Blockade of the Interaction of Leukotriene B₄ with Its Receptor Prevents Development of Autoimmune Uveitis

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PURPOSE. To investigate the role of leukotriene B₄ (LTB₄) and its receptor BLT1 in the pathogenesis of mouse uveitis.

METHODS. Experimental autoimmune uveitis (EAU) was induced in B10RIII mice by immunization of interphotoreceptor retinoid binding protein (IRBP; peptide sequence 161-180) or in C57BL/6 (B6) mice by transfer of activated T cells specific for IRBP1-20. The animals were then treated with and without the BLT1 receptor antagonist, CP105696, at the disease onset after immunization or at day 0 or day 6 after T-cell transfer. EAU was also induced in wild-type B6 (WT) and BLT1-deficient (BLT1⁻/⁻) mice by reciprocal transfer of the T cells from B6 to BLT1-deficient mice and vice versa. Clinical signs of inflammation and ocular histology were compared. The chemotactic activity of LTB₄ on naïve and IRBP-specific autoreactive T cells as well as effector leukocytes was examined.

RESULTS. The treatment of CP105696, greatly reduced the intensity of ongoing disease. IRBP1-20-specific T cells derived from wild-type B6 mice induced only mild uveitis in syngeneic BLT1-deficient mice and that IRBP1-20-specific T cells derived from BLT1⁻/⁻ mice induced milder disease in wild-type B6 mice than those derived from wild-type B6 mice, suggesting that expression of the LTB4 receptor on both activated autoreactive T cells and effector leukocytes was necessary for ocular inflammation to occur. Consistent with these data, transfer of autoreactive T cells from B6 mice to 5-lipoxygenase-deficient (5-LO⁻/⁻) mice, which have a functional defect in LTB₄ expression, also failed to induce uveitis in the recipient mice.

CONCLUSIONS. The results demonstrate a critical role for LTB₄ in ocular inflammation and in the development and progression of EAU and suggest a new potential target for therapeutic intervention in this disease. (Invest Ophthalmol Vis Sci. 2006; 47:1543–1549) DOI:10.1167/iovs.05-1238

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Uveitis is a common cause of human visual disability and blindness. Experimental autoimmune uveitis (EAU) can be elicited in rodents either by immunization with ocular antigens (Ags), such as retinal soluble-Ag (S-Ag),1,2 interphotoreceptor retinal-binding protein (IRBP),3,4 melanin-associated Ag,5,6 or myelin proteins,7–9 and peptides derived from these or by the adoptive transfer of uveitogenic T cells to syngeneic rodents,10–13 suggesting that uveitis is a T-cell-mediated organ-specific autoimmune disease. Animal models have been widely used to dissect the immunopathological mechanisms in uveitis and to develop preventive or therapeutic strategies.

Among the pathogenic events leading to development of uveitis, the trafficking and homing to the eye of specific uveitogenic T cells and the subsequent massive recruitment of inflammatory cells are crucial. Based on the observation that only a limited number of uveitogenic T cells are found in the inflamed eye and that nearly all the cells infiltrating the inflamed eye are polymorphonuclear leukocytes (PMNs) and macrophages, especially during the early phase of disease,14–20 we and others21,22 have hypothesized that the infiltrated autoreactive T cells interact with parenchymal cells of the eye and the reactivated autoreactive T cells release cytokines and chemokines, leading to the recruitment of large numbers of inflammatory leukocytes, which then amplify the process by contributing their own products, thus fueling an escalating inflammatory cascade, resulting in tissue damage and even visual loss. Although the molecular mechanisms that control the recruitment of these specific autoreactive T cells and infiltrated leukocytes are largely unknown, chemokine production and the interaction between chemokines and chemokine receptors are believed to be key events in inflammation. Leukotriene B₄ (LTB₄), a potent lipid inflammatory mediator rapidly generated at sites of inflammation, is derived from membrane phospholipids by the sequential actions of cytosolic phospholipase A₂ (PLA₂), 5-lipoxygenase (5-LO), and LTA₄ hydrolase.23 LTB₄ is a classic chemoattractant that triggers adherence and aggregation of leukocytes to the endothelium at nanomolar concentrations and recruits granulocytes and macrophages to the inflammation site. Recent studies have shown that LTB₄ is also a chemoattractant for T cells,24–26 creating a functional link between early innate and late adaptive immune responses to inflammation. Two LTB₄ receptors, BLT1 and BLT2, have been identified and characterized at the molecular level.27,28 Both are G-protein-coupled seven-membrane-domain receptors, the genes for which are located in very close proximity in the human and mouse genomes. The receptors differ in their affinity and specificity for LTB₄ and in their pattern of expressions. BLT1 is a high-affinity receptor specific for LTB4 and is expressed primarily in leukocytes, whereas BLT2 is a low-affinity receptor that also binds to other eicosanoids and is expressed ubiquitously.

In this study, we determined the effects of the specific BLT1 antagonist, CP105696,30,31 on the effector phase of uveitis induced by IRBP immunization and transfer of antigen-specific uveitogenic T cells. Using BLT1- and 5-LO-deficient mice and adoptive transfer of autoreactive T-cells, we demonstrated a dual role for LTB₄ in uveitis in facilitating the recruitment of
not only inflammatory leukocytes, but also autoreactive T cells, into the eye, thus playing an important role in the pathogenesis of autoimmune uveitis.

**MATERIALS AND METHODS**

**Mice**

Female BLT1-deficient mice on the B6 background were obtained from Bodduluri Haribabu (University of Louisville, KY), while wild-type C57BL/6, 5-LO-deficient mice on the H-2b background (B6.129S2-Alox5tm1Fun/J) and B10RIII (H-2r) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were used between 8 and 12 weeks of age. The animals were housed and maintained at the animal facilities of the University of Louisville. All animal studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Institutional approval was obtained and institutional guidelines regarding animal experimentation followed.

**Reagents**

The IRBP1-20 peptide consisting of residues 1-20 of human IRBP, (GPTHLFQSVLDMAKVLLD) and IRBP161-180 (SGIPYIISYLHPGNTILHVD), were synthesized by Sigma-Aldrich (St. Louis, MO). LTβ4 was purchased from Cayman Chemical (Ann Arbor, MI) and MO). LTB4 was purchased from Cayman Chemical (Ann Arbor, MI) and CCL21 from R&D (Minneapolis, MN). CP105696, the LTβ4 receptor antagonist, was kindly provided by Pfizer.

**Actively Induced and Adoptively Transferred Uveitis**

For active induction of disease in B10RIII mice, animals were immunized subcutaneously with 100 µL of an emulsion containing 50 µg of human 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, 0.3 µg of pertussis toxin was injected intraperitoneally (IP).

For adoptive transfer in B6 mice, recipient animals were injected IP with 0.2 mL of phosphate-buffered saline (PBS) containing 5 × 10^6 IRBP1-20-specific T cells, prepared as described previously. Clinical course of disease was assessed by funduscopcopy twice a week and graded as previously described. The pathology was confirmed by histology.

**CP105696 Treatment**

For treatment of adoptively transferred uveitis, mice were fed orally daily with 10 or 20 mg/kg of CP105696 dissolved in 0.5 mL of 0.5% methyl cellulose for 15 days beginning on day 0 or 6 after T cell injection. For treatment of actively induced uveitis, mice were treated identically, but treatment was started at disease onset (i.e., when clinical signs first appeared in the eye of any of the immunized mice). Control mice received the vehicle (0.5 mL of 0.5% methyl cellulose). For some experiments, CP105696 was added at different concentrations (from 100–1000 nM) to both wells of the chemotaxis chambers. Cells or without 10 nM of LTB4 or CCL21 was added to the lower wells. In some experiments, CP105696 was added at different concentrations (from 100–1000 nM) to both wells of the chemotaxis chambers. Cells that had migrated to the lower wells after 2 hours were collected, counted, and stained with antibodies against CD4, CD8, or Gr-1. The chemotactic index was calculated as the ratio of the number of migrating cells in chemotactic attractant-containing wells divided by the number of cells that migrated to medium alone. The percentage of the input cells that was recovered from the lower well was also calculated.

**Effect of Treatment with the BLT1 Antagonist CP105696**

We first determined whether treatment with the BLT1 agonist, CP105696, was able to suppress already established uveitis. B10RIII mice were immunized with a known uveitogenic peptide, IRBP161-180, and disease closely monitored by funduscoppy. The immunized mice were randomly divided into three groups (n = 6) and were fed with placebo or CP105696 at doses of 10 or 20 mg/kg starting when the first immunized mouse showed signs of inflammation in the eye. As shown in Figure 1A, disease severity in the treated mice was significantly reduced in a dose-dependent fashion.
Effect of CP105696 on the Severity of Transferred Uveitis

The inhibitory effects of CP105696 on the effector phase of uveitis was further examined in the adoptive transfer model of uveitis. In these studies, we transferred activated IRBP1-20-specific T cells from in vivo primed B6 mice to naïve syngeneic recipients, because the subsequent experiments were to be performed in BTL1-deficient mice with the B6 background. Groups (n = 6) of B6 mice injected IP with a pathogenic dose (5 × 10⁶) of IRBP1-20-specific T cells were fed placebo or 10-20 mg/kg per day of CP105696 or placebo for 15 days starting on the day of transfer (B) or at day 6 after transfer (C) (six mice per group). The EAU score is the mean ± SD for the group. Two separate experiments were performed with similar results.

Figure 1. Effect of CP105696 on the Severity of Transferred Uveitis

The inhibitory effects of CP105696 on the effector phase of uveitis was further examined in the adoptive transfer model of uveitis. In these studies, we transferred activated IRBP1-20-specific T cells from in vivo primed B6 mice to naïve syngeneic recipients, because the subsequent experiments were to be performed in BTL1-deficient mice with the B6 background. Groups (n = 6) of B6 mice injected IP with a pathogenic dose (5 × 10⁶) of IRBP1-20-specific T cells were fed placebo or 10-20 mg/kg per day of CP105696 from the time of T-cell transfer for 15 consecutive days. Disease severity was then monitored by funduscopy, and randomly selected animals from each group were subjected to pathologic diagnosis. As shown in Figure 1, disease severity in the CP105696-fed recipient mice was dramatically reduced during the 15-day treatment period. Of note, delayed treatment with CP105696, starting on day 6 after T-cell transfer and lasting 15 days, was equally effective (Fig. 1C). It should be noted that the clinical score gradually increased once treatment was stopped in the mice in which treatment started on the same day as T-cell transfer (Fig. 1B, day 0), but remained low after treatment was discontinued in the group in which treatment was delayed (Fig. 1C). These results indicate that blockade of the interaction of LTB4 with its receptor may prevent the trafficking of activated autoreactive T cells into the eye.

Histologic examination of the eyes at day 21 after T-cell transfer showed that recipient mice treated with placebo displayed damage in the photoreceptor layer, retinal detachment, and massive infiltration in the vitreous and retina (Fig. 2A), whereas mice treated with CP105696 showed a well-preserved retinal structure, with no, or only minimal, vitreous infiltrate (Fig. 2B). Infiltrating cells in the untreated inflamed eyes were mostly Gr-1-positive neutrophils (in R1 gate), T cells (in R2 gate), and a few NK cells, as analyzed by flow cytometry (Figs. 2C–F).
Susceptibility of BLT1−/− Mice to Transferred Disease

Next, we examined whether mice lacking BLT1 have altered disease susceptibility. The results showed that, whereas IRBP1-20-specific T cells from wild-type B6 mice transferred to wild-type B6 recipients induced severe and chronic clinical disease, the same T cells induced only very mild and transient clinical disease on transfer to BLT1−/− recipients (Fig. 3). We also examined whether IRBP1-20-specific T cells from immunized BLT1-deficient mice had a different disease-inducing ability to those from immunized wild-type B6 mice. As shown in Figure 3, IRBP1-20-specific T cells from BLT1−/− mice induced moderate uveitis in wild-type B6 recipients, both the incidence and severity of induced disease being lower than when IRBP1-20-specific T cells from immunized B6 were transferred to the same recipients. In addition, when IRBP1-20-specific T cells from immunized BLT1-deficient mice were adoptively transferred to BLT1-deficient recipients, no disease occurred. Similar results were obtained after transfer of T cells from immunized wild-type B6 mice to 5-LO-deficient mice, in which LTβ4 production is impaired.32

To exclude the possibility that the milder disease induced in B6 mouse by IRBP-specific T cells of BLT1−/− mice is caused by the degree of decreased activation of IRBP-specific T cells, we compared the proliferative response and IFN-γ production of IRBP-specific T cells between immunized B6 and BLT1−/− mice. Thus, the in vivo primed, IRBP-specific T cells were collected 10 days after immunization, enriched by passage through a nylon wool column, and exposed to graded doses of IRBP1-20 in a 96-well plate in the presence of syngeneic APCs. IRBP-specific T cells from BLT1−/− mice showed an intensity of proliferative response and IFN-γ production comparable to those in B6 mouse (Figs. 4A, 4B). In addition, autoantigen-specific T cells taken from BLT1−/− mice receiving transfers of IRBP1-20 T cells were capable of proliferation on re-exposure to IRBP1-20 (Fig. 4C).

Effect of LTβ4 on Activated IRBP-Specific and Naive T Cells

To determine the mechanism by which blockade of the interaction between the chemokine and its receptor interrupts the progress of inflammation and thus the development of autoimmune uveitis, we examined the chemoattractive effect of LTβ4 on autoimmune uveitogenic T cells, by using an in vitro chemotactic assay. In this experiment, the indicator cells were IRBP-specific T cells prepared from B6 mouse immunized 13 days earlier with IRBP-20 and stimulated for 2 days in vitro with immunizing peptide. As shown in Figure 5, LTβ4 induced chemotaxis of activated IRBP1-20-specific T cells, but not that of naive T cells, whereas the control chemokine CCL21 attracted IRBP-specific and naive T cells equally. In the presence of 10 nM LTβ4, the migration of IRBP1-20-specific T cells was approximately four times greater than in the absence of LTβ4. LTβ4 and CCL21 (both at 10 nM) were equally effective at attracting IRBP-specific T cells.

To confirm that the chemotactic activity of LTβ4 on autoimmune uveitogenic T cells is mediated by BLT1, we examined the migration of uveitogenic T cells derived from BLT1-deficient mice. As shown in Figure 5C, LTβ4 was not a chemoattractant for these cells, whereas CCL21 was.

The difference in the chemotactic effects of LTβ4 and CCL21 was analyzed further by testing the migration of draining lymph node cells and splenocytes from IRBP1-20-immunized B6 mice to a lower chamber containing 10 nM LTβ4 or CCL21. As shown in Figure 6A, before migration, the lymphocyte (gate R1)-to-PMN (gate R2) ratio was 0.73. After migration in the presence of LTβ4, most of migrated cells were granulocytes and the lymphocyte/PMN ratio was 0.28, whereas, in the presence of CCL21, most of the attracted cells were lymphocytes (R1/R2 ratio of 5.7). The results of the comparison of the chemoattractive effects of LTβ4 and CCL21 on CD4+, CD8+, NK, B, and Gr1+ cells are summarized in Figure 6B.

The results of blocking tests using CP105696 are shown in Figure 6C. CP105696 inhibited the LTβ4-induced migration of IRBP1-20-specific T cells in a dose-dependent manner, but had no effect on CCL21-induced migration. At a CP105696 concentration of 100 nM, migration of IRBP-specific T cells was inhibited by approximately 80% (Fig. 6C), whereas proliferation of the same cells was not affected (Fig. 6D).

**FIGURE 3.** Adoptive transfer-induced uveitis is suppressed in the absence of BLT1. (A) Stimulated T cells from IRBP1-20 immunized wild-type B6 or BLT1−/− mice were transferred to wild-type B6, BLT1−/−, or 5-LO−/− recipients as indicated, and disease was observed twice a week by funduscope. The data shown are the mean ± SE of the EAU scores at different times after adoptive transfer for two independent experiments (n = 5 each experiment). (B) Summarization of EAU induction by adoptive transfer of IRBP1-20 T cells derived from WT or BLT1−/− mice in BLT1−/− or 5-LO−/− mice.
Among the pathogenic events in uveitis, the migration of activated uveitogenic T cells to the eye and the subsequent recruitment of nonlymphoid inflammatory cells play a major role. LTB4 was initially identified as a metabolite of arachidonic acid produced by neutrophils and was found to be a potent chemoattractant for myeloid cells. The LTB4 receptor, BLT1, has been found to be expressed not only on neutrophils, macrophages, and eosinophils, but also on effector T cells.24,37,38 The early recruitment of effective T cells to the airway is impaired in the BLT1-deficient asthma model25,30 and inhibition of the LTB4 and BLT1 interaction suppresses eosinophil infiltration in a murine model of experimental allergic encephalomyelitis.36

We first examined whether LTB4 and BLT1 were involved in the pathogenesis of autoimmune uveitis by using the BLT1 antagonist CP105696. Our results showed that treatment of CP105696 suppressed the effector phase of disease in two disease models, either actively induced by Ag immunization or by adoptive transfer with activated autoreactive T cells. Most previous treatments protect against acute monophasic disease when administered before disease onset, but are less effective when disease has already become apparent53,59,40 whereas the major goal of clinical treatment is to impede disease progression, rather than prevent disease. It is therefore important to note that the therapeutic effect of the inhibition of binding of LTB4 to its receptor was significant on the progression of established uveitis. Furthermore, BLT1−/− mice were also much less susceptible to disease induction. Our results show that LTB4 and BLT1 are important chemoattractant molecules in ocular inflammation and the development of uveitis.

Using reciprocal transfer between wild-type B6 and BLT1−/− mice, we were able to show that BLT1 expression on both the autoreactive uveitogenic T cells and the bystander infiltrating cells of the inflammation was essential for pathogenesis. Thus, whereas IRBP1-20-specific T cells from immunized BLT1-deficient mice were less pathogenic than the corresponding T cells from wild-type mice on transfer to wild-type mice, BLT1-lacking recipient mice were less susceptible than wild-type mice to disease induction by transfer of IRBP1-20-specific T cells from immunized wild-type mice. It appears that blockade of the interaction between LTB4 and its receptor impedes disease development by preventing not only the entry of uveitogenic T cells into the eye, but also the further recruitment of inflammatory cells. This idea is supported by the in vitro experiments (Figs. 5, 6A, 6B) showing that LTB4 attracts
before mediators

A

B

C

D

LTB4 and CCL21 were summarized. (cell suspension and cells migrated into lower chambers containing

Greatly affected. However, when IRBP1-20 T cells derived from

Weaker disease, as the migration of the recipient’s cells is

Results are representative of those for four experiments.

Purified T cells were incubated with IRBP1-20 in the presence of

Chamber were counted after 2 hours.

Cytogram and the gate for the migrating cells. Lymphocytes (gate R1) and monocytes and PMNs (gate R2) are shown in the density plot. The numbers indicate the percentage of events in each gate. (B) Percentage of CD4⁺, CD8⁺, NK, B, and GR1⁺ cells in gates R1 and R2 of the original cell suspension and cells migrated into lower chambers containing LTB4 and CCL21 were summarized. (C, D) CP105696 specifically inhibits chemotaxis of uveitogenic T cells in response to LTB4. CP105696 at the indicated concentration was added to both the top wells containing blast uveitogenic T cells and the bottom wells containing LTB4 or CCL21, and the cells that migrated into the lower chamber were counted after 2 hours (C). In the proliferation assay, purified T cells were incubated with IRBP-20 in the presence of irradiated APCs and CP105696 at the indicated concentration (D). The results are representative of those for four experiments.

not only PMNs and macrophages, but also activated IRBP-specific T cells, even though it has a much lower effect on naïve T cells than does CCL21. CCL21 (a ligand for CCR7) attracted both resting and activated T cells. The differences of

Expression comparable to that in wild-type mice when the same immunization schedule is used (Figs. 4A, 4B). Furthermore, autoantigen-specific T cells taken from BLT1⁻/⁻ mice receiving transfers are capable of proliferation on re-exposure to autoantigens (Fig. 4C), indicating lymphoid function is unimpaired in these mice.

Blockade of the interaction between leukotrienes and their receptors is likely to be a successful new therapeutic strategy for some autoimmune diseases, including uveitis. As more aspects of the role of leukotrienes in the pathogenesis of autoimmune diseases are elucidated and the receptors better characterized, better strategies to target these molecules therapeutically can be expected.

References


