Anti-CD137 mAb Treatment Inhibits Experimental Autoimmune Uveitis by Limiting Expansion and Increasing Apoptotic Death of Uveitogenic T Cells

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PURPOSE. To explore the role of CD137 in the pathogenesis of experimental autoimmune uveitis (EAU) and to compare the inhibitory mechanism of anti-CD137 mAb with other costimulatory blockers.

METHODS. EAU was induced in B10RIII mice, either by immunization with a uveitogenic peptide, IRBP161-180, derived from the interphotoreceptor retinoid-binding protein, or by adoptive transfer of IRBP161-180-specific T cells. The effect of an agonistic anti-CD137 mAb (2A) on the in vivo induction of disease was studied. Subsequently, the mechanism by which anti-CD137 mAb inhibits uveitogenic T-cell activation was investigated, by using the adoptive transfer of T cells derived from anti-CD137 mAb–treated mice, and in vitro, using the proliferative response and apoptotic cell death of IRBP-specific T cells from anti-CD137 mAb–treated mice.

RESULTS. Administration of anti-CD137 mAb prevented the development of de novo induced uveitis, but not that induced by adoptive transfer of pathogenic T cells. Furthermore, anti-CD137 mAb treatment of the animals resulted in decreased expansion of uveitogenic T cells, accompanied by increased activated cell death and resistance to reininduction of uveitis.

CONCLUSIONS. CD137 plays a critical role in the induction, rather than the effector, phase of the disease. Different costimulatory molecules have different effects on the activation of autoreactive T cells by acting in different phases of T-cell activation. (Invest Ophthalmol Vis Sci. 2005;46:596–603) DOI:10.1167/iovs.04-0835

Uveitis is a common cause of human visual disability and blindness. Animal models of experimental uveitis (EAU) have been widely used to dissect the immunopathological mechanisms in uveitis and to develop preventive or therapeutic strategies. EAU can be elicited in rodents either by immunization with several different antigens—for example, retinal S antigen (S-Ag), interphotoreceptor retinoid-binding protein (IRBP), melamin-associated Ag (MAA), and myelin proteins—or by the adoptive transfer of uveitogenic T cells to syngeneic rodents, suggesting that uveitis is a T-cell-mediated, organ-specific autoimmune disease.

The activation of T cells, especially naive T cells, requires not only primary antigenic stimulation, but also costimulation by molecules on antigen-presenting cells (APCs), which provide essential signals for sustaining T-cell responses. Studies of T-cell activation have resulted in many new potential therapeutic approaches to the treatment of autoimmune diseases, one of which is the blocking of activation by the targeting of costimulatory molecules. However, it remains unclear whether the various costimulatory molecules are functionally different. Studies on the mechanisms by which individual costimulatory molecules exert their effects should help greatly in devising new therapies designed to control T-cell activation.

CD137 (4-1BB), a member of the tumor necrosis factor receptor (TNFR) superfamily, is an important costimulatory molecule in the immune response, mediating CD28-dependent and independent T-cell costimulation. This molecule, which is primarily expressed on the surface of activated T cells and NK cells, provides the costimulatory signal for both CD4+ and CD8+ T-cell-mediated immunity by binding to its ligand, CD137L, expressed on activated macrophages, dendritic cells, and T and B cells. The immune regulatory role of this molecule has been demonstrated in tumor rejection. In allogeneic immune responses, viral infection, and autoimmune diseases. In particular, the interaction of CD137 and CD137L plays a key role in the clonal expansion and survival of antiviral CD8+ effector T cells. It has been shown that CD8+ T-cell responses to viral infections are reduced in CD137L-deficient mice. In vivo, agonistic anti-CD137 mAb preferentially stimulates CD8 T cells that recognize and reject tumors and allograft transplants and it also protects CD8+ T cells from superantigen-induced cell death.

CD137 is implicated in immune responses mediated by CD4+ T cells, including alloimmune responses and inflammation. In CD137 transgenic mice, CD137 mediates primary CD4+ T-cell expansion and survival. In contrast to that, two different agonistic anti-CD137 mAbs inhibit T-cell-mediated tissue autoimmunity (experimental autoimmune encephalomyelitis [EAE]) and T-cell dependent antibody production (systemic lupus erythematosus [SLE]). The potential of anti-CD137 mAb in the treatment of T-cell-mediated autoimmune diseases is extended in our uveitis model. In this study, we tested the effect of one agonistic anti-CD137 mAb, 2A, on the development of EAU in B10RIII mice and found that it suppressed the activation of uveitogenic T cells and prevented the development of actively induced uveitis and the reinduction of uveitis on reimmunization with the same antigen. However, it did not prevent the development of uveitis induced by the adoptive transfer of uveitogenic T cells. These studies suggest...
that anti-CD137 mAb acts during the induction, rather than the effector, phase of the disease. In contrast to CTLA4-Fc protein, which binds to B7, blocks CD28–B7 interaction, and inhibits the early phase of T-cell activation, anti-CD137 mAb enhanced the early phase, but inhibited the later phase, of T-cell activation, indicating that different costimulatory molecules may function differently and sequentially at different stages of T-cell activation.

**Materials and Methods**

**Reagents and Animals**

The generation and production of the anti-CD137 mAb (2A) has been described elsewhere. Isotype-matched control rat IgG2a was obtained from Sigma-Aldrich (St. Louis, MO). The human IRBP161-180 peptide was synthesized by Invitrogen (Carlsbad, CA).

Pathogen-free female B10RIII mice (6 to 8 weeks old), purchased from Jackson Laboratory (Bar Harbor, ME), were housed and maintained at the animal facilities of the University of Louisville. Animals were managed in accordance with the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

All T cells were cultured in RPMI 1640 medium (Invitrogen-Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Invitrogen-Gibco), 5×10⁻⁵ M 2-mercaptoethanol, and 100 μg/mL penicillin/streptomycin.

**Actively Induced and Adoptively Transferred Uveitis**

For active induction of disease, B10RIII mice were immunized subcutaneously with 100 μL of an emulsion containing 100 μg of IRBP161-180 peptide and 500 μg of Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) in incomplete Freund’s adjuvant (Sigma-Aldrich), distributed over six spots on the tail base and flank. Concurrently, 0.4 μg of pertussis toxin (PTX; Sigma-Aldrich) was injected intraperitoneally (IP). Some mice were rechallenged with IRBP161-180 on day 30 by using the same protocol.

For adoptive transfer, unless otherwise stated, recipient mice were injected IP with 6×10⁵ IRBP161-180-specific T cells, prepared as described previously, in 0.2 mL of PBS.

**Anti-CD137 mAb Treatment**

For treatment of actively induced uveitis, unless otherwise stated, mice received 200 μg of rat anti-mouse CD137 mAb or control rat IgG on day 0, the day of immunization with IRBP161-180. For treatment of adoptively transferred uveitis, a single IP injection of 200 μg anti-CD137 mAb or control rat IgG was injected IP on the day of transfer.

**CTLA4-Fc Treatment**

Mice (n = 5) received 200 μg of CTLA4-Fc (kindly provided by Philip Morgan, Pharmacia, St. Louis, MO) or control wild type CTLA4-Fc (mutant B7 binding domain) on day 0, the day of immunization with hIRBP161-180.

**Clinical Observation of Uveitis by Indirect Fundoscopy**

For fundoscopic examination, pupils were dilated with a 0.5% ophthalmic solution of tropicamide. The mice were anesthetized with a cocktail of anesthetics (ketamine + xylazine) before the procedure. The eyes were examined with a stereomicroscope with coaxial illumination. Grading of disease was performed by using a scoring system described previously.

**Histology and Immunohistochemistry**

Whole eyes were collected and prepared for histopathological evaluation at the end of the experiment (day 21 or 51 postinjection [PI]) for histopathology of EAU in mice immunized with IRBP peptide and antigen-immunized mice or day 15 after T-cell transfer). The eyes were immersed for 1 hour in 4% phosphate-buffered glutaraldehyde and then transferred to 10% phosphate-buffered formaldehyde until processed. The fixed and dehydrated tissues were embedded in methacrylate, and then 5-μm sections were cut through the pupillary–optic nerve plane and stained with hematoxylin and eosin. The presence or absence of disease was evaluated blind by examining six sections cut at different levels of each eye specimen. The severity of EAU was scored on a scale of 0 to 4 (no disease) to 4 (maximum disease) in half-point increments, as described previously.

**Cell Proliferation and Cytokine Assays**

APCs (irradiated syngeneic spleen cells, 2×10⁵/well) were preincubated in 96-well, flat-bottomed microtiter plates with 0 to 10 μg/mL of 2-A
A single injection of 200 μg anti-CD137 mAb does not protect against development of actively induced uveitis. B10RIII mice were immunized with 100 μg of IRBP161-180 on day 0 and injected IP with a single injection of 200 μg anti-CD137 mAb or control rat IgG on days 6 or 12 PI. Eyes were collected on day 21 PI. The EAU score is the mean ± SD for the group (n = 6). Two separate experiments were performed with similar results.

IRBP161-180 for 1 hour, and then nylon wool–enriched lymph nodes or spleen T cells (3 × 10⁵/well) were added. After 48-hour incubation, a fraction of the culture supernatant was analyzed for IL-2, IL-4, IL-10, and IFN-γ production, by using ELISA kits (R&D Systems, Minneapolis, MN). [3H]thymidine incorporation during the last 8 hours was assessed with a microplate scintillation counter (Packard Instruments, Meriden, CT). The proliferative response was expressed as the mean counts per minute ± SD of triplicate determinations.

Statistic Analyses
The data are expressed as the mean ± SD. Each experiment was repeated at least two or three times. Student’s t-test was used to analyze the results.

RESULTS

Anti-CD137 mAb Protection against Uveitis Induced by Immunization with the Uveitogenic Peptide IRBP161-180
To determine the role of CD137 in the development of uveitis, B10RIII mice were immunized with IRBP161-180 on day 0 and injected IP with 100 μg anti-CD137 mAb or isotype-matched control rat IgG on each of days 0, 3, 6, and 9. As shown in Figures 1A and 1B, all 10 mice treated with the control IgG showed development of moderate to severe EAU, whereas 6 of the 10 anti-CD137 mAb–treated animals were protected, with the other 4 having mild disease. Similar results were obtained when mice (n = 8) were given a single dose of 200 μg anti-CD137 mAb on day 0 (Fig. 1C). When the histopathology of EAU in mice immunized with IRBP peptide and treated with anti-CD137 mAb or control rat IgG was compared, those injected with control IgG showed loss of the photoreceptor layer, retinal detachment, and the presence of inflammatory cells in the vitreous and retina (Fig. 1D), whereas those treated with anti-CD137 mAb showed a well-preserved retinal structure (Fig. 1E). Thus, the anti-CD137 mAb–treated animals had no or mild uveitis, whereas all animals treated with control rat IgG remained fully susceptible to disease induction.

Effect of Anti-CD137 mAb on Ongoing Actively Induced EAU
Because CD137 is upregulated on activated T cells, we examined whether antibody treatment alters the disease course and severity when given after, rather than before, initiation of the autoimmune reaction—that is, on day 6 PI (during T-cell priming) or on day 12 PI (at disease onset), when autoreactive T cells have already been activated. As shown in Figure 2, a single injection of 200 μg anti-CD137 mAb on day 6 or 12 PI did not protect against EAU assessed on day 21.

Effect of Anti-CD137 mAb on the Development of Adoptively Transferred Uveitis
To determine whether antibody treatment acted on the induction and/or effector phase of the disease, we tested whether anti-CD137 mAb treatment could block the development of adoptively transferred uveitis generated by transfer of IRBP161-180-specific T cells. When groups (n = 6) of naive mice were simultaneously injected IP with a pathogenic dose (6 × 10⁶) of IRBP161-180-specific T cells and 200 μg (or even higher dose, 300 μg per mouse) anti-CD137 mAb or control IgG, none of the anti-CD137 mAb–treated animals showed significant amelioration of clinical symptoms, including disease onset and severity, when monitored by fundoscopy and pathology examination (Fig. 3).

Failure to Induce Uveitis by T Cells Derived from the Mice Treated with Anti-CD137 mAb
We then investigated whether T cells capable of inducing EAU were still present and functional in anti-CD137 mAb–treated mice when transferred into naive B10RIII mice. Donor B10RIII mice were immunized with 100 μg IRBP161-180 emulsified in CFA, and, at 10 to 14 days PI, nylon wool–purified T cells were prepared from their DLN and spleens and cultured with IRBP161-180 for 2 days, and then 6 × 10⁶ cells were transferred IP into naive B10RIII mice, which were injected IP with 200 μg anti-CD137 mAb or control IgG on the day of transfer (six mice per group). Eyes were monitored twice per week by fundoscopic examination. The mean disease score ± SD was determined for all the eyes of one group of six mice (A). Histology was performed on eyes of mice killed 15 days after transfer. Data are the mean disease score of all eyes of one group (B).
mice. Nylon wool-purified T cells from the draining lymph nodes (DLNs) and spleen of IRBP161-180-immunized donor mice injected with 200 μg anti-CD137 mAb or control IgG on day 0 were prepared 10 to 14 days after immunization and cultured in vitro for 2 days with IRBP161-180, and then 1 × 10^7 T cells were adoptively transferred into groups of six naive syngeneic B10RIII mice. As shown in Table 1A, only two of the mice receiving T cells from anti-CD137 mAb–treated immunized mice had mild EAU at 15 days after transfer, whereas five of the mice receiving T cells from control IgG-treated mice had much more severe disease. We conclude that anti-CD137 mAb had a long-term inhibitory effect on IRBP161-180–specific T cells.

To determine further whether long-lasting protection was established in mice treated with anti-CD137 mAb, IRBP161-180-immunized animals treated on day 0 with 200 μg anti-CD137 mAb or control IgG were rechallenged with IRBP161-180 on day 30, during or after resolution of the first attack, and then reassessed on day 51. As shown in Table 1B, five of the six animals in the group treated with control IgG had high EAU scores (grades 2 to 3), whereas only one of the five anti-CD137 mAb–treated mice had mild uveitis (grade 0.5) after rechallenge, showing that the administration of agonistic anti-CD137 mAb inhibited the autoreactive T-cell response. Spleen cells harvested from these two groups of mice on day 51 were also tested for proliferation and IFN-γ production in response to IRBP. As shown in Figure 4, the group treated with anti-CD137 mAb had a lower proliferative response and produced less IFN-γ than the control IgG-treated group. Thus, the disease scores and cellular responses indicated that anti-CD137 mAb treatment protected mice from a second episode of EAU and resulted in long-lasting tolerance.

### Effect of Anti-CD137 mAb on the Expansion of Activated Uveogenic T Cells

To determine whether anti-CD137 mAb treatment impaired the specific T-cell responses induced by antigen immunization, we tested the proliferative response of DLN T cells to the immunizing IRBP161-180 peptide in vitro. DLN cells were isolated from B10RIII mice 10 to 12 days after immunization with IRBP161-180 peptide and injection of 200 μg anti-CD137 mAb or control IgG on day 0. When the in vivo–primed nylon wool–enriched T cells were stimulated with graded doses of IRBP161-180, the cells from anti-CD137 mAb–treated mice showed a very low response to the peptide compared with those from control IgG-treated animals (Fig. 5A).

### Difference in Inhibitory Effect of Anti-CD137 mAb from That in CTLA4-Fc-Mediated Inhibition of T-Cell Activation

We have reported that CTLA-4 Fc, a fusion protein consisting of the extracellular domain of human CTLA4 coupled to mouse IgG2a Fc, which binds to B7 and blocks the CD28–B7 interaction, inhibits antigen-specific autoreactive T-cell functions, thereby preventing the development of antigen-induced uveitis.41 To examine whether CTLA-4Fc and anti-CD137 mAb blocks disease development by different effects on T-cell activation, we compared the activation of IRBP161-180–specific T cells at various days PI in IRBP161-180–immunized mice treated on day 0 with a single dose of 200 μg of CTLA4-Fc or anti-CD137 mAb. As shown in Figure 5C, DLN T cells from either CTLA4-Fc-treated or anti-CD137 mAb–treated mice prepared at day 10 PI showed significantly depressed proliferative T-cell responses to the immunizing peptide compared with those from control IgG-treated mice. However, when T-cell proliferation was assessed at day 5 PI, only T cells from CTLA4-Fc-treated mice showed a decreased response, whereas the response of T cells from anti-CD137 mAb–treated mice to both immunizing peptide (IRBP161-180) and purified protein derivative of Tuberculin (PPD, a component in the CFA) was unex-
 Unexpectedly increased (Figs. 5B, 5F). In addition, 5 days PI, anti-CD137 mAb–treated DLN cells produced higher levels of IFN-γ and IL-2 than control-treated DLN cells when cultured with IRBP161-180 peptide in vitro. However, 10 days PI, anti-CD137 mAb–treated DLN cells produced much less IFN-γ and IL-2 than control-treated DLN cells (Figs. 5D, 5E). We also measured the production of IL-4 and IL-10 by the DLN cells collected on days 5 and 10 from mice treated with either control rat IgG or anti-CD137 mAb. These cytokines were under detectable levels (data not shown).

These results indicate that anti-CD137 mAb treatment has multiple effects on T-cell activation, augmenting the activation of IRBP-specific T cells—particularly, Th1 cell functions—during the early stage and downregulating T-cell functions during the later stage, whereas CTLA4-Fc treatment inhibits APC–T-cell interaction during both the early and late stages of T-cell priming in vivo.

**Increased Apoptotic Death of Activated Autoreactive T Cells in Anti-CD137 mAb–Treated Recipient Mice**

Based on the observation that anti-CD137 mAb treatment resulted in enhancement of the IRBP-specific T-cell response during the early stage of T-cell priming in vivo and in a decreased T-cell response in the late stage, we hypothesized that the augmented early T-cell response by an agonistic anti-CD137 mAb may be associated with increased activation-induced T-cell death (AICD). We therefore examined whether uveitogenic T cells from animals treated with anti-CD137 mAb under- or control IgG on day 0 were prepared on day 10 PI and incubated with IRBP161-180 and T-cell-free APCs in 24-well plates, then tested 36 and 60 hours later for apoptosis, by using annexin V.
observed. Collectively, these findings suggest this approach may have potential for deleting autoreactive T cells in autoimmune diseases in animal models.41–43 Antagonist antibodies or specific T-cell activation and induces tolerance of autoreactive T cells. An alternative mechanism for the anti-CD137 mAb-mediated inhibition of active uveitis is that it may initially activate T cells and then lead to AICD, suggesting a dual role, depending on the activation status of different T-cell populations.48,49 We found that T cells from anti-CD137 mAb–treated animals showed increased antigen-specific proliferation during the early stages of T-cell activation in vivo (i.e., at day 5 PI), but decreased proliferation at day 10 PI (Fig. 5). Our additional studies have shown that the proliferation response toward PPD (a component in the CFA) was also augmented in recipient mice of anti-CD137 antibody at day 5 post-immunization. T cells from mice treated with anti-CD137 mAb were found to be susceptible to AICD (Fig. 6). The mechanism of the agonistic effect of anti-CD137 antibody remains largely unknown.

Multiple mechanisms may be involved in the prevention of the development of uveitis by anti-CD137 mAb treatment. T cells from anti-CD137 mAb–treated mice failed to adoptively transfer uveitis, suggesting either that the number of IRBP161-180-reactive T cells was decreased because of cell death (Fig. 6) or that regulatory T cells were upregulated by anti-CD137 mAb treatment.50 The latter will be further confirmed by our system. Previous studies have shown that different costimulatory molecules can have a synergistic effect on the activation of pathogenic T cells. Thus, prolongation of skin graft survival is not seen in CD137L-deficient mice, but is seen in CD137L-deficient/CD28-deficient mice.50 We have reported that treatment of Lewis rats with CTLA4-Fc inhibits the development of actively induced uveitis by blocking the CD28–B7 interaction14 and that a similar protective effect is seen using LTβR-Fc, a fusion protein blocking the binding of LIGHT to LTβR or HVEM, newly identified costimulatory molecules of the TNFR family.57 We have shown that the blocking effect of LTβR-Fc differs from that of CTLA4-Fc, as CTLA4-Ig is more effective against antigen-specific activation of in vivo primed T cells than against CD3-mediated nonspecific T-cell activation, whereas LTβR-Fc inhibits antigen-specific and nonspecific T-cell responses equally well.57 In the present report, we demonstrated that anti-CD137 mAb 2A had a similar protective effect on autoimmune uveitis, but, in contrast to CTLA4-Fc and LTβR-Fc, which, respectively, inhibit T-cell activation in the early phase or in the early and late phases, anti-CD137 mAb enhanced T-cell activation in the early phase and then induced AICD. These results imply that different costimulatory molecules have different effects on the activation of autoreactive T cells by...
acting at different phases of T-cell activation and possibly on different subsets of autoreactive T cells and/or at different disease phases.

In summary, the biological complexity of the action of various costimulatory molecules in T-cell activation is now being recognized. Future studies should reveal whether T-cell activation involves cascading costimulatory pathways and whether the involvement of different costimulatory interactions results in different biological consequences. The manipulation of various costimulatory molecules may have a synergistic effect in the treatment of autoimmune diseases.

References
13. Salomon B, Bluestone JA. Complexities of CD28/CD137 interactions results in different biological consequences. The manipu-lation of various costimulatory molecules may have a syner-gistic effect in the treatment of autoimmune diseases.


