Requirement of B7-Mediated Costimulation in the Induction of Experimental Autoimmune Anterior Uveitis

Hui Shao,1,2 Ming-Dar Woon,1 Satoshi Nakamura,3 Jeong-Hyeon Sohn,2 Phillip A. Morton,4 Nalini S. Bora,2 and Henry J. Kaplan4

PURPOSE. To study the role of costimulatory signaling through the CD28/B7 interaction in experimental autoimmune anterior uveitis (EAAU).

METHODS. Naive Lewis rats were immunized with insoluble melanin-associated antigen (MAA) derived from bovine iris and ciliary body. CTLA4-Fc, a recombinant protein comprised of the extracellular domain of human CTLA4 bound to mouse IgG2a Fc, was used to block the CD28-B7 interaction. A mutant version (CTLA4-Fc-mutant) was used as a control. The effect of CTLA4-Fc on the in vivo induction of disease with MAA was studied. Subsequently, the mechanism by which CTLA4-Fc blocked the interaction of CD28 and B7 was investigated in vivo, using the adoptive transfer of T cells derived from CTLA4-Fc-treated rats, and in vitro, using the proliferative response and cytokine production of MAA-T cells in the presence of CTLA4-Fc.

RESULTS. CTLA4-Fc markedly reduced the incidence and severity of EAAU in Lewis rats after sensitization with MAA. The adoptive transfer of sensitized T cells from CTLA4-Fc–treated donors did not induce EAAU in naïve recipients. CTLA4-Fc inhibited the expansion of antigen-specific MAA-T cells and the production of TNF-α.

CONCLUSIONS. The costimulatory signal delivered through CD28-B7 is required for the induction and pathogenesis of EAAU. In the absence of this signal, antigen-specific expansion of MAA reactive T cells as well as production of TNF-α is inhibited. Abrogation of this costimulatory signal may be an important therapeutic option for EAAU. (Invest Ophthalmol Vis Sci. 2001;42:2016–2021)

A cute anterior uveitis (AAU), the most common form of uveitis, is an intraocular inflammatory disease of the anterior uvea of the eye. Although a single episode of the disease usually does not result in permanent visual loss, the recurrent nature of AAU is associated with significant clinical complications such as cystoid macular edema, cataract formation, and glaucoma. The cause of AAU is unknown. However, it is thought to be an autoimmune disease similar to the experimental model of autoimmune anterior uveitis (EAAU).

Broekhuysen and colleagues, in 1991, first described an organ-specific autoimmune disease in the Lewis rat induced by a single injection of melanin-associated antigen (MAA).1 Although they termed this disease EAAU, it subsequently became apparent that the experimental model that most closely resembled AAU was induced solely by a single injection of MAA derived from the bovine iris and ciliary body without melanin-associated antigen from the choroidal and retinal pigment epithelium.2,3 Histopathologic examination of eyes with EAAU reveal a severely inflamed iris and ciliary body infiltrated by mostly mononuclear cells with minimal choroidal and no retinal involvement. Immunohistochemical study of inflamed eyes4 and the adoptive transfer of AAU by activated T cells3 suggest that EAAU is a T-cell–mediated autoimmune disease. However, the precise mechanism by which autoimmunity to ocular antigens develops remains to be determined.

Recently, the role of costimulatory signaling in the induction of autoreactive T cells in autoimmune diseases has been intensively studied. The activation and differentiation of T cells require both antigen/MHC recognition and costimulatory signals.5 The signal delivered by the T-cell reactivity determines the antigen specificity of the response. The second signal, termed costimulation, is provided by accessory molecules on antigen-presenting cells (APCs) and appears to be necessary for functional T-cell activation. There are several receptor–ligand pairs that can provide costimulation. However, the signal provided by the B7 family of cell surface molecules, with its receptors on T cells, CD28, and CTLA-4, seems to be dominant for T-cell activation.6-8

At least two members of the B7 family of CD28 ligands have been defined, B7-1 (CD80) and B7-2 (CD86).9-12 These molecules, although homologous, are each capable of providing costimulation to T cells for proliferation and IL-2 production. A mouse genetically deficient for B7-1 is essentially immunocompetent.9 Likewise, T cells from a mouse deficient for CD28 cannot produce IL-2 after stimulation with anti-CD3 antibody.13 A mouse deficient for CTLA-4 has a fetal phenotype analogous a lymphoproliferative disorder.14 Thus, the costimulatory signal derived through this receptor ligand is important for the development of an effective immunoresponse.

CTLA4-Fc, a recombinant fusion protein consisting of the extracellular domain of human CTLA4 fused to mouse IgG2a Fc, binds to both B7-1 and B7-2 and can thereby block the interaction between B7 and CD28 or CTLA-4.15 Administration of CTLA4-Ig prevented rat cardiac allograft rejection and pancreatic islet cell xenograft rejection in mice.16,17 In both instances, it appeared the mechanism of suppression involved the induction of antigen-specific tolerance. The costimulation provided by B7 also appears to be important for the development of autoimmunity. CTLA4-Ig treatment, initiated the day before immunization of mice with myelin basic protein (MBP)...
and continued for 3 weeks, prevented development of the clinical and histologic manifestations of experimental allergic encephalomyelitis (EAE). CTLA4-Ig treatment is also effective if initiated 10 days postimmunization with MBP, when clinical disease is event. In the adoptive transfer model of EAE, moreover, treatment of donor cells with CTLA4-Ig during ex vivo exposure to MBP inhibited T-cell proliferation and IL-2 secretion in response to MBP and prevented transfer of EAE to recipients. As is the case with EAE, treatment with CTLA4-Ig prevented the development of disease in other animal models of organ-specific autoimmunity—for example, collagen-induced arthritis in the BB/Wor rat, autoimmune oophoritis induced by zona pellucida glycoprotein ZP23 peptide, and experimental antiligemular basement mem-

brane autoimmune glomerulonephritis. A recent clinical study suggested a potential therapeutic use for this novel immunomodulatory approach in T-cell-mediated diseases. T-cell costimulation in patients with psoriasis vulgaris was blocked using CTLA4-Ig and was observed to have a beneficial effect.

Using an animal model of AAU, we investigated the role of ongoing T-cell costimulation in the development and pathogenesis of ocular autoimmunity. In this article, we have shown that interference with the B7 costulatory signal through CD28 by CTLA4-Fc reduced both the incidence and the severity of intraocular inflammation in EAAU. Furthermore, CTLA4-Fc resulted in a profound decrease in the proliferative response and TNF-α production by a rat MAA-specific T-cell line. Our results suggest a critical role for B7-mediated costimulation in the development of intraocular inflammation and identifies a promising therapeutic approach for the treatment of autoimmune uveitis.

**METHODS**

All animal studies conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Institutional approval was obtained, and institutional guidelines regarding animal experimentation were followed.

**Rats**

Pathogen-free male Lewis rats aged 5 to 6 weeks (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were housed and maintained at the animal facility of Washington University Medical School.

**CTLA4-Fc**

Soluble recombinant proteins comprising the extracellular domain of wild-type human CTLA4 or a mutated form of CTLA4 lacking B7 binding activity (control) were obtained as fusion proteins to mouse IgG2a Fc. In the experimental panel, 100 μg of CTLA4-Fc was injected intraperitoneally on days −1, 0, +1, +2, and every other day after MAA injection, for a total of eight doses (0.8 mg). The control panel received identical injections with 100 μg of insoluble MAA, isolated from bovine iris and ciliary body, in the hind footpad with a single dose. Animals were examined daily after 12 days postimmunization for clinical signs of acute anterior uveitis by slit-lamp biomicroscopy and graded using criteria previously published. Animals typically develop acute inflammation localized to the anterior uvea by day 14 to 18 postimmunization, which peaks around day 20 to 22, and spontaneously resolves by day 30.

**Animal Model of EAAU**

The animal model of EAAU in Lewis rats has been previously described. Briefly, Lewis rats 5 to 6 weeks of age were immunized with 100 μg of insoluble MAA, isolated from bovine iris and ciliary body, in the hind footpad with a single dose. Animals were examined daily after 12 days postimmunization for clinical signs of acute anterior uveitis by slit-lamp biomicroscopy and graded using criteria previously published. Animals typically develop acute inflammation localized to the anterior uvea by day 14 to 18 postimmunization, which peaks around day 20 to 22, and spontaneously resolves by day 30.

**Establishment and Maintenance of MAA-Specific T-Cell Lines**

The method described by Rozenszajn et al. was modified and used to generate MAA-specific T-cell line. Draining popliteal lymph node cells were collected at day 16 postimmunization with MAA. Single cell suspensions were placed into dishes and incubated for 4 to 6 hours to separate T cells from the adherent macrophages and B cells. The nonadherent cell population including lymphocytes were mixed at a concentration of 5 × 10⁶ cells/ml with 2.5 × 10⁶ cells/ml of naive syngeneic, irradiated (2500 rads) accessory spleen cells and 5 μg/ml of soluble MAA in RPMI-1640 (supplemented with antibiotics, 50 μM 2-ME, and 3% syngeneic rat serum). The cells were cultured in 24-well tissue culture plates for 4 days at 37°C, 5% CO₂ and then for 72 hours in 24-well dishes with 5% conditioned medium (CM). The CM containing IL-2 was obtained from the supernatants of Lewis rat spleen cells stimulated with Con A (5 μg/ml) in a culture medium containing complete RPMI-1640 and 10% inactivated human pooled serum. Cells (2 × 10⁶ cells/ml) were restimulated with MAA (5 μg/ml) in the presence of 5 × 10⁶ syngeneic, irradiated antigen presenting spleen cells for 4 days and underwent continued expansion in medium containing IL-2. T-cell lines were maintained with repetitive stimulation and expansion cycles. After three cycles of expansion, cells were used to assess antigen-specific proliferative responses.

**Proliferative Response of Cell Lines to Antigen**

T cells (2.5 × 10⁶ per well) were cultured with or without 5 μg/ml MAA in the presence of irradiated naive spleen cells (4 × 10⁹ per well) at 57°C for 72 hours. To test whether the inhibitory effect of CTLA4-Fc could be reversed by addition of IL-2, 5% or 10% of CM was added into the wells with CTLA4-Fc (10 μg/ml). [³H]Thymidine incorporation was assessed during the last 16 hours by a microplate scintillation counter (Packard, Meriden, CT). The proliferative response was expressed as mean cpm ± SD of the triplicate determination.

**ELISA**

MAA T cells (1 × 10⁹ per well) in 96-well plate were cultured with irradiated syngeneic naive spleen cells (2 × 10⁶ cells/well) in the presence of 10 μg/ml of CTLA4-Fc or mutant CTLA4-Fc. Culture supernatants were collected after 48 hours. TNF-α released in the supernatant was assayed with the R&D (Minneapolis, MN) ELISA kit according to the manufacturer’s instruction.

**Statistical Analysis**

Data were analyzed and compared using Student's t-test, and differences were considered statistically significant with P < 0.05.

**Results**

CTLA4-Fc on Incidence and Severity of EAAU

The role of B7 costimulation in EAAU in vivo was studied by observing the clinical effect of CTLA4-Fc treatment on the incidence and severity of the disease. Animals were injected with 100 μg CTLA4-Fc (experimental group) or CTLA4-Fc mutant (control group) intraperitoneally on days −1, 0, +1,
Adoptive Transfer of MAA-T Cells from Rats Treated with CTLA4-Fc

We next explored whether MAA-responsive T cells from CTLA4-Fc–treated animals were able to transfer intraocular inflammation. First, we investigated whether T cells capable of inducing EAAU were still present and functional in CTLA4-Fc–treated rats. Draining popliteal lymph node cells from CTLA4-Fc–treated rats were cultured in vitro for 3 days with MAA, treated with CTLA4-Fc. No inflammation was observed in the anterior chamber, iris, or ciliary body of recipient rats treated with CTLA4-Fc. In contrast, the anterior uveitis associated with EAAU was effectively prevented by treatment of the recipient rat with CTLA4-Fc during the induction of disease. This result suggested that the CD28-B7 interaction is required for the initiation of EAAU.

CTLA4-Fc on MAA-Specific T-Cell Proliferation

MAA-responsive T-cell lines were cultured in vitro with MAA and CTLA4-Fc to address whether blockade of the CD28-B7 interaction inhibited expansion of MAA-responsive T cells was the mechanism of inhibition of disease. The proliferative response of rat MAA-specific T cells was evaluated in vitro in the presence of 10 μg/ml of CTLA4-Fc (experimental group) or

| Table 1. Effects of CTLA4-Fc on the Clinical Course of EAAU |
|-----------------|-----------------|-----------------|-----------------|
| Treatment*     | Incidence       | Mild            | Severe          | Day of Onset†‡  |
| CTLA4-Fc mutant| 16/16           | 0              | 16             | 17 ± 1          |
| CTLA4-Fc        | 2/16            | 2              | 0              | 18 ± 0          |

* Treatment given on days −1, 0, 1, 2, 4, 6, 8, and 10 after induction with 100 μg insoluble MAA. The incidence of EAAU is given as the number of positive eyes/the number of total eyes, judged by slit-lamp biomicroscopy.
† Mean ± SD.

CTLA4-Fc on MAA-Specific T-Cell Proliferation

MMA-responsive T-cell lines were cultured in vitro with MAA and CTLA4-Fc to address whether blockade of the CD28-B7 interaction inhibited expansion of MAA-responsive T cells was the mechanism of inhibition of disease. The proliferative response of rat MAA-specific T cells was evaluated in vitro in the presence of 10 μg/ml of CTLA4-Fc (experimental group) or

| Table 2. EAAU in Naïve Rats Induced by Adoptive Transfer of Lymphocytes from Either Control (Mutant CTLA4-Fc) or CTLA4-Fc–treated Donors |
|-----------------|-----------------|-----------------|-----------------|
| Treatment*     | Incidence       | Mild            | Severe          | Day of Onset†‡  |
| Control treated donor cells | 16/16 | 0 | 16 | 7 ± 1  |
| CTLA4-Fc–treated donor cells | 0/18 | 0 | 0 | 0  |
| Mixture of cells | 10/10 | 0 | 10 | 5 ± 1  |

* Naïve Lewis rats received lymphocytes derived from control (mutant CTLA4-Fc)-treated or CTLA4-Fc–treated rats as described in Material and Methods. The incidence of EAAU is given as the number of positive eyes/the number of total eyes judged by slit-lamp biomicroscopy.
† Mean ± SD.
mutant CTLA4-Fc (control group). Representative results of the proliferative response, as determined by [3H]thymidine incorporation, are shown in Figure 3. The proliferative response of the MAA-specific T-cell line was significantly inhibited by CTLA4-Fc ($P < 0.001$) but not by mutant CTLA4-Fc. The inhibition of the proliferative response by CTLA4-Fc could be reversed by coculture with IL-2 in a dose-dependent fashion. These results suggest the effect of CTLA4-Fc in this model is due to inhibition of the expansion of MAA-T cells that cause EAAU.

**CTLA4-Fc on TNF-α Production of MAA-Specific T Cells**

TNF-α is an important proinflammatory cytokine in EAAU. To examine whether CD28-B7 blockade also inhibited the production of TNF-α, supernatants from T cells stimulated with MAA in the presence of CTLA4-Fc or its mutant were assessed for TNF-α by ELISA. As shown in Figure 4, CTLA4-Fc significantly reduced TNF-α secretion.
FIGURE 4. Effect of CTLA4-Fc on the TNF-α production of a rat MAA-specific T-cell line. T cells were plated in triplicate in 96-well plates with irradiated naive syngeneic spleen cells. They were cultured in the presence of medium, MAA, MAA + CTLA4-Fc, or CTLA4-Fc mutant for 2 days. TNF-α in the supernatants was determined by ELISA. Results are presented as the mean; error bars, SD.

DISCUSSION

T-cell costimulation caused by interaction between CD28 and B7 appears to be required for the development of many experimental autoimmune diseases. However, the requirement for the CD28-B7 interaction between MAA-responsive T cells and APCs in the development and pathogenesis of EAAU in the Lewis rat has not been studied. Previous work in other rat models of autoimmunity has demonstrated that CD28-B7 costimulatory blockade by CTLA4-Ig is effective in inhibiting disease. The systemic administration of CTLA4-Ig was effective in preventing and treating EAE induced in the Lewis rat. Administration of CTLA4-Ig from the time of immunization prevented the development of collagen type II–induced arthritis, whereas treatment during ongoing disease resulted in amelioration of arthritis. CTLA4-Ig treatment ameliorated experimental autoimmune glomerulonephritis in the WKY rat by reducing autoantibody production and cellular infiltration of glomeruli. Lately, Silver and colleagues demonstrated that blockade of costimulation through B7-CD28 inhibited experimental autoimmune uveoretinitis (EAAU) but did not reduce long-term tolerance. Similarly, in our experiments CTLA4-Fc administered during the priming of MAA-T cells prevented the induction of intraocular inflammation. Since CTLA4-Fc blocks the CD28 interaction with both B7-1 and B7-2, our observations suggest that a costimulatory signal delivered through the CD28-B7 interaction between T cells and APCs is necessary for the induction of EAAU.

Although previous studies demonstrated that CTLA4-Fc treatment in vivo predominantly produced immunosuppression, the mechanism by which CTLA4-Fc inhibits autoimmunity in different animal models remains to be determined. We observed in our adoptive transfer experiments that T cells from CTLA4-Fc-treated rats, even after in vitro stimulation with MAA, were not able to adoptively transfer disease in naive recipients. These results suggest that CTLA4-Fc treatment results in the nonresponsiveness of MAA-specific T cells. Additionally, adoptive transfer experiments were performed in which lymphoid cells from both control (CTLA4-Fc mutant) and CTLA4-Fc-treated rats when combined before injection. These experiments did not prevent the induction of EAAU. The time course and severity of disease were similar to that observed after the adoptive transfer of T cells from control-treated rats alone. Thus, it appears that blockade of the CD28-B7 interaction during the induction of EAAU did not result in the generation of antigen-specific regulatory (i.e., suppressor) T cells. Regulatory cells are now known to express CTLA-4 so their function may also be inhibited by CTLA4-Fc. The consequences of this for long-term antigen specific regulation within the patient remain to be further investigated.

In vitro experiments have demonstrated that antigen-reactive T cells are rendered anergic if they are exposed to the peptide-MHC complex on APCs which lack B7 molecules. The anergic T cells failed to expand and differentiate into functional T cells. In vivo experiments in autoimmune models of disease and solid organ transplantation also suggest that CTLA4-Fc can induce long-term unresponsiveness to autoantigens, as well as donor-specific tolerance, presumably through T-cell anergy. In our in vitro experiment CTLA4-Fc inhibited the MAA-responsive T-cell proliferation and this inhibition was reversed by the addition of IL-2. This result suggests that CD28-B7 blockade inhibits MAA-specific T-cell expansion by inhibition of IL-2 production. In addition, the expansion and effector functions of autoreactive T cells can be sufficiently controlled by short-term CD28 blockade, which probably explains why T cells from rats treated with CTLA4-Fc were unable to induce disease in naive rats by adoptive transfer. These conclusions are consistent with the observations recently reported in EAU where it was found that B7-CD28 blockade prevented generation of effector T cells.

CTLA4-Fc treatment does not always induce T-cell anergy in autoimmune models of disease. In murine EAE, for example, CTLA4-Fc treatment prevented induction of disease, but T cells from immunized CTLA4-Fc–treated animals transferred EAE to naive recipients. Administration of CTLA4-Fc at the time of immunization reduced production of IFN-γ and IL-2 (secreted by Th1 cells) in the central nervous system but not IL-4 and IL-10 (secreted by Th2 cells). These results indicated that B7 blockade by CTLA4-Fc may not just only inhibit a T-cell autoimmune response but also alter the dynamic balance of T-cell subsets and their secreted products. Previous studies in EAAU have shown that the production of TNF-α during the induction of disease. Administration of CTLA4-Fc significantly inhibited the in vitro production of TNF-α and may be a mechanism by which EAAU is inhibited.

Differences in the kinetics and the level of expression of B7-1 and B7-2 on APCs have been reported during the generation of an immune response. Consequently, the effect observed after blocking B7-1 or B7-2 in various models of autoimmune disease may differ. The expression pattern and kinetics of B7-1 and B7-2 on APCs within the eye, an immunologically privileged site, may very well differ from that of a conventional site. Furthermore, the cell type expressing B7-1 or B7-2 may be important in determining the type of immune response generated. Because CTLA4-Fc binds both B7-1 and B7-2 on the APC, our current data suggest an essential role for B7-CD28–mediated costimulation in the induction of uveitis. However, the precise role of costimulatory signals on APCs in the draining lymph node and the eye is currently being studied.

Blockade of CD28-B7 costimulation has the potential for inhibition of the T-cell–mediated autoimmune response in AAV in humans, regardless of the specific autoantigen(s) involved. Thus, blockade of B7-mediated costimulation by CTLA4-Fc may be a potential therapeutic approach in uveitis.
Acknowledgments

The authors are grateful to Berlinda McMahan for her histology expertise and Russ Hornbeck for his photographs.

References


