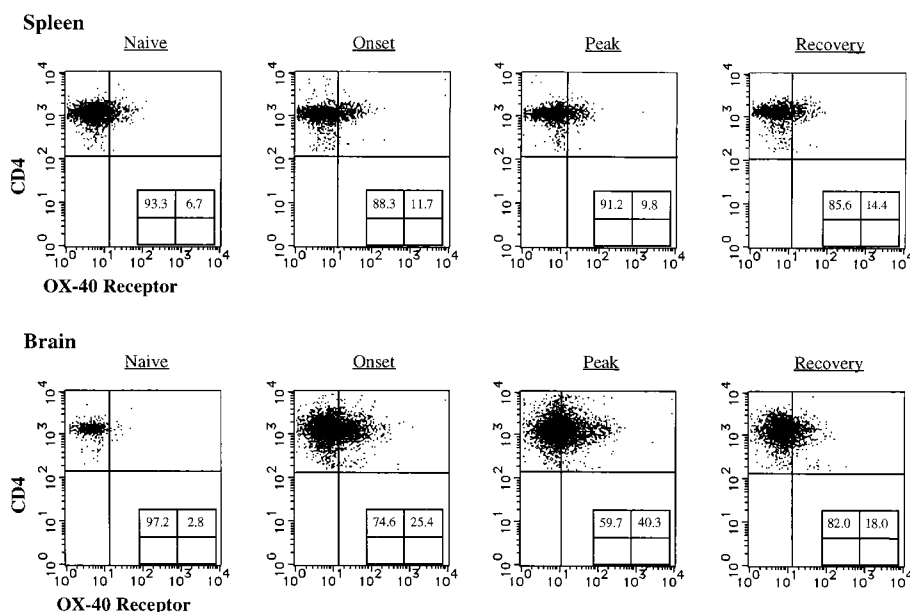
Proliferation assays

The *in vitro* proliferative responses of PLP_{139–151}-specific T cell lines were determined in 96-well microtiter plates as reported previously (26, 27). Cells were incubated for 72 h with Ag with the following cell concentrations: PLP_{139–151}-specific T cell lines 2×10^4 cells per well with 2×10^5 syngeneic irradiated thymocytes per well or 6000 syngeneic CD11b⁺ positive brain macrophage/microglia per well as APC. Tritiated thymidine was added for the last 18 h of culture, and the cpm of incorporated label were determined in triplicate wells by standard liquid scintillation techniques.

Flow microfluorometry analysis

Immunofluorescence analysis of cells was performed on a FACScan (Becton Dickinson, Mountain View, CA) as described previously (21, 25). T cell lines and cells isolated from different organs were incubated with the specific mAbs for 15 min at 4°C in staining buffer (PBS with 3% newborn calf serum and 0.1% sodium azide), washed twice, resuspended in 300 µl of staining buffer containing 5 µg/ml of propidium iodide for dead cell discrimination, and directly analyzed. Each sample was stained with an isotype-matched control.

FIGURE 1. Kinetics of OX-40R expression on CD4⁺ T cells in spleen and brain following active induction of EAE. The dot plots were gated on CD4 positive cells that were analyzed for OX-40R expression. Cells were isolated from the brain and spleen at the indicated stages of R-EAE (2–3 mice per group). Disease stages of EAE are as follows: onset (disease score 1, day 1 of disease, 13 days after immunization); peak (disease score 8–9, day 3 of disease, 13 days after immunization); recovery (disease score 0, 19 days after immunization). It should be noted that the same cells were analyzed in Fig. 4.



Monoclonal Abs

mAbs used for FACS analysis were as follows: CD4 (clone GK1.5, rat IgG2b), Mac-1 (CD11b) (clone M1/70, rat IgG2b), and anti-IA^s (clone OX-6, mouse IgG1) were purchased from PharMingen (San Diego, CA). The goat anti-human IgG (Fc-specific) secondary Ab used to detect T cells stained with recombinant human-Ig fusion proteins was obtained from Sigma (St. Louis, MO).

Recombinant Ig fusion proteins for FACS staining and in vivo treatment

The soluble human OX-40R:Fc-Ig recombinant protein was produced by deleting the membrane-spanning portion of the OX-40R molecule and fusing the gene to the Fc portion of human Ig. The human OX-40R:Ig protein has been shown to bind to the murine OX-40L (8). This protein was supplied by Cantab Pharmaceuticals (Cambridge, U.K.). The soluble murine OX-40L:Fc-Ig was used to detect the murine OX-40R, and the cell-staining procedure was as described for the OX-40R:Ig construct. This protein was supplied by Cantab Pharmaceuticals. CTLA-4:Fc-Ig was supplied by Smith Kline-Beecham (King of Prussia, PA) and was used to detect CD80 and/or CD86 in Fig. 3A and for some of the in vivo experiments discussed in detail below. The lymphotoxin-β receptor:Fc-Ig fusion protein was used to detect membrane-bound lymphotoxin-β and was supplied by Carl Ware (La Jolla for Allergy and Immunology, San Diego, CA). All fusion protein staining was accomplished using 1 μg of fusion protein to stain 1 × 10⁶ cells and a goat anti-human FITC (Caltag, South San Francisco, CA) was used to detect staining of the recombinant fusion proteins. Where specified, human Ig (IgG1 isotype) was used as a control for staining as well as in the in vivo treatment experiments (Smith-Kline Beecham). For the in vivo experiments, OX-40R:Fc-Ig, CTLA-4:Fc-Ig, and human Ig were given i.p. in 0.2 ml of saline at the specified concentrations for the individual experiments.

Results

Kinetics of OX40R expression on CD4⁺ T cells from CNS in actively induced EAE

It has previously been reported that the OX-40R was preferentially expressed on autoantigen-specific CD4⁺ T cells isolated from the CNS of Lewis rats with EAE (16, 17, 21). Therefore, we used a soluble murine OX-40L:Ig chimeric protein to stain T cells isolated from the brains of SJL mice with R-EAE for the presence of OX-40R. Fig. 1 depicts a time course of OX-40R expression on CD4⁺ T cells isolated from the spleen and brain of mice with actively induced EAE, compared with T cells from naive mice. Naive animals had very few CD4⁺ T cells in the brain, and they expressed little or no OX-40R. By

comparison, CD4⁺ T cells isolated from the brains of mice with actively induced EAE expressed significant levels of OX-40R. In a representative experiment (Fig. 1), at disease onset (day 1 of disease, 13 days after immunization) 25% of CD4⁺ cells expressed OX-40R, which increased to 40% at peak disease (day 3 of disease, 13 days after immunization), decreased to 18% at disease recovery (17–20 days after immunization), and increased to 34% at severe disease relapse (21–28 days after immunization) (relapse time point was part of a different experiment; data not shown). It should be noted that at peak disease ~10–15% of the CD11b⁺ cells also express the CD4 Ag but do not express the OX-40R. Therefore, at peak disease the percentages of CD4⁺ T cells expressing OX-40R may be underestimated due to contaminating CD4⁺ macrophages. T cells isolated from the spinal cord showed similar trends to those observed in the brain (data not shown). However, the number of infiltrating cells obtained from a diseased spinal cord was 10-fold less than that obtained from a diseased brain. Therefore, brains from mice with EAE were used in preference to spinal cord in subsequent experiments. By comparison, in nonneural tissue such as the spleen, examination of CD4⁺/OX-40R⁺ T cells obtained from naive spleens and spleens from animals with active EAE demonstrated only a slight increase in OX-40R expression with disease onset (11.7% vs 6.7%). Interestingly, there was little variation of OX-40R expression in the spleen during the time course of disease (onset, 11.7%; peak of disease, 9.8%; and disease recovery, 14.4%).

Characterization of CD11b cells from CNS in actively induced EAE

To characterize the cell type within the CNS capable of presenting Ag to the CD4⁺/OX-40R⁺ T cells, we first examined MHC class II expression at various time points of actively induced EAE. In normal brain, ~93% of the cells expressed CD11b. However, <2% of these CD11b⁺ cells expressed MHC class II (Fig. 2). The CD11b⁺ cells found in the naive brain were CD45^{low}, which is a phenotype consistent with the presence of brain microglia and not bone marrow derived macrophages (data not shown) (28, 29). Upon examination of the brain infiltrates obtained from animals at various time points of

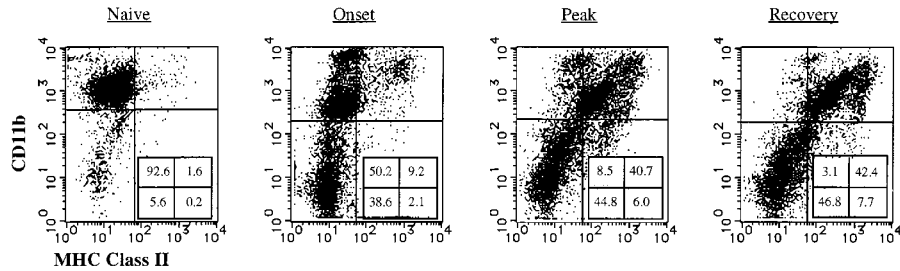


FIGURE 2. Kinetics of MHC class II expression on CD11b⁺ cells isolated from the CNS following active induction of EAE. The dot plots show total CNS mononuclear cells isolated from brains at the indicated stages of R-EAE (2–3 mice per group). Disease stages of EAE are as follows: onset (disease score 1–2, day 1 of disease, 11 days after immunization), peak (disease score 7–9, day 4 of disease, 16 days after immunization), recovery (disease score 0, 20 days after immunization).

active EAE, it is clear that with disease onset a significant population of CD11b negative cells appear (40% at disease onset (see Fig. 2), most of which are CD4⁺ T cells). However, in the CD11b⁺ cell population there is a dramatic increase in the percentage of cells expressing MHC class II. At disease onset, 15% of the CD11b⁺ cells express MHC class II, which increased to 83% at peak disease (Fig. 2). Interestingly, there is no decrease of MHC class II expression by CD11b⁺ cells following recovery from active disease (93%). At peak disease, 62% of the CD11b⁺ cells also expressed the CD45^{high} phenotype, a characteristic staining profile of bone marrow-derived macrophages (data not shown) (28, 29).

To further characterize CNS-isolated CD11b⁺ cells in animals with actively induced EAE, we examined cell surface expression of costimulatory molecules. Brain mononuclear cells were isolated from animals with severe clinical EAE and stained with several soluble chimeric fusion proteins (CTLA-4:Ig, OX-40R:Ig, or lymphotoxin-β receptor:Ig) or human Ig as an isotype control. As shown in Fig. 3, the CD11b⁺ cells isolated from the brain of an-

imals with severe disease expressed CD80 and/or CD86 (70%) and OX-40L (67%) but no surface lymphotoxin-β. Others have also reported selective up-regulation of B7.1 (CD80) expression within the CNS of mice with EAE (30).

Results from Fig. 1 suggested that, in the brain, the percentage of CD4⁺ T cells expressing OX-40R correlated with active EAE disease progression. Therefore, in the same mice, we determined if OX-40L expression by CD11b⁺ cells also correlated with disease progression. As demonstrated in Fig. 4, brain microglia cells obtained from naive brain were negative for OX-40L expression. By comparison, CD11b⁺ cells isolated from the brains of animals exhibiting actively induced EAE were shown to express significant levels of OX-40L. Expression of OX-40L by CD11b⁺ cells dramatically increased at disease onset (day 1 of disease, 13 days after immunization; 58% of CD11b⁺ cells) and stayed relatively the same at the peak of disease (day 3 of disease, 13 days after immunization; 52%). OX-40L expression was undetectable at disease recovery (17–20 days after immunization; < 1%), in contrast to MHC class II expression, which remained elevated. Interestingly, OX-40L expression on CD11b⁺ brain APC reappeared during clinical relapses (43% express OX-40L) (relapse time point was part of a different experiment; data not shown). There was little or no expression of OX-40L in spleens of animals with active disease (Fig. 4).

To confirm expression of OX-40L on APC from the brains of animals with actively induced EAE, we performed RT-PCR to detect OX-40L mRNA and Western blot analysis to detect OX-40L protein. RNA was extracted from CNS mononuclear cells isolated from animals with EAE. Following PCR amplification with OX-40L-specific primers, a band of the correct m.w. for OX-40L was visualized. In confirmation of our flow cytometry data, OX-40L RNA expression was highest at the onset and peak of disease and less abundant during recovery (data not shown). Western blot analysis of OX-40L protein synthesis by mononuclear cells in the CNS of animals with actively induced EAE also confirmed our previous findings (data not shown). CNS mononuclear cells and splenocytes were isolated from animals at the acute phase of EAE, and the cell surface proteins were passed over an OX-40R column. The CNS-derived lysate showed a single band at 40 kDa, which was identical in m.w. when compared with the lysate from an OX-40L transfected cell line. No band was detected when a protein lysate from splenocytes isolated from animals with EAE was passed over the same column (data not shown). Taken together, these data confirm preferential expression of the OX-40L within the CNS of animals with clinical signs of EAE.

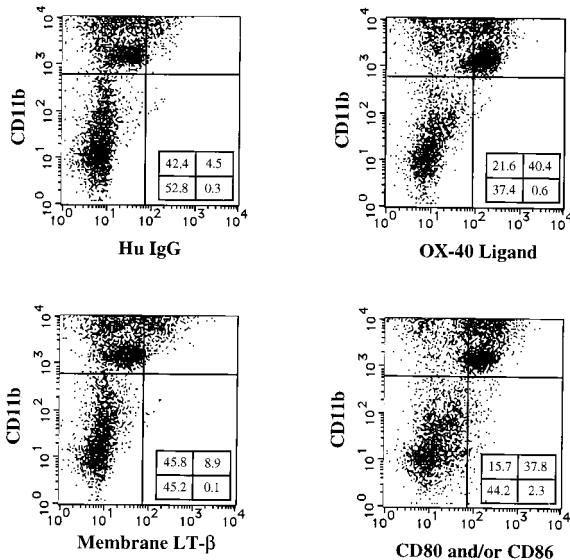
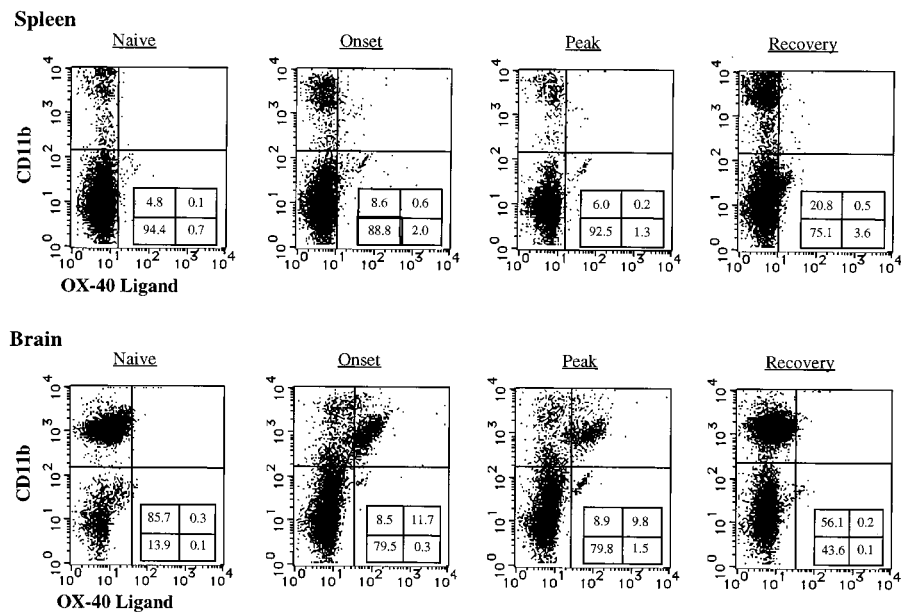


FIGURE 3. Surface phenotype of CD11b⁺ cells isolated from the CNS of animals with severe disease following active induction of EAE. Cells were isolated from the brains of mice at the peak of clinical signs of EAE (disease score 6–8, day 3 of disease, 14 days after immunization). The dot plots show total CNS mononuclear cells stained with CD11b phycoerythrin and counterstained as indicated.

FIGURE 4. Kinetics of OX-40L expression on CD11b⁺ cells in spleen and brain following active induction of EAE. The dot plots show spleen cells and total CNS mononuclear cells isolated from the brain and spleen at the indicated stages of R-EAE (2–3 mice per group). Disease stages of EAE are as follows: onset (disease score 1, day 1 of disease, 13 days after immunization), peak (disease score 8–9, day 3 of disease, 13 days after immunization), recovery (disease score 0, 19 days after immunization). It should be noted that the same cells were analyzed in Fig. 1.



OX-40R-specific inhibition of *in vitro* T cell proliferation

Results from Figs. 1 and 4 suggest that OX-40L may be important for costimulating myelin-specific T cells found within the CNS of animals with actively induced EAE. To determine whether OX-40L expression by CD11b⁺ cells isolated from the CNS of mice with EAE could act as APC and provide costimulation, we isolated these cells from the brains of animals exhibiting active EAE and used them in a T cell proliferation assays. Brain APC were assessed for their ability to present Ag to PLP_{139–151}-specific T cells in the presence or absence of soluble costimulatory blocking reagents (CTLA-4:Ig and OX-40R:Ig). Irradiated thymocytes (used as control APC) or brain macrophages were placed in culture in the presence of CTLA-4:Ig, OX-40R:Ig, or CTLA-4:Ig and OX-40R:Ig for 1 h. The APC were added to PLP_{139–151}-specific T cells in the presence of specific peptide. The proliferation results are shown in Fig. 5.

Using irradiated thymocytes as APC (at an APC:T cell ratio of 10:1) (Fig. 5A), soluble CTLA-4 inhibited T cell proliferation by 22% when present at 2 μ g/ml and by 71% when present at 40 μ g/ml. T cell proliferation was not inhibited in the presence of 2 μ g/ml of OX-40R:Ig but was inhibited at 40 μ g/ml (48%). The combination of CTLA-4:Ig and OX-40R:Ig at 2 μ g/ml inhibited T cell proliferation by 22%, and when present at 40 μ g/ml it decreased proliferation by 95%. Human IgG was added at 80 μ g/ml to the assay and did not significantly inhibit proliferation.

Using irradiated brain CD11b⁺ cells (> 80% positive) (Fig. 5B) as APC (at an APC:T cell ratio of 0.3:1), soluble CTLA-4 at 2 μ g/ml inhibited Ag-specific T cell proliferation by 26% and by 32% at 40 μ g/ml. Soluble OX-40R:Ig, when present at 2 μ g/ml, inhibited T cell proliferation by 15% and at 40 μ g/ml inhibited T cell proliferation by 71%. The combination of CTLA-4:Ig and OX-40R:Ig at 2 μ g/ml inhibited T cell proliferation by 44% and higher concentrations (40 μ g/ml) inhibited proliferation by 91%. When Abs directed against MHC class II (OX-6) were added, T cell proliferation was completely inhibited.

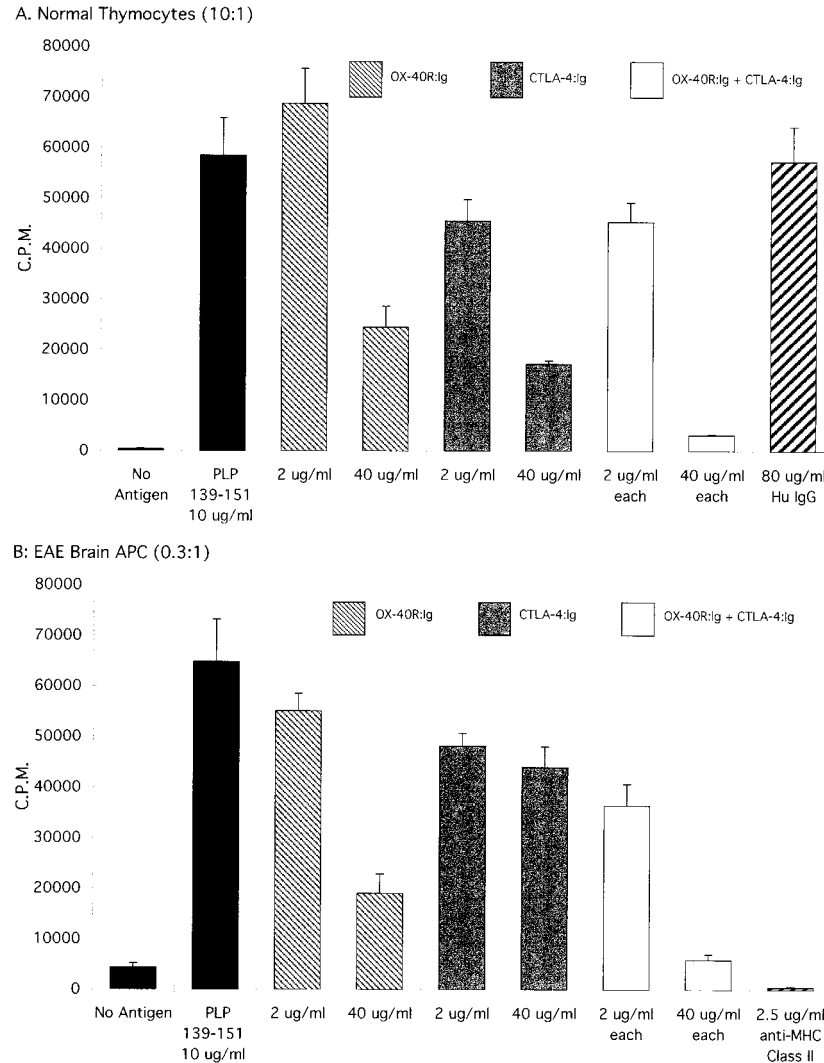
In vivo therapy of actively induced EAE with soluble OX-40R:Ig

The *in vitro* proliferation data, as well as FACS analysis (Figs. 1–5), suggested that it might be possible to dampen an ongoing immune response within the CNS of animals with EAE by

blocking OX-40R/OX-40L interaction. Therefore, we treated mice with OX-40R:Ig to bind the OX-40L present on APC and prevent OX-40R-specific signaling. Animals were immunized with PLP_{139–151} and CFA and treated with 400 μ g of OX-40R:Ig or saline on the first day of disease onset and for two days thereafter. Fig. 6 and Table I (Expt. 1), demonstrate that the group treated with OX-40R:Ig had less severe clinical signs of disease compared with the control group and recovered more quickly from the acute phase of disease, although both groups did relapse (Fig. 6). Table I shows two additional treatment experiments (Expts. 2 and 3). In experiment 2, animals were treated with OX-40R:Ig, CTLA-4:Ig, OX-40R:Ig, and CTLA-4:Ig or saline. Treatment was initiated 10 days after active immunization and continued daily for 5 days. Both OX-40R:Ig and CTLA-4:Ig groups showed decreased disease severity, and the combination showed no enhancement over OX-40R:Ig or CTLA-4:Ig treatment alone. In addition, 80% of the animals receiving saline died due to severity of disease, whereas all of the treatment groups survived. Experiment 3 compared OX-40R:Ig treatment to a human Ig isotype control. Similar to experiment 2, 50% of the control animals died, whereas all of the OX-40R:Ig group survived, and the control group had more severe disease in comparison to the OX-40R:Ig-treated group.

Treatment with OX-40R:Ig at disease onset had little or no effect on subsequent disease relapses. Therefore, studies were initiated to determine whether soluble OX-40R:Ig or CTLA-4:Ig administered at the time of recovery from acute EAE altered the percentage of animals that relapsed. Animals were treated daily following recovery from EAE with 100 μ g of OX-40R:Ig, CTLA-4:Ig or human Ig for 14 days. OX-40R:Ig was able to prevent relapses during the time of treatment (14 days), but once the treatment was stopped, the animals relapsed (data not shown). CTLA-4:Ig was able to prevent relapses during and after the time of treatment, while the control (human Ig) animals relapsed (data not shown). Animals were analyzed for the presence of Abs specific for OX-40R:Ig, CTLA-4:Ig, and hu:Ig after the experiment was concluded. Mice treated with both human Ig and OX-40R:Ig did have Abs specific for the Ig component of OX-40R:Ig and human IgG. The mice treated with CTLA-4:Ig did not develop any Abs capable of binding to CTLA-4:Ig, OX-40R, human IgG (data not shown). It is

FIGURE 5. OX-40R:Ig can inhibit the proliferation of PLP-specific T cell lines in vitro. The same PLP T cell line was used in panels A and B. A, Normal thymocytes were used to present PLP_{139–151} (10 μg/ml) to T cells in the presence of OX-40:Ig, CTLA-4:Ig, or the combination of OX-40:Ig and CTLA-4:Ig. The ratio of thymocytes to PLP-specific T cells was 10:1. B, CD11b⁺ cells were isolated from the brains of mice exhibiting active EAE (days 1–4 of disease, 14 days after immunization) and used to present PLP_{139–151} (10 μg/ml) to T cells in the presence of OX-40:Ig, CTLA-4:Ig, or the combination of OX-40:Ig and CTLA-4:Ig. The ratio of CD11b⁺ cells to PLP-specific T cells was 0.3:1. CD11b⁺ cells were enriched by positive selection using FITC-labeled CD11b and anti-FITC magnetic beads (> 80% purity). Human Ig (Hu Ig) was used as a negative control for OX-40:Ig and CTLA-4:Ig (panel A). An Ab that interferes with MHC class II presentation of peptide to CD4⁺ T cells (OX-6) was added as an indication of maximal inhibition of T cell proliferation (panel B). In both experiments the PLP-specific T cells and APC were incubated with 10 μg of PLP peptide and the indicated blocking reagents for 72 h. Tritiated thymidine was added during the last 18 h of in vitro culture.



possible that the development of anti-OX-40R:Ig Abs caused the clearance of OX-40R:Ig and resulted in reduced treatment efficacy of R-EAE. This is in direct contrast to treatment with CTLA-4:Ig,

which did not lead to the generation of anti-CTLA-4:Ig Abs and was able to prevent disease relapse.

In vivo therapy of adoptively transferred EAE with soluble OX-40R:Ig

We next assessed whether treatment with OX-40R:Ig during the acute phase of adoptive (passive) EAE had a therapeutic effect. Fig. 7 and Table II (Expt. A) show that the OX-40R:Ig-treated mice had less severe disease than the human IgG-treated mice. In experiment B (Table II), the mice were injected with OX-40R:Ig for 5 consecutive days after disease onset. Forty percent of the control animals died due to severe EAE, while all of the OX-40R:Ig-treated group survived and had a lower maximum disease score.

Discussion

We have shown that blocking OX-40R engagement by the OX-40L during T cell activation in vitro and in vivo led to a decrease in T cell proliferation and a diminution in clinical signs of EAE. The OX-40R and its ligand were selectively expressed at the site of inflammation on CD4⁺ T cells and CD11b⁺ cells, respectively. The selective expression of the OX-40R and its ligand within the CNS suggested that OX-40R-mediated treatment was occurring by blocking T cell activation primarily at the site of inflammation. We and others have demonstrated the selective up-regulation of the OX-40R on CD4⁺ T cells isolated from the site of inflammation in

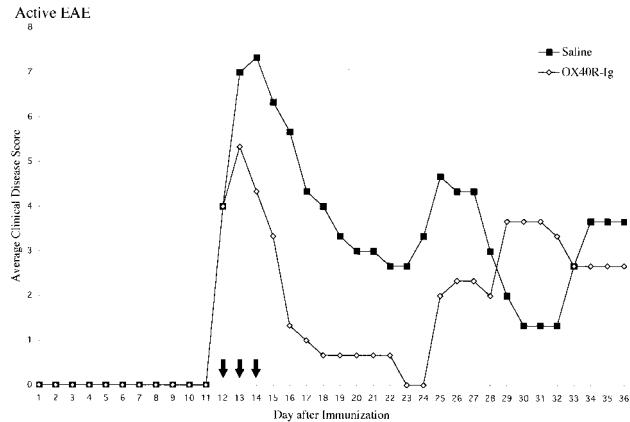


FIGURE 6. EAE was induced in female SJL mice by s.c. injection of 150 μg of PLP peptide emulsified in CFA. Treatment regimen was initiated after animals exhibited clinical signs of disease (day 1) and was administered daily for 3 days (400 μg/day). The arrows on the graph indicate the days the treatment was administered. Additional data on these mice are presented in Table I, experiment 1.

Table I. Disease severity of actively induced EAE is reduced by in vivo treatment with soluble OX-40R:Ig and CTLA-4:Ig

Expt.	Treatment ^a	Treatment Regimen	Max. Disease Severity Score	10-Day Cumulative Disease Score ^d	EAE Deaths
Expt. 1		Day of Disease ^b			
n = 3	OX-40R:Ig (400 µg/day)	1, 2, 3	5.3	22 ± 11	0/3
n = 3	Saline	1, 2, 3	7.3	48 ± 16	0/3
Expt. 2		Day After Immunization ^c			
n = 5	OX-40R:Ig (400 µg/day)	10, 11, 12, 13	6.6		0/5 ^g
n = 5	CTLA-4:Ig (400 µg/day)	10, 11, 12, 13	6.2		0/5 ^g
n = 5	OX-40R:Ig + CTLA-4:Ig (200 µg + 200 µg/day)	10, 11, 12, 13	5.8		0/5 ^g
n = 5	Saline	10, 11, 12, 13	8.8	NA ^e	4/5 ^f
Expt. 3		Day of Disease ^b			
n = 6	OX-40R:Ig (400 µg/day)	1, 2, 3, 4, 5	6.3		0/6
n = 6	Hu-IgG (400 µg/day)	1, 2, 3, 4, 5	8.5	NA ^e	3/6 ^f

^a OX-40R:Ig, CTLA-4:Ig, and Hu IgG were diluted in sterile saline at the appropriate concentration to deliver the indicated amount in 0.2 ml of saline (i.p. injection).
^b At the first sign of actively induced EAE, animals were paired according to clinical disease score and then split equally between the two treatment groups. Groups were then treated as indicated.
^c Animals were randomly assigned to individual treatment groups on day 10 after immunization before disease onset. Groups were then treated as indicated.
^d The 10-day cumulative disease score is determined by adding the daily clinical disease score of an individual animal for the first 10 days of disease.
^e Not applicable because not all animals survived to day 10 of disease.
^f Animals died of disease despite receiving gelatin squares and daily injections of saline alone or as a diluent of Hu IgG.
^g Significant relative to saline treatment group ($p = 0.03$), as determined by comparison of proportions.

rheumatoid arthritis, inflammatory bowel disease, GVHD, and cancer (31). Therefore, blocking OX-40R-mediated costimulation in vivo might be useful in treating a variety of CD4⁺ T cell-mediated human autoimmune disorders, without causing peripheral immune suppression.

Our data show that blocking OX-40R/OX-40L interaction during the acute phase of disease did not prevent animals from having clinical relapses. This suggests that the T cells that cause clinical relapses were not affected by the initial blockade of OX-40R/OX-40L-mediated T cell costimulation. There are two possible expla-

nations for the cause of clinical relapses in EAE: 1) The T cells that are initially activated in the CNS recirculate to the periphery, become reactivated, and cross the blood-brain barrier once again leading to a clinical relapse. 2) Naive T cells from the periphery are activated by myelin Ags after the acute phase of disease and migrate to the CNS leading to the relapse. If T cells were able to circulate out of the CNS, then treating with OX-40R:Ig did not induce anergy or cause T cell death at the site of inflammation. However, if peripheral naive T cells stimulated by myelin Ags caused the relapse, then the T cells responsible for the acute phase of EAE within the CNS could have been anergized by treatment with OX-40R:Ig. We are currently exploring whether T cell activation blocked by OX-40R:Ig can cause T cell anergy or activated induced cell death.

T cells isolated from inflammatory lesions in EAE have an activated memory phenotype (IL-2R⁺, CD45RB^{low}, CD44^{high}, Mel-14^{low}) (32). We hypothesize that T cells invading the CNS in active EAE have been Ag primed in the draining lymph nodes, and within 10–12 days after stimulation circulate out of the lymph node and penetrate the CNS. These cells have been characterized as short-term memory “effector” T cells, and upon encountering Ag within the CNS produce a burst of cytokines that in turn leads to clinical paralysis (21). In a TCR transgenic model (33), we are currently investigating the stage of CD4⁺ T cell development (naive or effector) during which the OX-40R costimulatory event is biologically relevant (Gramaglia et al. (34)). Using a MHC class II transfected fibroblast (DCEK) (35) that is capable of presenting Ag to the TCR transgenic T cells, we have cotransfected the OX-40L or B7.1 gene products individually or in combination. The OX-40L transfected DCEK line did not induce proliferation or IL-2 production in naive T cells, but provided potent costimulation to both Th1 and Th2 effector T cells. In contrast, the DCEK line

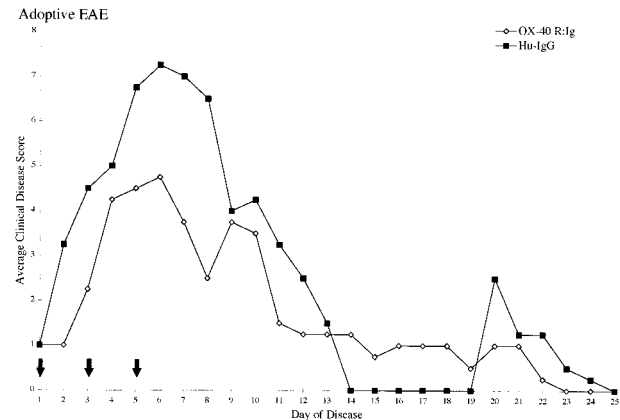


FIGURE 7. Adoptive EAE was induced in female SJL mice by i.p. injection of 5×10^6 syngeneic PLP_{139–151}-specific T cells. On the first day of clinical disease, animals received the treatment indicated by i.p. injection (400 µg/day). The arrows on the graph indicate the days the treatment was administered. Additional data on these mice are presented in Table II, experiment A.

Table II. Disease severity of adoptively transferred EAE is reduced by in vivo treatment with soluble OX-40R:Ig

Number of Cells Transferred ^a	Animal Number	Treatment ^b	Treatment Regimen ^c (day of disease)	Max. Disease Severity Score	10-day Cumulative Disease Score ^d	EAE Deaths
Expt. A						
5 × 10 ⁶	n = 4	OX-40R:Ig (150 μg/day)	1, 3, 5	5.0	31 ± 14.9 ^g	0/4
5 × 10 ⁶	n = 4	Hu IgG (150 μg/day)	1, 3, 5	7.5	49.5 ± 12.8	0/4
Expt. B						
5 × 10 ⁶	n = 5	OX-40R:Ig (150 μg/day)	1, 2, 3, 4, 5	5.4		0/5
5 × 10 ⁶	n = 5	Hu IgG (150 μg/day)	1, 2, 3, 4, 5	7.6	NA ^e	2/5 ^f

^a EAE was adoptively induced by the transfer of PLP_{139–151}-specific T cell lines, after activation with specific peptide (5 μg/ml) and syngeneic irradiated thymocytes.
^b OX-40R:Ig and Hu IgG were diluted in sterile saline at the appropriate concentration to deliver the indicated amount in 0.2 ml of saline (i.p. injection).
^c At the first sign of adoptively induced EAE, animals were paired according to clinical disease score and then split equally between the two treatment groups. Groups were then treated as indicated.
^d The 10-day cumulative disease score is determined by adding the daily clinical disease score of an individual animal for the first 10 days of disease.
^e Not applicable because two animals did not survive to day 10 of disease.
^f Animals died of disease despite receiving gelatin squares and daily injections of saline as a diluent of Hu IgG.
^g Significant relative to Hu IgG treatment group (*p* = 0.03), as determined by Student's *t* test.

transfected with OX-40L and B7.1 provided a potent signal for naive T cell activation and also induced potent costimulation for effector T cells. In the Lewis rat, we have also shown that the OX-40R provides costimulation for long-term MBP-specific T cell lines (9). The data from the TCR transgenics and the Lewis rat model suggest that the OX-40R appears to be biologically active on effector or memory effector T cells and can synergize with B7.1 to stimulate naive T cells. The data presented here suggest that the T cells invading the CNS in animals with EAE (memory effector T cells) are sensitive to OX-40R costimulation and therefore responded to the OX-40R:Ig treatment. An alternative interpretation of the treatment results is that cross-linking the OX-40L on CNS APC induces inhibitory cytokines that can down-regulate T cell function.

The OX-40L was originally described as a protein that was up-regulated by the transcriptional transactivating human T cell lymphotropic virus, type I (HTLV-I) gene product tax (13). Expression of the OX-40L seems to be quite restricted to APC found within inflammatory sites or APC that have been activated in vitro (14, 15). Therefore, it appears that OX-40R-mediated costimulation in vivo may be limited by expression of the OX-40L. We have found that the OX-40L is coexpressed with CD80 and/or CD86 on the CD11b⁺ cells isolated from the CNS of animals with EAE (Fig. 3). This would suggest that if these CD11b⁺ cells were presenting Ag to OX-40⁺ T cells in the CNS, the T cells would receive a combination of costimulatory signals through the OX-40R and CD28. Our in vitro studies with the DCEK transfectants showed that the combination of B7 and OX-40L provided a stronger costimulus than either signal by itself. Blocking both signals showed the greatest inhibition of T cell proliferation in vitro (Fig. 5), but we saw no enhanced amelioration of EAE when both CTLA-4:Ig and OX-40R:Ig were administered together vs OX-40R:Ig alone. Whether the combination of both costimulatory signals are additive in vivo, or the sequential engagement of CD28 and OX-40R leads to cytokine production and T cell proliferation, is unknown. If sequential engagement of both costimulatory receptors is necessary in vivo, then blocking one of the receptors

would lead to decreased inflammation, thereby making it unnecessary to block both receptors.

Others have shown that in vivo injections of an anti-OX-40R Ab in animals with chronic GVHD exacerbates the clinical signs of disease (36). In chronic GVHD, the administration of anti-OX-40R Abs converted the disease to acute GVHD (36). We have shown that OX-40R⁺ T cells from animals with acute GVHD were the cells that recognize alloantigen in vivo. We have also observed a similar pattern of OX-40R expression in bone marrow transplant patients who have GVHD (T. Tittle, unpublished observations). Currently, we have identified a CD11b⁺ cell population in the peripheral blood and lymph nodes of animals with GVHD that expresses the OX-40L (T. Tittle, unpublished observations). Future experiments will be aimed at tempering clinical signs of GVHD by targeting OX-40L⁺ cells by in vivo administration of OX-40R:Ig.

Our data suggest that the CD11b⁺/MHC class II⁺ cells isolated from the CNS of animals with EAE are presenting Ag to myelin-specific CD4⁺ T cells. Alternatively, myelin-specific T cells could be stimulated by the endothelial cells found at the blood-brain barrier. Others have shown that endothelial cells at the site of blood-brain barrier disruption have an activated phenotype and up-regulate MHC class II (37). Activated endothelial cells have recently been shown to express the OX-40L (15). Activated endothelial cells at the blood-brain barrier may be presenting Ag to T cells that are in the process of penetrating the CNS. If this is the case, then costimulation could occur through an OX-40R/OX-40L-mediated step. Inhibition of inflammation in our EAE model may occur by blocking a T cell endothelial cell interaction with the OX-40R:Ig.

The colocalization of OX-40L-bearing macrophages/microglia and OX-40R⁺ T cells within the inflamed CNS tissue of animals with EAE suggests an important role for OX-40R/OX-40L interaction in the immunopathology of EAE. Blockade of OX-40R/OX-40L interaction both prevented T cell activation in vitro and most likely led to the in vivo amelioration of clinical signs in EAE. A significant advantage of using OX-40R and OX-40L as targets for intervention is their transient expression restricted to activated

T cells and activated APC. This feature allows targeting of T cells and macrophages at the inflammatory site without affecting the peripheral T cell repertoire, thereby avoiding global immune suppression.

Our success in treating EAE suggests that targeting OX-40R/OX-40L interactions may be useful in treating multiple sclerosis and other autoimmune diseases. We have previously demonstrated that T cells in the joints of patients with rheumatoid arthritis selectively express OX-40R (21). As in EAE, targeting activated T cells and APCs within the sites of inflammation in human autoimmune diseases may be a promising approach to immunoselective regulation of the disease process, using reagents that recognize the OX-40L or the OX-40R.

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