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Suppression of Established Experimental Autoimmune Uveitis by Anti-LFA-1 α Ab

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Abstract

PURPOSE—To identify costimulatory molecules that are important in the effector phase of experimental autoimmune uveitis (EAU).

METHODS—EAU was induced in C57BL/6 (B6) mice by transfer of activated T cells specific for the interphotoreceptor-binding protein (IRBP) 1–20 peptide. The animals were then treated with and without anti-leukocyte function associated antigen (LFA)-1 α mAb, at day 0 or 10 (disease onset) after T-cell transfer. Clinical signs of inflammation, ocular histology, and infiltrated inflammatory cells in the eye were compared. The primary and secondary proliferative responses of uveitogenic CD4 and CD8 T cells were tested after treatment with costimulatory blockers in vivo and in vitro. Moreover, the abilities of uveitogenic T cell trafficking and their interaction with retinal astrocytes were examined.

RESULTS—Anti-LFA-1 α Abs caused significant suppression of disease when administered either at the time of effector uveitogenic T cell transfer or at disease onset. Studies of the mechanisms by which anti-LFA-1 α Ab inhibits the effector phase of uveitis demonstrated that it blocks multiple pathogenic events of uveitis mediated by IRBP-specific uveitogenic T cells, including the activation of T cells outside and inside the eye and the trafficking of activated autoreactive T cells into the inflammatory site. In addition, Ab treatment selectively suppressed the activation and expansion of pathogenic, but not regulatory, T cells in vivo.

CONCLUSIONS—Anti-LFA-1 α Abs are potent inhibitors of established autoimmune uveitis and that such treatment may be applicable not only for the prevention, but also the treatment, of T-cell-mediated autoimmune diseases.

Experimental autoimmune uveitis (EAU) is a well-characterized model that is useful in the study of human idiopathic uveitis.^{1–3} Although both genetic and environmental factors⁴ are important in the pathogenesis of uveitis, studies in rodents have demonstrated that the transfer of nonactivated or partially activated autoreactive T cells does not cause disease,^{3,5} suggesting that only activated autoreactive T cells are pathogenic. The inhibition of autoreactive T-cell activation therefore becomes a primary therapeutic goal. Given that T-cell activation requires two signals, one from ligation of T-cell receptors (TCRs) by complexed antigen-MHC molecules and the other from costimulatory molecules,⁶ it is generally believed that blockade of costimulation may effectively prevent T-cell activation, especially when multiple and/or undefined autoantigens are involved in a single disease. Nevertheless, studies by other laboratories and our own have shown that, although many costimulatory molecule blockers effectively prevent disease when administered before disease onset, they are less effective after

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disease onset.^{7–14} Furthermore, although many treatments have a therapeutic effect on disease induced by active immunization, they are less effective in treating disease induced by adoptive transfer in which the autoreactive T cells are subjected to in vitro activation and the activated T cells may be more resistant to costimulatory blockade.¹⁵ Since the major goal of clinical treatment is to impede disease progression, it is important to identify costimulatory signals, the blocking of which is effective in the treatment of already initiated diseases.

Leukocyte function associated antigen (LFA)-1 was one of the first cell-surface heterodimeric integrins to be discovered.^{16–18} It interacts primarily with intracellular adhesion molecule (ICAM)-1, but also with ICAM-2 and -3, and junctional adhesion molecule (JAM)-1.¹⁹ As an one of the major molecules involved in the immunologic synapse, LFA-1 rapidly clusters after T-cell ligation, optimizing T cell–antigen-presenting cell (APC) contact and increasing the number of ligated TCRs.^{20–23} Various studies have shown that LFA-1 has multiple roles in immune responses, such as cell adhesion,²⁴ T-cell activation,²⁵ and the trafficking of leukocyte populations.^{26–28} LFA-1 therefore appears to be an attractive target for therapies aimed at attenuating clinical disease mediated by activated T cells. Antibodies interfering with the LFA-1/ICAM-1 interaction have been extensively evaluated in numerous preclinical studies on transplantation and autoimmune diseases,^{29–37} including animal models of uveitis.^{38–40}

In the present study, we tested the effect of a panel of costimulatory molecule-specific Abs or the fusion protein, CTLA4-FC. Our results showed that, although many of these could block the function of activated autoreactive T cells isolated from antigen-immunized recipient mice (primary T-cell response), only anti-LFA-1 α Ab inhibited the proliferative response of autoimmune T cells isolated from animals with adoptively induced EAU and of (interphotoreceptor-binding protein) IRBP-specific T-cell lines (secondary T-cell response). Of importance, in vivo injection of anti-LFA 1 α Ab greatly reduced the incidence and severity of disease induced by adoptive transfer of in vitro activated T cells reactive with the peptide IRBP1-20 and suppressed disease development, even when administered after disease onset. The inhibitory effect of anti-LFA-1 α Ab during the effector phase of disease was shown to be due to its blocking effector T-cell activation in peripheral lymphoid organs and inside the eye and preventing the trafficking of pathogenic T cells into the target organ.

MATERIALS AND METHODS

Animals and Reagents

Pathogen-free female C57BL/6 mice (8–10 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and were housed and maintained in the animal facilities of the University of Louisville. All animal studies conformed to the ARVO statement on the Use of Animals in Ophthalmic and Vision Research. Institutional approval was obtained, and all procedures adhered to institutional guidelines regarding animal experimentation.

All antibodies against costimulatory molecules were purchased from eBioscience (San Diego, CA). CTLA4-Fc was kindly provided as a gift by Philip Morgan (Pfizer, St. Louis, MO). Ascites containing anti-LFA-1 α monoclonal antibody (mAb; FD441, anti-LFA-1 α , CD11a) from Rac1-deficient mice was produced by Taconic Farms (Germantown, NY).

Preparation of IRBP1-20-Specific T Cells

Briefly, to prepare T cells, donor mice were immunized with subcutaneous injection of 200 μ L of an emulsion containing 200 μ g of IRBP1-20 (amino acids 1-20 of IRBP; Sigma-Aldrich, St. Louis, MO) and 500 μ g of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI) in incomplete Freund's adjuvant (Sigma-Aldrich), distributed over six spots at the tail base and

on the flank. T cells were isolated at 13 days after immunization, from lymph nodes and spleen cells by passage through a nylon wool column, and then 1×10^7 cells in 1 mL of complete medium (CM; RPMI 1640 medium containing 10% fetal bovine serum, 2 mM growth medium [Glutamax II; Invitrogen-Gibco, Grand Island, NY], 100 IU/mL penicillin, and 100 μ g/mL streptomycin; Sigma-Aldrich) in a six-well plate (Corning Costar, Corning, NY) were stimulated with 10 μ g/mL of IRBP1-20 in the presence of 1×10^7 irradiated syngeneic spleen cells as APCs. After 2 days, the activated lymphoblasts were isolated by gradient centrifugation (Lymphoprep; Robbins Scientific, Mountain View, CA) and cultured in CM supplemented with 15% IL-2-containing medium (supernatant from Con A-stimulated rat spleen cells).⁵

Adoptive Transfer of EAU

Uveitis was induced in naïve B6 mice by adoptive transfer of 5×10^6 IRBP1-20-specific T cells, as described previously.^{5,12,41} starting at day 8 after transfer, the animals were examined every 3 days by funduscopy for clinical signs of uveitis. Funduscopy evaluation for longitudinal follow-up of disease was performed using a binocular microscope after pupil dilation using 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions. The incidence and severity of EAU were graded on a scale of 0 to 4 in half-point increments using previously described criteria,⁵ based on the type, number, and size of lesions present.

Pathologic Examination

Inflammation of the eye was confirmed by histopathology. Whole eyes were collected, immersed for 1 hour in 4% phosphate-buffered glutaraldehyde, and transferred to 10% phosphate-buffered formaldehyde until processed. The fixed and dehydrated tissue was embedded in methacrylate, and 5- μ m sections were cut through the pupillary-optic nerve plane and stained with hematoxylin and eosin. Presence or absence of disease was evaluated by blind examination of six sections cut at different planes in each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, as described previously.⁵

Purification of CD4 and CD8 T Cells

Purified CD4 and CD8 T cells were prepared from the draining lymph nodes and spleen of IRBP-immunized B6 mice using a CD4 or CD8 isolation kit (Miltenyi Biotec Inc., Auburn, CA).⁴² T cells from the lymph node and spleen cells, purified by passage over nylon wool, were incubated for 10 minutes at 4°C with a cocktail of biotin-conjugated Abs against CD8 (CD8a, Ly-2) or CD4 (CD4, L3T4) T cells, B cells (CD45R, B220), NK cells (CD49b, DX5), hematopoietic cells (CD11b, Mac-1), and erythroid cells (Ter119), then for 15 minutes at 4°C with anti-biotin microbeads. The cells were separated into bound and non-bound on a magnetic separator column (auto-MACS; Miltenyi Biotec, Inc.), which was washed with 15 mL of medium according to the manufacturer's protocol, and the flow-through fraction containing CD4- or CD8-enriched cells were collected. The purity of the isolated cell fraction was determined by flow cytometric analysis using fluorescein isothiocyanate (FITC)-conjugated anti-TCR antibodies and phycoerythrin (PE)-conjugated anti-CD8 or CD4 antibodies (BD Bioscience, San Jose, CA).

CFSE Staining

The cells were washed and resuspended at a density of 50×10^6 cells/mL in serum-free RPMI 1640 medium; incubated at 37°C for 10 minutes, with gentle shaking, with a final concentration of 10 μ M CFSE (carboxy-fluorescein diacetate, succinimidyl ester; Invitrogen-Molecular Probes, Eugene, OR); washed twice with CM; and resuspended in CM.

Immunofluorescence Flow Cytometry

Aliquots (2×10^5 cells) were double-stained with combinations of FITC- or PE-conjugated mAbs against mouse TCR, CD4 or CD8, CD44, or CD62L. Data collection and analysis were performed on a flow cytometer (FACSCalibur using CellQuest software; BD Biosciences).

Proliferation and Suppressor Cell Assays

T cells (4×10^5 cells/well), prepared from IRBP1-20-immunized B6 mice or from mice after IRBP1-20-specific T-cell transfer treated or untreated with costimulatory molecule blockers, were cultured at 37°C for 48 hours in a total volume of 200 μ L with or without IRBP1-20 in the presence of irradiated syngeneic spleen APCs (1×10^5).

For in vitro suppression assay, a total of 3×10^5 CD4⁺CD25⁻ T cells from IRBP1-20-immunized mice were cultured in triplicate with (1×10^5) APCs, IRBP1-20 peptide and the indicated number of CD4⁺CD25⁻ cells from IRBP1-20-specific, T-cell-transferred mice, with or without the treatment of anti-LFA-1 α Ab. Proliferation was measured by [³H]thymidine incorporation (1 μ Ci for the last 8 hours of culture), with a microplate scintillation counter (PerkinElmer, Meriden, CT).

Enzyme-Linked Immunosorbent Assay

IL-2, IL-3, IL-10, IFN- γ , and TNF- α were measured using commercially available ELISA plates (R&D Systems, Minneapolis, MN).

Real-Time Quantitative RT-PCR Assay

Total RNA from the eyes of B6 mice was extracted using an RNA isolation kit (Invitrogen, Carlsbad, CA), treated with DNase I (GE Healthcare, Piscataway, NJ), and reverse transcribed into cDNA using an MMLV-RT kit (Invitrogen). Each cDNA sample was amplified for the gene of interest and β -actin (*TaqMan* assays; Mx3000P system; Stratagene, La Jolla, CA). The concentration of the gene of interest was determined using the comparative threshold cycle number and normalized to that of the internal β -actin control. The primers and probes used were β -actin, forward primer, 5'-ATCTACGAGGGCTAT-GCTCTCC-3', reverse primer, 5'-ACGCTCGGTCAGGATCTTCAT-3', probe, 5'-CCTGCGTCTGGACCTG-GCTGGC-3'; IL-17, forward primer, 5'-TGAGTCCAGGGAGAGCTTCATC-3', reverse primer, 5'-GGACACGCT-GAGCTTTGAGG-3', probe, 5'-CGCTGCTGCCCTTCACTGTAGC-CGC-3'; IFN- γ , forward primer, 5'-ATGAGTATTGCCAAGTTTGAGGTC-3', reverse primer, 5'-TCTCTTCCCCACCCGAATC-3', probe, 5'-CCACAG-GTCCAGCGCCAAG-CATTC-3', TNF- α , forward primer, 5'-AAATGGC-CTCCCTCTCATCAG-3', reverse primer, 5'-GCTTGCTCACTCGAATTTTG-AGAAG-3', probe, 5'-ATGGCCCAGACCCCTCAC-ACTCAGA-3'; IL-6, forward primer, 5'-CCTTCTTGGGACTGATGCTG-3', reverse primer, 5'-TCT-GTTGGGAGTGGTATCCTC-3', and probe, 5'-ACCACGGCCTTCCCT-ACTTCACAA-3'.

Isolation of Cells from Inflamed Eyes

Cells were isolated as described previously.^{41,43} Eyes were collected at day 23 after transfer after PBS perfusion, and a cell suspension was prepared by digestion for 10 minutes at 37°C with collagenase (1 mg/mL) and DNase (100 μ g/mL) in RPMI 1640 containing 10% FCS. The cells were washed and resuspended in staining buffer (PBS containing 3% FCS and 0.1% sodium azide). To identify inflammatory cells, rat anti-mouse mAbs against TCR (T cells); CD161 (NK cells); CD4, CD8, and Mac-1 (macrophages); or Gr-1 (neutrophils) were used for staining, and the cells were analyzed by flow cytometry.

Primary Mice Retinal Astrocyte Isolation and Culture

Eyes from B6 mice (4–6 weeks old) were collected, the connective tissue removed, and the eyes immersed in Ca^{2+} -, Mg^{2+} -free PBS containing 100 IU/mL of penicillin and 100 of $\mu\text{g}/\text{mL}$ streptomycin on ice for 3 hours. The anterior half of the eye and the vitreous were removed and discarded, and the retina was digested for 15 minutes at 37°C with 0.25% trypsin/1 mM EDTA. Digestion was terminated by addition of CM, and the cells centrifuged at 400g for 5 minutes and dissociated by gentle trituration through a fire-polished Pasteur pipette. After three washes, the cells were resuspended in CM and seeded on poly-D-lysine coated six-well plates at a density of 1.5×10^7 cells/well. After 14 days' incubation, the purity of the astrocytes was more than 95%, as assessed by staining with Ab against glial fibrillary acidic protein.

Coculture of Astrocytes and T Cells

The retinal astrocyte (RAC) monolayer was incubated with IRBP1-20-specific T cells (3×10^5) in the presence of IRBP1-20, with or without the indicated blockers for 48 hours, then the culture supernatants were collected for cytokine assay by ELISA (R&D Systems). To exclude the possibility that the cytokines were produced by the astrocytes, rather than the T cells, the astrocytes were treated with mitomycin C (100 $\mu\text{g}/\text{mL}$) for 1 hour at 37°C before being mixed with the responder T cells.

RESULTS

Effect of Anti-LFA-1 α Ab on Responses of IRBP1-20-Specific T Cells

We have shown that several costimulatory molecule blockers can prevent acute autoimmune disease induced by active immunization if applied in the inducing phase of disease, but they are less effective in ameliorating ongoing disease,^{12–14} suggesting that most costimulatory molecules are essential for the activation of naïve Ag-specific T cells (primary T-cell response), but are not necessary for the reactivation of effector T cells (secondary T-cell response). Because inhibition of effector autoreactive T cells is of clinical significance, we wished to identify costimulatory molecule blockers that are effective in both the primary and secondary T-cell responses. As shown in Figure 1, whereas the primary responses of *in vivo* primed T cells were blocked by a number of anti-costimulatory Ab/fusion proteins (Fig. 1A), such as B7.1, CTLA4-Fc, CD40, and LFA-1 α , the secondary response of IRBP1-20-specific T cells isolated from mice with tEAU induced by adoptive transfer was only effectively blocked by anti-LFA-1 α Ab (Fig. 1B). Established IRBP-specific T-cell lines were also resistant to all these blockers, except anti-LFA-1 α Ab (data not shown).

Effect of Anti-LFA-1 α Ab on the Function of CD4 and CD8 IRBP-Specific T Cells

We have reported that both CD4 and CD8 autoreactive T cells are involved in the pathogenesis of uveitis.^{42,44} To determine whether anti-LFA-1 α Ab has an inhibitory effect on both CD4 and CD8 IRBP-specific T cells, CD4 and CD8 responder T cells from the draining lymph nodes and spleen of IRBP1-20-immunized mice at day 13 after immunization were prepared by cell sorting (MACS; Miltenyi) and exposed to irradiated APCs and immunizing peptide in the absence or presence of anti-LFA-1 α (10 $\mu\text{g}/\text{mL}$). Figure 2A shows that the antigen-induced proliferative responses of both CD4 and CD8 IRBP-specific T cells were inhibited by anti-LFA-1 α Ab. As control experiments, anti-B7.1 and anti-CD40 mAbs inhibited the CD4 T-cell response, but not that of the CD8 T cells, and anti-ICOSL and anti-OX40L mAbs inhibited the CD8 T cell, but not the CD4 T-cell, response (data not shown,¹⁵). To determine whether anti-LFA-1 α Ab affected cytokine production by CD4 and CD8 IRBP-specific T cells, supernatants from the CD4 and CD8 responder T cells were collected at 48 hours after antigen stimulation. As shown in Figure 2B, anti-LFA-1 α Ab caused significant inhibition of the production of pro- and anti-inflammatory cytokines by CD4 responder T cells, whereas, with CD8 T cells,

production of the proinflammatory cytokines (IL-2, IFN- γ , and IL-3), but not that of the anti-inflammatory cytokine (IL-10), was significantly inhibited.

Effect of Anti-LFA-1 α Ab on the Severity of Uveitis Induced by Adoptive Transfer of IRBP1-20-Specific T Cells

In the T-cell-induced uveitis model (tEAU), the pathogenic T cells used to induce EAU have to be activated by in vitro stimulation before transfer to recipient mice. This allows us to test the importance of pathogenic factors that control the migration of antigen-specific T cells into the eye and the reactivation of infiltrated antigen-specific T cells in the target organ, rather than the activation of autoreactive T cells in the periphery. To determine whether anti-LFA-1 α Ab treatment could protect against tEAU, naïve mice were injected on day 0 with in vitro stimulated IRBP1-20 T cells derived from in vivo primed syngeneic B6 mice and were then randomly divided into two groups: one receiving anti-LFA-1 α -Ab and the other an isotype-matched Ig as the control. The antibodies were administered at a dose of 150 μ g per mouse on days 0, 5, and 10 after T-cell transfer. Compared with the mice treated with control Ig, the anti-LFA-1 α -treated mice ($n = 12$) developed much milder ocular inflammation as determined by funduscopy (Fig. 3A). Most important, when treatment with anti-LFA-1 α Ab was started on day 10 (disease onset), it was equally effective (Fig. 3B).

Histologic examination of the eyes at day 23 after transfer in animals treated three times starting on day 0 showed that recipient mice treated with control Ab exhibited damage to the photoreceptor layer and infiltration in the vitreous and retina (Fig. 3C), whereas those treated with anti-LFA-1 α Ab had a well-preserved retinal structure, with no, or only minimal, vitreous infiltrate (Fig. 3D).

Effect of Anti-LFA-1 α Ab on IRBP1-20-Specific T-Cell Migration into the Eye and on Inflammatory Cell Infiltration

One of the major pathogenic events in uveitis is the trafficking of activated autoreactive T cells into the autoimmune organ. To determine whether LFA-1 α mediates T-cell trafficking into the target organ, we examined IRBP1-20-specific T-cell trafficking into the eye after anti-LFA-1 α Ab treatment. In these experiments, the recipient mice were randomly divided into three groups that were treated with a single injection of anti-LFA-1 α Ab, CTLA-4Fc, or isotype-matched control Ab on the same day (day 0) as the adoptive transfer of a pathogenic dose of IRBP1-20-specific T cells prelabeled with CFSE. On day 3 after T-cell transfer, we harvested recipient eyes, after perfusion, and assessed entry of the injected CFSE-labeled T cells into the eye by flow cytometry. As shown in Figure 4A, despite the low percentages, a well-defined CFSE-labeled donor T-cell population was found in the eyes of control Ig-treated or CTLA4-Fc-treated recipients, but was markedly decreased in the eyes of anti-LFA-1 α Ab-treated mice.

In another study at day 23 after transfer in mice treated with three injections of anti-LFA-1 α Ab or control Ig starting on day 0, the anti-LFA-1 α Ab-treated mice showed a profound reduction in the percentage of infiltrated T cells (both CD4 and CD8 cells), NK cells (CD161+), and neutrophils (Gr-1+) in the eye, whereas NK T cells (TCR+CD161+) and macrophages (Mac-1) were minimally affected by antibody treatment (Fig. 4B), consistent with the clinical EAU scores shown in Figure 3. In contrast, mice treated with control Ig showed extensive inflammation (Fig. 4B). Moreover, the cytokine-producing ability of the eye-infiltrating T cells isolated from these anti-LFA-1 α -treated mice (IFN- γ , IL-17, and IL-6) was significantly decreased, as measured by real-time PCR (Fig. 4C), whereas the production of TNF- α , expressed by macrophages, was not affected.

Responsiveness of IRBP1-20-Specific T Cells from Anti-LFA-1 α Ab-Treated Mice to IRBP1-20

To determine whether *in vivo* anti-LFA-1 α Ab treatment inhibited the proliferation and expansion of effector IRBP1-20-specific T cells, as *in vitro*, we isolated T cells at day 23 after transfer from the spleen of mice with tEAU treated with three injections of control Ig or anti-LFA-1 α Ab starting on day 0 and stimulated them with IRBP1-20 in the presence of APCs. Significant depression of the proliferative response was seen using T cells from the mice treated with anti-LFA-1 α Ab (Fig. 5A). Consistent with this, T cells expressing the activation marker CD44^{hi}CD62L^{low} were decreased by approximately 50% in the anti-LFA-1 α Ab-treated mice compared to the control Ig-treated mice (Fig. 5B). The calculation is based on the following formula: (% of CD44⁺CD62⁻ T cells in untreated mice – % of CD44⁺CD62⁻ T cells in treated mice)/% of CD44⁺CD62⁻ T cells in untreated mice.

Effect of Anti-LFA-1 α Ab on the Interaction between RAC and Autoreactive T Cells

Reactivation of autoreactive T cells in the autoimmune organ is believed to be a critical event in the pathogenesis of T-cell-mediated autoimmune disease. The expression of ICAM-1/LFA-1 molecules was reported to be increased in the eye of several models of uveitis.^{40,45,46} To determine whether LFA-1 α is involved in the reactivation of uveitogenic T cells by the parenchymal cells of the eye, we isolated RAC and measured the cytokine production by IRBP1-20-specific T cells *in vitro* in the presence of Ag presented by cultured RACs. The assay was performed in 96-well plates, in which RAC monolayers were incubated with IRBP1-20 (5 μ g/mL), with or without various costimulatory molecule blockers, including anti-LFA-1 α Ab, and then IRBP1-20-specific T cells were seeded at a T cell-to-RAC ratio of 4:1. As shown in Figure 6, IRBP1-20-specific T cells stimulated by RACs produced large amounts of TNF- α in the presence of IRBP1-20. However, the antigen-presenting capacity of the astrocytes was significantly blocked by anti-LFA-1 α Ab and partially blocked by CTLA4-Fc, but not by antibodies against CD80, CD86, ICOSL, or 4-1BBL.

The Number and Function of CD4⁺CD25⁺ T_{reg} Cells in Anti-LFA-1 α Ab-Treated Mice

To determine whether LFA-1 α has a similar inhibitory effect on CD4⁺CD25⁺ regulatory T cells, we adoptively transferred mice with IRBP1-20-specific T cells and treated them three times starting on day 0 with control Ig or anti-LFA-1 α , then the numbers of CD4⁺CD25⁺ and CD25⁺FoxP3⁺ T cells in the recipient mice were assessed at day 23 after transfer. As shown in Figure 7A, the anti-LFA-1 α Ab-treated mice had a higher percentage of regulatory T cells (CD4⁺CD25⁺) in the spleen than control mice. In addition, functional tests showed that the CD4⁺CD25⁺ T cells isolated from tEAU mice treated with anti-LFA-1 α Ab had increased, rather than decreased, suppressor activity on the proliferative response of CD4⁺CD25⁻ responder T cells from EAU mice (Fig. 7B).

DISCUSSION

Animal experiments have demonstrated that, in many autoimmune diseases, only newly activated autoreactive T cells can adoptively transfer disease,^{5,47} suggesting that the activation status of the autoreactive T cells is more important than the number of autoreactive T cells in the pathogenesis of disease. Many laboratories, including our own, have therefore attempted to block the activation of autoreactive T cells by acting on costimulation, because full T-cell activation requires both an Ag-specific signal through the TCR and an Ag-nonspecific costimulatory signal through costimulatory molecules. The results of these studies have demonstrated that, in disease induced by active immunization, several costimulatory molecule blockers are effective in preventing disease development if given before disease onset, but most do not ameliorate ongoing disease.^{12–14} Moreover, they have little effect on adoptively transferred disease, in which the disease is induced by transfer of *in vitro* activated autoreactive T cells.^{12–14} Given that treatment of an established autoimmune disease is more relevant to

human disease, the discovery of treatments effective on established disease is of interest. In the present study, we showed that anti-LFA-1 α Ab effectively suppressed tEAU, either when administered at the time of effector T cell transfer or later, after disease onset (Fig. 3), indicating that LFA-1 α is an important costimulatory molecule for effector T cell response and functions.

The major pathogenic events of T cell-mediated autoimmune diseases, such as EAU, are initiated by activation of autoreactive T cells in the periphery.^{4,48} The activated T cells gain an increased capability to enter the target organ.⁴⁹ After entry, autoreactive T cells are reactivated by interacting with MHC II antigen-expressing parenchymal cells,⁴⁵ which produce proinflammatory cytokines and chemokines, which cause further infiltration of inflammatory cells, resulting in tissue damage. To explore why anti-LFA-1 α Ab was more effective than other blockers in suppressing adoptively transferred disease and established EAU, we reasoned that it may inhibit the trafficking of autoreactive T cells into the eye and interfere with the reactivation of infiltrated T cells in the eye, as well as inhibiting the activation of autoreactive T cells in the periphery.

In this article and previous studies,¹⁵ we have compared the mechanisms of the inhibitory effects of anti-LFA-1 α Ab on established uveitis with those of costimulatory blockers that only prevent disease induction—for example, CTLA-4-Fc, a recombinant fusion protein that blocks the interaction of CD28 on T cells with its B7 ligands on APCs. In the present study, using an adoptive-transfer uveitis model, in which uveitis is induced by transfer of in vitro activated IRBP1-20-specific T cells into naïve mice, we compared the ability of anti-LFA-1 α Ab and CTLA4-Fc to block the migration of activated autoreactive T cells into the eye. Our results (Fig. 4) showed that the early trafficking of IRBP1-20-specific T cells into the eye was blocked by anti-LFA-1 α Ab, but not by CTLA4-Fc.

We also compared the ability of costimulatory molecule blockers to interfere with the interaction between autoreactive T cells and the parenchymal cells of the eye. Our results showed that, although anti-LFA-1 α antibody blocked the activation of IRBP1-20-specific T cells by peripheral (splenic) APCs or eye-derived astrocytes, CTLA4-Fc preferentially inhibited the activation of T cells by peripheral APCs (Figs. 1, 6). These observations indicate that, in addition to being a costimulatory molecule blocker, anti-LFA-1 α Ab blocks the entry of autoreactive T cells into the eye and interferes with the interaction between T cells and local parenchymal cells, thereby interfering with multiple pathways that are essential for autoreactive T cells to cause disease.

We have reported that both CD4 and CD8 autoreactive T cells are involved in the pathogenesis of EAU.^{5,44} Moreover, CD8 autoreactive T cells differ from their CD4 counterparts in that they must be activated⁵⁰ and must undergo costimulation to be activated.¹⁵ Among the costimulatory molecules tested, B7.1, B7.2, and CD40 are more important in CD4 IRBP1-20-specific T-cell responses, whereas ICOSL, OX40L, and 4-1BBL are dominant costimulatory molecules for CD8 IRBP1-20-specific T-cell activation.¹⁵ Our present results showed that LFA-1 α is required for the activation of both CD4 and CD8 T cells. It is likely that this ability to block both major sets of pathogenic T cells explains why anti-LFA-1 α Ab is more effective than the other agents in the treatment of ongoing disease.

It is interesting to note that anti-LFA-1 α Ab treatment inhibited effector but not regulatory T cell activation in vitro. The percentage of CD25⁺CD4⁺ cells in anti-LFA-1 α -treated mice was even higher than that in untreated mice (Fig. 7A), and purified CD25⁺CD4⁺ T cells from anti-LFA-1 α -treated mice completely blocked the proliferative response of IRBP1-20-specific effector T cells (Fig. 7B). Anti-LFA-1 α Ab may induce apoptosis of effector T cells. However, we did not observe an apoptotic effect on these cells in our in vitro studies (e.g., Figs. 1, 2). In addition, we did not observe apoptosis of effector T cells in vivo with TUNEL (data not shown).

The negative result of the TUNEL studies implies that there was either no apoptotic effect from the Ab or that dead cells were removed by phagocytosis, which is difficult to prove. It is likely that pathogenic and regulatory T cells require different costimulatory patterns for their activation. In addition, the dose and timing of anti-LFA-1 α Ab treatment used in this study may preferentially interfere with the ICAM/LFA interaction on pathogenic T cells, rather than that on regulatory T cells. The net effect of treatment results in the inhibition of pathogenic T cells and an increase in regulatory T cells. Indeed, our previous studies have shown that CD4 and CD8 autoreactive T cells rely on a different pattern of costimulatory molecules for their activation.¹⁵

Taken together, our results show that LFA-1 α is a crucial costimulatory molecule for effector T-cell activation. This molecule is also essential for the “rolling” and “homing” of the activated autoreactive T cells to the disease organ and for their interaction with the “target cells” in the parenchyma of the diseased organ.^{19,51,52} Compared with other costimulatory molecules that are necessary for naïve T-cell priming, LFA-1 α is also necessary for the multiple events involved in the pathogenesis of the effector phase of uveitis, such as effector T-cell reactivation in the periphery and inside the eye and migration to the eye. The regulatory tolerance induced in vivo by anti-LFA-1 α Ab reported herein and recently by others,^{19,53,54} together with the availability of a new generation of humanized anti-LFA-1 α mAbs efalizumab, which can be used clinically^{19,55} open a new phase for a potential immunotherapy, which may find its greatest application as an agent that augments other therapies.

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References

1. Mochizuki M, Kuwabara T, McAllister C, Nussenblatt RB, Gery I. Adoptive transfer of experimental autoimmune uveoretinitis in rats: immunopathogenic mechanisms and histologic features. *Invest Ophthalmol Vis Sci* 1985;26:1–9. [PubMed: 3967952]
2. Gregerson DS, Obritsch WF, Fling SP, Cameron JD. S-antigen-specific rat T cell lines recognize peptide fragments of S-antigen and mediate experimental autoimmune uveoretinitis and pinealitis. *J Immunol* 1986;136:2875–2882. [PubMed: 2420873]
3. Shao H, Shi H, Kaplan HJ, Sun D. Chronic recurrent autoimmune uveitis with progressive photoreceptor damage induced in rats by transfer of IRBP-specific T cells. *J Neuroimmunol* 2005;163:102–109. [PubMed: 15885312]
4. Caspi RR. Immunogenetic aspects of clinical and experimental uveitis. *Reg Immunol* 1992;4:321–330. [PubMed: 1290750]
5. Shao H, Liao T, Ke Y, Shi H, Kaplan HJ, Sun D. Severe chronic experimental autoimmune uveitis (EAU) of the C57BL/6 mouse induced by adoptive transfer of IRBP1–20-specific T cells. *Exp Eye Res* 2006;82:323–331. [PubMed: 16125173]
6. Dubey C, Croft M, Swain SL. Costimulatory requirements of naive CD4⁺ T cells. ICAM-1 or B7-1 can costimulate naive CD4 T cell activation but both are required for optimum response. *J Immunol* 1995;155:45–57. [PubMed: 7541426]
7. Riley JL, June CH. The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation. *Blood* 2005;105:13–21. [PubMed: 15353480]
8. Adorini L. Immunotherapeutic approaches in multiple sclerosis. *J Neurol Sci* 2004;223:13–24. [PubMed: 15261555]

9. Liossis SN, Sfrikakis PP. Costimulation blockade in the treatment of rheumatic diseases. *Biodrugs* 2004;18:95–102. [PubMed: 15046525]
10. Pollard KM, Arnush M, Hultman P, Kono DH. Costimulation requirements of induced murine systemic autoimmune disease. *J Immunol* 2004;173:5880–5887. [PubMed: 15494542]
11. Stuart RW, Racke MK. Targeting T cell costimulation in autoimmune disease. *Expert Opin Ther Targets* 2002;6:275–289. [PubMed: 12223069]
12. Shao H, Fu Y, Liao T, et al. Anti-CD137 mAb treatment inhibits experimental autoimmune uveitis by limiting expansion and increasing apoptotic death of uveitogenic T cells. *Invest Ophthalmol Vis Sci* 2005;46:596–603. [PubMed: 15671287]
13. Shao H, Fu Y, Song L, Sun S, Kaplan HJ, Sun D. Lymphotoxin beta receptor-Ig fusion protein treatment blocks actively induced, but not adoptively transferred, uveitis in Lewis rats. *Eur J Immunol* 2003;33:1736–1743. [PubMed: 12778492]
14. Shao H, Woon MD, Nakamura S, et al. Requirement of B7-mediated costimulation in the induction of experimental autoimmune anterior uveitis. *Invest Ophthalmol Vis Sci* 2001;42:2016–2021. [PubMed: 11481266]
15. Zhang P, Sun D, Ke Y, Kaplan HJ, Shao H. The net effect of costimulatory blockers is dependent on the subset and activation status of the autoreactive T cells. *J Immunol* 2007;178:474–479. [PubMed: 17182586]
16. Kurzinger K, Reynolds T, Germain RN, Davignon D, Martz E, Springer TA. A novel lymphocyte function-associated antigen (LFA-1): cellular distribution, quantitative expression, and structure. *J Immunol* 1981;127:596–602. [PubMed: 6788846]
17. Davignon D, Martz E, Reynolds T, Kurzinger K, Springer TA. Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blockade of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. *J Immunol* 1981;127:590–595. [PubMed: 6166678]
18. Davignon D, Martz E, Reynolds T, Kurzinger K, Springer TA. Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from Lys-2,3 that participates in T lymphocyte-mediated killing. *Proc Natl Acad Sci USA* 1981;78:4535–4539. [PubMed: 7027264]
19. Nicolls MR, Gill RG. LFA-1 (CD11a) as a therapeutic target. *Am J Transplant* 2006;6:27–36. [PubMed: 16433753]
20. Grakoui A, Bromley SK, Sumen C, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science* 1999;285:221–227. [PubMed: 10398592]
21. Lee KH, Holdorf AD, Dustin ML, Chan AC, Allen PM, Shaw AS. T cell receptor signaling precedes immunological synapse formation. *Science* 2002;295:1539–1542. [PubMed: 11859198]
22. Monks CRF, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 1998;395:82–86. [PubMed: 9738502]
23. Qi SY, Groves JT, Chakraborty AK. Synaptic pattern formation during cellular recognition. *Proc Natl Acad Sci USA* 2001;98:6548–6553. [PubMed: 11371622]
24. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994;76:301–314. [PubMed: 7507411]
25. Bachmann MF, Kall-Faienza K, Schmits R, et al. Distinct Roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* 1997;7:549–557. [PubMed: 9354475]
26. Kavanaugh AF, Lightfoot E, Lipsky PE, Oppenheimermarks N. Role of Cd11/Cd18 in adhesion and transendothelial migration of T-cells: analysis utilizing Cd18-deficient T-cell clones. *J Immunol* 1991;146:4149–4156. [PubMed: 1710241]
27. Hamann A, Jablonskiwestrich D, Duijvestijn A, et al. Evidence for an accessory role of Lfa-1 in lymphocyte-high endothelium interaction during homing. *J Immunol* 1988;140:693–699. [PubMed: 3276776]
28. Warnock RA, Askari S, Butcher EC, Andrian UH. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J Exp Med* 1998;187:205–216. [PubMed: 9432978]
29. Guerette B, Skuk D, Celestin F, et al. Prevention by anti-LFA-1 of acute myoblast death following transplantation. *J Immunol* 1997;159:2522–2531. [PubMed: 9278346]

30. Anderson ME, Siahaan TJ. Targeting ICAM-1/LFA-1 interaction for controlling autoimmune diseases: designing peptide and small molecule inhibitors. *Peptides* 2003;24:487–501. [PubMed: 12732350]
31. Bertry-Coussot L, Lucas B, Danel C, et al. Long-term reversal of established autoimmunity upon transient blockade of the LFA-1/intercellular adhesion molecule-1 pathway. *J Immunol* 2002;168:3641–3648. [PubMed: 11907130]
32. Bruner RJ, Farag SS. Monoclonal antibodies for the prevention and treatment of graft-versus-host disease. *Semin Oncol* 2003;30:509–519. [PubMed: 12939720]
33. Calhoun RF, Oppat WF, Duffy B, Mohanakumar T. Intercellular adhesion molecule-1/leukocyte function associated antigen-1 blockade inhibits alloantigen specific human T cell effector functions without inducing anergy. *Transplantation* 1999;68:1144–1152. [PubMed: 10551644]
34. Connolly MK, Kitchens EA, Chan B, Jardieu P, Wofsy D. Treatment of murine lupus with monoclonal antibodies to lymphocyte function-associated antigen-1: dose-dependent inhibition of autoantibody production and blockade of the immune response to therapy. *Clin Immunol Immunopathol* 1994;72:198–203. [PubMed: 8050193]
35. Hasegawa Y, Yokono K, Taki T, et al. Prevention of autoimmune insulin-dependent diabetes in non-obese diabetic mice by anti-LFA-1 and anti-ICAM-1 mAb. *Int Immunol* 1994;6:831–838. [PubMed: 7916204]
36. Kevil CG, Hicks MJ, He X, et al. Loss of LFA-1, but not Mac-1, protects MRL/MpJ-Fas(lpr) mice from autoimmune disease. *Am J Pathol* 2004;165:609–616. [PubMed: 15277234]
37. Yusuf-Makagiansar H, Anderson ME, Yakovleva TV, Murray JS, Siahaan TJ. Inhibition of LFA-1/ICAM-1 and VLA-4/VCAM-1 as a therapeutic approach to inflammation and autoimmune diseases. *Med Res Rev* 2002;22:146–167. [PubMed: 11857637]
38. Ando K, Fujino Y, Mochizuki M. Effects of monoclonal antibodies directed at cell surface molecules on murine experimental autoimmune uveoretinitis. *Graefes Arch Clin Exp Ophthalmol* 1999;237:848–854. [PubMed: 10502060]
39. Uchio E, Kijima M, Tanaka S, Ohno S. Suppression of experimental uveitis with monoclonal antibodies to ICAM-1 and LFA-1. *Invest Ophthalmol Vis Sci* 1994;35:2626–2631. [PubMed: 7909311]
40. Whitcup SM, DeBarge LR, Caspi RR, Harning R, Nussenblatt RB, Chan CC. Monoclonal antibodies against ICAM-1 (CD54) and LFA-1 (CD11a/CD18) inhibit experimental autoimmune uveitis. *Clin Immunol Immunopathol* 1993;67:143–150. [PubMed: 8100190]
41. Liao T, Ke Y, Shao WH, et al. Blockade of the interaction of leukotriene b4 with its receptor prevents development of autoimmune uveitis. *Invest Ophthalmol Vis Sci* 2006;47:1543–1549. [PubMed: 16565390]
42. Shao H, Peng Y, Liao T, et al. A shared epitope of the interphotoreceptor retinoid-binding protein recognized by the CD4+ and CD8+ autoreactive T cells. *J Immunol* 2005;175:1851–1857. [PubMed: 16034128]
43. Shao H, Lei S, Sun SL, Xiang J, Kaplan HJ, Sun D. CpG-containing oligodeoxynucleotide 1826 converts the weak uveitogenic rat interphotoreceptor retinoid-binding protein peptide 1181–1191 into a strong uveitogen. *J Immunol* 2003;171:4780–4785. [PubMed: 14568955]
44. Shao H, Sun SL, Kaplan HJ, Sun D. Characterization of rat CD8+ uveitogenic T cells specific for interphotoreceptor retinal-binding protein 1177–1191. *J Immunol* 2004;173:2849–2854. [PubMed: 15295004]
45. Forrester JV, Liversidge J, Dua HS. Regulation of the local immune response by retinal cells. *Curr Eye Res* 1990;9(suppl):183–191. [PubMed: 2200644]
46. Kim MC, Kabeer NH, Tandhasetti MT, Kaplan HJ, Bora NS. Immunohistochemical studies on melanin associated antigen (MAA) induced experimental autoimmune anterior uveitis (EAAU). *Curr Eye Res* 1995;14:703–710. [PubMed: 8529406]
47. Sun D, Le J, Yang S, Malotkey M, Coleclough C. Major role of antigen-presenting cells in the response of rat encephalitogenic T cells to myelin basic proteins. *J Immunol* 1993;151:111–118. [PubMed: 7686928]
48. Caspi RR. Immune mechanisms in uveitis. *Springer Semin Immunopathol* 1999;21:113–124. [PubMed: 10457585]

49. Dullforce PA, Seitz GW, Garman KL, et al. Antigen-specific accumulation of naive, memory and effector CD4 T cells during anterior uveitis monitored by intravital microscopy. *Cell Immunol* 2006;239:49–60. [PubMed: 16712823]
50. Peng Y, Shao H, Ke Y, et al. In vitro activation of CD8 interphotoreceptor retinoid-binding protein-specific T cells requires not only antigenic stimulation but also exogenous growth factors. *J Immunol* 2006;176:5006–5014. [PubMed: 16585597]
51. Hogg N, Smith A, McDowall A, et al. How T cells use LFA-1 to attach and migrate. *Immunol Lett* 2004;92:51–54. [PubMed: 15081527]
52. Xu H, Manivannan A, Jiang HR, et al. Recruitment of IFN-gamma-producing (Th1-like) cells into the inflamed retina in vivo is preferentially regulated by P-selectin glycoprotein ligand 1:P/E-selectin interactions. *J Immunol* 2004;172:3215–3224. [PubMed: 14978129]
53. Go S, Fleischmann A, Lantz O, et al. Anti-LFA-1 antibody postpones T-cell receptor triggering while preserving generation of regulatory T cells in T-cell receptor anti-HY transgenic mice. *Transplantation* 2006;82:119–126. [PubMed: 16861951]
54. Lunsford KE, Koester MA, Eiring AM, Horne PH, Gao D, Bumgardner GL. Targeting LFA-1 and CD154 suppresses the in vivo activation and development of cytolytic (CD4-independent) CD8+ T cells. *J Immunol* 2005;175:7855–7866. [PubMed: 16339521]
55. Pietrzak A, Podhorecka M, Chodorowska G, Rolinski J, Urban J. Efalizumab in the treatment of psoriasis. *Ann Univ Mariae Curie Sklodowska [Med]* 2003;58:174–178.

FIGURE 1.

Of the costimulatory molecule blockers tested, only anti-LFA-1 α Ab suppressed both the in vitro primary and secondary response of autoreactive T cells. T cells (4×10^5 cells/well) from IRBP1-20-immunized mice (**A**) or from IRBP1-20-specific T-cell-transferred mice (**B**) were incubated with APCs and IRBP1-20 ($10 \mu\text{g/mL}$), in the presence or absence of blocking agents ($10 \mu\text{g/mL}$), and T-cell proliferation was measured by ^3H thymidine uptake. The values are the mean $\pm$ SD of the results for triplicate wells in a single experiment. The experiment was repeated three times with similar results.

FIGURE 2.

Anti-LFA-1 α Ab suppressed the in vitro activation of IRBP1-20-specific CD4 and CD8 T cells. (A) Proliferative response: purified CD4 and CD8 cells from IRBP1-20-immunized B6 mice were incubated with irradiated APCs and IRBP1-20 (10 μ g/mL) in the presence of anti-LFA-1 α Ab or isotype control Ig (10 μ g/mL). (B) Cytokine production: cell culture was performed and then the culture supernatants were collected at 48 hours and assayed for IL-2, IFN- γ , IL-3, and IL-10 by ELISA.

FIGURE 3.

The effector phase of EAU was suppressed by anti-LFA-1 α Ab. (**A**, **B**) IRBP1-20-specific T cells from the draining lymph nodes and spleens of donor B6 mice immunized with IRBP1-20 were transferred to naïve mice, which were injected three times with 150 μ g/mouse of anti-LFA-1 α Ab or isotype control Ab at 5-day intervals starting on the day of transfer (**A**) or at day 10 after transfer (disease onset) (**B**, six mice per group). The animals were examined every 3 days for clinical signs of uveitis by funduscopy, starting at day 8 after transfer. The experiment was repeated twice. The EAU score was expressed as the mean $\pm$ SEM. *Significant differences ($P < 0.05$) determined with repeated-measures ANOVA (**C**, **D**) Histology of the eye at day 23 after transfer in a tEAU mouse treated with isotype control Ab (**C**) or anti-LFA-1 α Ab (**D**) starting on the day of transfer. Hematoxylin and eosin; original magnification, $\times 40$.

FIGURE 4.

LFA-1 α was shown to be essential for the recruitment of autoreactive T cells in the tEAU model. (A) IRBP1-20-specific T cells labeled with CFSE were transferred into hosts that were treated with a single injection of anti-LFA-1 α Ab, CTLA-4Fc, or isotype control Ig (150 μ g/mouse) on the same day (day 0). On day 3, single cell suspensions from the eyes were analyzed by flow cytometry using PE-conjugated anti-TCR antibodies. The experiment was performed three times with similar results. (B) Analysis of infiltrating leukocytes in the eye of tEAU mice treated three times with anti-LFA-1 α Ab or isotype control Ig starting on day 0. Eyes were collected from PBS-perfused tEAU mice at day 23 after transfer and eye-infiltrating cells prepared by enzyme digestion, then double-stained with the indicated FITC- and PE-conjugated mAbs, and analyzed by flow cytometry. The percentage of positive cells is indicated on the gated cells. The experiment was performed five times with similar results. (C) Cytokine expression by effector T cells is reduced in anti-LFA-1 α Ab-treated eyes. Real-time PCR analysis was performed on cDNA prepared from DNase-treated RNA extracted, on day 23 after transfer, from the eyes of tEAU mice treated three times with anti-LFA-1 α Ab or control

Ig, starting on day 0, and the cytokine mRNA levels measured. The results are from a pool of RNA from five mice and are representative of results in two independent experiments.

FIGURE 5.

In vivo treatment of uveitis induced by IRBP1-20-specific T cells using anti-LFA-1 α mAb. (A) Splenic T cells from IRBP1-20-specific T cell-transferred mice treated three times with anti-LFA mAbs or control Ig starting on day 0 were collected at day 23 after transfer and stimulated with various doses of IRBP1-20, and the proliferative response was measured by ³[H]thymidine uptake. (B) Some of the cells were double stained with PE-conjugated anti-CD44 and FITC-conjugated anti-CD62L mAbs and analyzed by flow cytometry. The indicated number is the percentage of CD44^{hi}CD62L⁻ cells.

FIGURE 6.

Reactivation of autoreactive T cells by retinal astrocytes was inhibited by anti-LFA-1 α Ab. An RAC monolayer was cultured with IRBP1-20-specific T cells and IRBP1-20 peptide, with or without the indicated blockers. Twenty-four hours later, the supernatants were collected and assessed for TNF- α production. The data are the mean $\pm$ SD of the results in triplicate wells in a single experiment. The experiment was repeated three times with similar results.

FIGURE 7.

LFA-1 α blockade increased the number and function of CD4⁺CD25⁺ T_{reg} cells. **(A)** At day 23 after transfer, splenic T cells from tEAU mice treated three times with anti-LFA-1 α Ab or control Ig starting on day 0 were isolated and stained with FITC- or PE-conjugated Abs against CD25 and either CD4 or FoxP3 and the percentage of CD4⁺CD25⁺ T cells or FoxP3⁺CD25⁺ cells assessed. The data are representative of those for two independent experiments. **(B, C)** CD4⁺CD25⁻ T regulatory cells from tEAU mice not treated **(B)** or treated with anti-LFA-1 α Ab as above **(C)** and/or CD4⁺CD25⁻ T effector cells from Ag-immunized mice at day 10 after immunization were further purified and stimulated with IRBP1-20 separately or together at the indicated ratio of 1:0.5 (CD4⁺CD25⁻ to CD4⁺CD25⁺), and proliferation was measured. The data are the mean $\pm$ SD of the results in triplicate wells in a single experiment. The experiment was repeated three times with similar results.