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Disruption of CD40/CD40-Ligand Interactions in a Retinal Autoimmunity Model Results in Protection without Tolerance

Lee M. Bagenstose,1,2* Rajeev K. Agarwal,2* Phyllis B. Silver,† David M. Harlan,†
Steven C. Hoffmann,‡ Robert L. Kampen,‡ Chi-Chao Chan,* and Rachel R. Caspi3*

We examined the role of CD40/CD40L interactions on the development of experimental autoimmune uveoretinitis (EAU), a cell-mediated, Th1-driven autoimmune disease that serves as a model for autoimmune uveitis in humans. EAU-susceptible B10.RII1 mice immunized with the retinal autoantigen interphotoreceptor retinoid binding protein in CFA and treated with anti-CD40L Ab (MR1) had reduced incidence and severity of disease. Real-time PCR analysis revealed that the innate and adaptive responses of protected mice were reduced, without an obvious shift toward a Th2 cytokine profile. In contrast to some other reports, no evidence was found for regulatory cells in adoptive transfer experiments. To determine whether CD40L blockade resulted in long-term tolerance, mice protected by treatment with MR1 Ab were rechallenged for uveitis after circulating MR1 Ab levels dropped below the detection limit of ELISA. MR1-treated mice developed severe EAU and strong cellular responses to interphotoreceptor retinoid binding protein, comparable to those of control mice. These responses were higher than in mice that had not received the primary immunization concurrently with anti-CD40L treatment. We conclude that 1) CD40/CD40-Ligand interaction is required for EAU and its disruption prevents disease development; 2) CD40L blockade inhibits the innate response to immunization and reduces priming, but does not result in immune deviation; and 3) protection is dependent on persistence of anti-CD40L Abs, and long-term tolerance is not induced. Furthermore, immunological memory develops under cover of CD40L blockade causing enhanced responses upon rechallenge. Taken together, our data suggest that ongoing CD40/CD40L blockade might be required to maintain a therapeutic effect against uveitis.

We demonstrate that these interactions are critical for EAU development. Although modulating CD40/CD40L interaction by anti-CD40L prevents EAU induction, we could find no evidence suggesting that regulatory cells were induced, nor that the treatment promoted robust long-lived tolerance. Rather, we found that continued circulating anti-CD40L Ab was required to protect against EAU in this restimulation model.

Materials and Methods

Mice

EAU-susceptible B10.RIII mice (H-2b) were purchased from The Jackson Laboratory. Mice were used between ages 8 and 12 wk. Treatment of animals was in compliance with Institutional Guidelines and all animal study protocols were approved by an Institutional Review Board.

Ags, Abs, and reagents

Native bovine IRBP was prepared as described (27–29). CFA was from Sigma-Aldrich, and RPMI 1640 medium was from BioWhittaker and was supplemented as described (30). MR1, a hamster anti-mouse CD40L mAb, was produced in ascites and purified by ion exchange HPLC as previously described (31, 32). Control hamster Ig was from Jackson ImmunoResearch Laboratories.

Real-time PCR analysis of cytokine expression

Inguinal lymph nodes were collected on day 4 after immunization for RNA purification. Tissue was snap frozen in liquid nitrogen within 1 min of collection and stored at −80°C until homogenized (Power Gen 35 homogenizer; Fisher Scientific) in 1 ml of TRIzol reagent (Invitrogen Life Technologies), incubated for 5 min on ice, and extracted with chloroform. Samples were then precipitated with isopropanol and washed with 70% ethanol. Total RNA (3 μg) was converted to cDNA with random hexamers and AMV reverse transcriptase (Roche Molecular Biochemicals) from commercially available kits according to manufacturer’s instruction. The expression of RNA transcripts for IL-4, IL-5, IL-12p40, TNF-α, IFN-γ; CD80, CD86, and CD152 were determined by real-time PCR as previously described (33). Control hamster Ig was used as a template for real-time PCR using commercially available gene primers and probes (Applied Biosystems). The reaction contained forward and reverse primers (900 nM each) and FAM-labeled probes (125 nM). In addition, forward and reverse primers for 18S ribosomal RNA (50 nM each of 2,7-dimethyl-6-fluorescein FAM-labeled probes) were used. Reactions were run on a Stratagene Mx3005P real-time PCR system and was reported as an n-fold difference of the experimental sample relative to a pool of B10.RIII nonimmunized naive inguinal lymph nodes.

EAU induction, treatment, and scoring

Mice were immunized s.c. in the thighs and base of the tail with 25 μg of native bovine IRBP (33). The Ag was emulsified in 0.2 ml of emulsion in CFA supplemented with Mycobacterium tuberculosis strain H37RA to 2.5 mg/ml. Mice were treated with 200 μg of either MR1 or hamster-Ig on days −1, 1, and 3. Clinical disease was evaluated by fundoscopy. Eyes were harvested for histopathology 21 days after immunization or 7–14 days after adoptive transfer. Disease was scored in a masked fashion by ophthalmitic pathologist C.C. Chan (National Eye Institute, Bethesda, MD), on a scale from 0 (no disease) to 4 (maximum disease) in half-point increments, according to a semiquantitative system previously described (33, 34).

Adoptive transfer of lymphocytes

Early transfer protocol. The early adoptive transfer experiments were modeled after the protocol of Homann et al. (35). Groups of donor and recipient B10.RIII mice were immunized with 25 μg of IRBP on day 0 as described earlier. Donor mice received treatment with 200 μg of MR1 or hamster-Ig on days −1, 1, and 3. Three of seven donor mice were sacrificed on day 3 and spleens were harvested. Equal numbers of CBA/CaJ mice were implanted, unmanipulated splenocytes (−50 × 10^6/mouse, one donor per two recipients) were infused by i.v. route into immunized recipients. Remaining donors (four per group) and recipients were fundoscopied for disease induction and were sacrificed 2 wk postinfusion for histopathological evaluation of EAU.

Late transfer protocol. Groups of B10.RIII donor mice were immunized with 25 μg of IRBP in CFA as described on day 0, and received treatment with 200 μg of either MR1 or hamster isotype control on days −1, 1, and 3. To confirm disease in donor mice, EAU was assessed by fundoscopy 16 days after immunization. Donor mice were sacrificed on day 21 postimmunization and spleen cells were isolated, washed in RPMI 1640, and transferred i.p. into naive recipient mice at −70 × 10^6 cells/mouse. Two days after adoptive transfer, recipient mice were immunized with 25 μg of IRBP in CFA. One week after the T cell transfer, disease was assessed by fundoscopy in these recipient mice, and the mice were then euthanized. Eyes collected from donor and recipient mice were fixed and sectioned for histopathological examination.

Rechallenge experiments

Two groups of six B10.RIII mice were treated with MR1 and two groups with hamster-Ig, respectively, as described earlier. One half of MR1-treated and hamster-Ig-treated mice were immunized (25 μg of IRBP in CFA) and were fundoscopied for disease development. To confirm that MR1 Abs had been cleared from circulation, ELISA was performed on sera samples using Ab pairs from BD Pharmingen. All MR1-treated mice (immunized and unimmunized) were bled on wk 3, 6, 12, and 17 to test for the presence of MR1 Abs. On wk 17, mice showed undetectable levels of MR1 Ab titers. All groups were given a second immunization (25 μg of IRBP in CFA), and mice were evaluated for development of EAU by fundoscopy and histopathology at the time of termination of the experiment. Delayed-type hypersensitivity (DTH) response to immunizing Ag was also recorded as described below.

Determination of immunological responses

DTH to IRBP was evaluated by the ear swelling assay (36). For Ag-specific lymphocyte proliferation and cytokine production in primary cultures, the spleen and draining lymph nodes (inguinal and iliac) of individual mice (4–5 per group) were collected 3 wk after immunization. Lymphoid cells from each mouse were plated individually or were pooled within the group, as indicated, and were incubated with graded doses of Ag in triplicate. 0.2-ml cultures, essentially as described (30). Proliferation was determined by [3H]thymidine uptake. Cytokines were quantitated in 48-h Ag-stimulated supernatants using the Pierce Multiplex SearchLight Arrays technology (www.SearchLightOnline.com) (37).

Reproducibility and statistical analyses

Statistical analysis of EAU scores was done using the Snedecor and Cochran 𝜉 test for linear trend in proportions (nonparametric, frequency-based) (38). Each mouse (average of both eyes) was treated as a statistical event. DTH, lymphocyte proliferation, and cytokine data were analyzed by independent 𝑡 tests. Values for 𝑝 < 0.05 were considered significant.

Results

Anti-CD40L mAb (MR1) inhibits EAU induction

To investigate whether interfering with the CD40/CD40L interaction would influence EAU induction, we injected IRBP into groups of five B10.RIII mice and treated some with a neutralizing mAb to CD40L (MR1). Control mice treated with hamster Ig and untreated control mice developed high EAU scores, typified by retinal detachment, vasculitis, retinitis, vitritis, and photoreceptor cell damage, whereas the MR1-treated group was completely protected from disease (Fig. 1a). Representative histopathology of MR1-treated and control mice is shown in Fig. 1, b and c, respectively.

Immunological responses in MR1-treated mice

We first checked the effect of blocking CD40/CD40L interaction on the innate response by analyzing the draining lymph nodes ex vivo without any in vitro culture or manipulation. This innate response is likely to be primarily elicited by the bacterial components in the adventit and creates the milieu in which the autopathogenic adaptive response is elicited. Inguinal lymph nodes from mice immunized with a uvenezitic protocol of IRBP in CFA, and treated with MR1, or with control hamster Ig, were...
collected on day 4 after immunization and were immediately snap-frozen. RNA was extracted and cytokine gene expression was analyzed by real-time PCR in comparison to baseline (lymph nodes of unimmunized mice) as described in Materials and Methods. The expression of each cytokine is shown as a fold increase over baseline. The analysis revealed that anti-CD40L treatment resulted in lower mRNA levels for most cytokines (Fig. 2) with the exception of IL-4 (whose absolute values were very low to begin with). In addition, we observed significantly lower mRNA levels for the costimulatory molecules CD80 (B7-1), CD86 (B7-2), CD152 (CTLA4), as well as CD40L (CD154) itself.

We next examined the adaptive immune responses of these mice 21 days after immunization. Mice injected with IRBP in their ear lobes 48 h earlier were assessed for a DTH response, and their lymphoid cells were collected for analysis of proliferation and Ag-specific cytokine production. Compared with B10.RIII mice receiving the hamster Ig control, MR1-treated mice displayed a significantly reduced DTH response to IRBP (Fig. 3a). Ag-specific proliferation to IRBP of cells taken from spleen or from lymph nodes draining the site of immunization was slightly (but statistically significantly) reduced (Fig. 3b), as were some, but not all,
IRBP-elicted cytokine responses. Production of the prototypic proinflammatory cytokines IFN-\(\gamma\), IL-6, and TNF-\(\alpha\) was reduced (Fig. 4) but titers of IL-5, IL-10, IL-13, and IL-12 p70 in these supernatants were essentially unchanged (data not shown). Notably, both IFN-\(\gamma\) and IL-4 type were reduced to a similar extent, suggesting that there was no shift in the Th1/Th2 balance. Interestingly, we found that splenic T cell proliferative and cytokine responses in protected mice were practically unchanged (data not shown).

Taken together, these results suggest that although interfering with CD40/CD40L interactions in IRBP-immunized B10.RIII mice effectively prevents EAU and measurably curtails a number of innate response parameters, a significant adaptive response to IRBP does develop despite the MR1 treatment.

**Anti-CD40L-induced protection from EAU does not involve regulatory cells**

To address the question whether anti-CD40L protection in EAU was mediated by regulatory cells, we adoptively transferred cells from IRBP-immunized and MR1-treated mice into naive recipients, then subsequently induced EAU using one of two techniques. We call these two the 1) late transfer and 2) early transfer protocols, as cells are transferred during the induction (afferent) or expression (effferent) stage of disease.

In the late transfer protocol, we transferred cells from mice confirmed to have been protected by MR1. Donor mice immunized with IRBP and treated either with MR1 or hamster isotype control were allowed to develop EAU (confirmed by fundoscopy on day 16 and by histopathology on day 21). As before, IRBP-immunized control mice developed significant disease, whereas MR1-treated mice were protected (Fig. 5a). Spleen cells were collected on day 21, and up to 70 \(\times\) 10\(^6\) splenocytes were directly transferred into naive syngeneic recipients. These mice were then challenged 2 days later with a standard uveitogenic regimen of IRBP. Recipients of cells from protected donors developed equivalent disease to recipients of hamster Ig-treated donors, although the donors themselves from MR1-treated donors developed disease equivalent to recipients of hamster Ig-treated donors, although the donors themselves from MR1-treated donors would have been protected had they been allowed to continue to the efferent phase of disease (Fig. 5b).

**FIGURE 5.** CD40 blockade does not induce regulatory cells. a, Late transfer protocol. Groups of donor B10.RIII mice were immunized with 25 \(\mu\)g of IRBP on day 0 and received Ab treatment with 200 \(\mu\)g of either anti-CD40L or a hamster isotype control on days −1, 1, and 3 of treatment. Donor mice were sacrificed at day 21 of disease and 70 \(\times\) 10\(^6\) splenocytes from these donors were directly transferred i.p. into naive B10.RIII recipients. Two days after adoptive transfer, recipient mice were immunized with a standard regimen of IRBP. Recipient mice were sacrificed after 1 wk of transfer. Eyes were harvested from donors on day 21 and from recipients 1 wk after adoptive transfer. The EAU score is the average of all the mice in the group. b, Early transfer protocol. Groups of hamster Ig-treated or anti-CD40L-treated B10.RIII donor mice were immunized with IRBP in parallel to untreated recipient mice. Five days after IRBP immunization, unsorted splenocytes from immunized/treated donor mice were transferred to immunized recipients at the same stage of disease development. In addition, control groups of B10.RIII mice that were immunized and treated with hamster Ig or anti-CD40L in parallel to donor groups were allowed to continue to day 21 to confirm induction of disease and protection by anti-CD40L treatment. The data are from a representative experiment. Statistically significant differences from the respective control are indicated.

Our early transfer protocol was modeled after Homann et al. (35), who reported successful transfer of suppression in a virally induced diabetes model. In brief, groups of hamster Ig-treated or anti-CD40L-treated donor mice were immunized with IRBP in parallel with recipient mice not pretreated with any Ab. Five days after IRBP immunization, total splenocytes from the donor mice were transferred to recipient mice (at the equivalent stage of disease development). As a control, some mice were immunized and treated with hamster Ig or anti-CD40L and were followed to day 21 to confirm that the donors would have responded to immunization and treatment as expected. As before, recipients of cells from MR1-treated donors developed disease equivalent to recipients of hamster Ig-treated donors, although the donors themselves would have been protected had they been allowed to continue to the efferent phase of disease (Fig. 5b).

**Anti-CD40L treatment protects from first episode of uveitis, but does not result in tolerance**

Although our data is not consistent with regulatory cells accounting for the observed protection, other investigators have reported that anti-CD40L treatment can induce immunological tolerance (39, 40). We therefore asked whether anti-CD40L could result in long-term protection against disease, as manifested by resistance to a second uveitogenic challenge (i.e., after anti-CD40L had been cleared from the serum). Groups of B10.RIII mice were treated...
with anti-CD40L, isotype control Ig, or left untreated. Some mice were immunized with IRBP, whereas others were unimmunized. We examined their eyes by fundoscopy to evaluate for disease, and blood was drawn weekly to assess MR1 Ab persistence. As before, anti-CD40L-treated B10.RIII mice immunized with IRBP were completely protected from EAU, whereas untreated and isotype control-treated mice developed significant disease (Fig. 6a). Serum hamster Ig levels became undetectable 17 wk after the initial immunization and treatment, whereupon all mice (the MR1-treated ones still negative for EAU) were rechallenged with a second uveitogenic IRBP immunization. Mice that had initially been protected by anti-CD40L mAb therapy developed severe disease equivalent to or higher than primary immunization controls (Fig. 6b). Further, DTH responses in the Ag rechallenged mice were even higher than in mice immunized only once (Fig. 6c). These data strongly suggest that tolerance was not induced by anti-CD40L; rather, that circulating anti-CD40L Abs are required for continued EAU protection.

**Discussion**

CD40/CD40L interactions are critical for a variety of immune functions including APC activation, Ab production, CD4+ and CD8+ T cell priming, effector cell maturation, and enhancement of inflammation and chemotraction (reviewed in Refs. 14 and 41). Interventions interfering with the CD40/CD40L interaction have been examined in a number of models, from transplantation, through autoimmune diabetes (spontaneous and lymphocytic CMV-induced), autoimmune gnomerulonephritis, experimental lupus erythematosus, through EAE, an induced disease model that shares similar mechanisms with EAU. Diverse mechanisms have been reported, even in the same model, including inhibition of autoreactive T effector priming via T cell-APC interference (42, 43), inhibition of Th1 effector function/expansion/migration (21, 44), immune deviation/cytokine blockade (45, 46), induction of anergy and/or deletion (47, 48), and elicitation of regulatory cells (35, 49).

The effect of this type of intervention in the EAU model, which represents human autoimmune uveitis, had not been previously examined. In the present study, disruption of CD40/CD40L interactions by administration of an anti-CD40L mAb completely protected against EAU and suppressed most related immunological responses. For instance, draining lymph node cellular responses ex vivo, 4 days after IRBP immunization (largely representing the innate response), revealed that anti-CD40L treatment, compared with control, reduced mRNA expression for the costimulatory molecules and essentially all measured cytokines and chemokines. Three weeks down the line, examination of the adaptive response that developed in this milieu revealed reduced DTH and cellular responses, but much less than we expect from the draining lymph node APC activation parameters. Our data do not address the question whether this finding is a result of reduced priming (as suggested by decreased draining lymph node APC activation parameters) or of Fc-dependent Ag-specific T cell depletion (50).

To further elucidate the mechanism of protection, we asked whether regulatory cells might be involved. We assessed whether EAU resistance could be transferred from protected donors to nontolerized recipients. The straightforward approach of transferring splenocytes from MR1-treated donors, challenged for EAU and were protected, revealed no evidence suggesting transferable regulatory cells. Because Homann et al. (35) demonstrated spleen-resident regulatory cells 5 days after Ag challenge in the lymphocytic CMV diabetes model, we performed a similar experiment in our model. Again using this approach, we found no evidence for transferable protection, speaking against a mechanism involving regulatory cells in the EAU model.

Our findings are mostly in line with those of Howard et al. (43, 51) in their EAE model. They found disease inhibition, and a reduced number of effector cells (but no effector cell phenotype changes) after CD40 blockade, and like our results, they found no evidence for regulatory cells. Our results are also reminiscent of findings in autoimmune myasthenia gravis reported by Im et al. (52), as they also found no regulatory cells, although they did find a diminished Th1 response. In contrast, Samoilova et al. (45) found evidence of Th2 skewing following anti-CD40L treatment to prevent EAE.
We next asked whether the observed protection from disease in fact reflected immunological tolerance as other studies have concluded following prolonged periods of disease-free survival (53). We reasoned that the initial Ag depot may largely dissipate over a period of weeks or months, reducing the antigenic stimulus. Therefore, we elected to use a more stringent definition, and rechallenged the protected mice with a second uveitogenic immunization, after the treatment Abs had cleared. We found that treated mice had measurable circulating hamster IgG for up to 17 wk. Mice challenged after the treatment Ab was cleared, with a second IRBP immunization, not only developed full-blown disease but in fact showed evidence of enhanced EAU scores and immunological responses compared with parallel mice receiving their first immunization. Therefore, not only had anti-CD40L not induced tolerance, it permitted immunological memory to be induced. Notably, mice that were rechallenged after 7 wk, before MR1 Abs had been fully cleared from the circulation, were protected (our unpublished observations). Taken together, these results indicate that continued protection in this model is dependent on persistent anti-CD40L levels. This interpretation is also supported by the readily detectable IRBP-induced cellular proliferative and cytokine responses ex vivo 3 wk after mice were immunized, despite the fact that the animals were essentially disease free.

Our results are reminiscent of previous observations in which we prevented EAU by interfering with B7-CD28 interactions. As with the CD40/CD40L interaction, although disease was prevented, we found no protection from rechallenge and even acquisition of immunological memory to IRBP despite the treatment (10). A characteristic feature of the EAU model, in contrast to models of transplantation and diabetes, is strong stimulation of innate immunity due to use of CFA. We propose that innate stimuli, eliciting strong immunological “danger” signals, can counteract the tolerogenic effect of costimulatory blockade, prevent acquisition of tolerance, and permit priming and acquisition of immunological memory. This should be taken into account when considering use of agents that modulate costimulatory signaling in situations in which innate immune elements are likely to be engaged. Finally, our data indicate that efficacy of this therapeutic paradigm would depend on repeated treatments to maintain continued blockade of CD40/CD40L interaction.

Disclosures

The authors have no financial conflict of interest.

References


