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Therapeutic effect of CTLA4-Ig on a murine model of primary biliary cirrhosis

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Abstract

Collectively, the data in both humans and murine models of human primary biliary cirrhosis (PBC) suggest that activated T cells, particularly CD8 T cells, play a critical role in biliary cell destruction. Under physiological conditions, T cell activation involves two critical signals that involve the MHC and a set of co-stimulatory molecules which include a receptor on T cells coined cytotoxic T lymphocyte antigen 4 (CTLA-4). Germane to the studies reported herein, signaling via CTLA-4 has the potential to modulate co-stimulation and induce inhibitory signals. In this study we have taken advantage of our well-defined murine model of PBC in which mice are immunized with 2-octynoic acid coupled to BSA, leading to the production of high titer anti-mitochondrial autoantibodies and portal cellular infiltrates. To investigate the potential of CTLA-4 Ig as an immunotherapeutic agent, we treated mice both before and after induction of autoimmune cholangitis. Firstly, we demonstrate that CTLA-4 Ig treatment begun one day before 2-OA-BSA immunization, completely inhibits the manifestations of cholangitis, including AMA production, intra-hepatic T cell infiltrates and bile duct damage. However, and more critically, treatment with CTLA-4 Ig initiated after the development of autoimmune cholangitis in previously immunized mice, also resulted in significant therapeutic benefit, including reduced intra-hepatic T cell infiltrates and biliary cell damage, although AMA levels were not altered. These data suggest that
an optimized regimen with CTLA-4 Ig has the potential to serve as an investigative therapeutic tool in patients with PBC.

Keywords
Primary biliary cirrhosis; cytotoxic T lymphocyte antigen 4; Abatacept autoimmunity; cholangitis

Introduction

Primary biliary cirrhosis (PBC) is a chronic autoimmune liver disease characterized by non-suppurative destructive cholangitis and cholestasis, as well as progressive development of fibrosis, cirrhosis, leading eventually to liver failure (1). Previous studies have suggested a critical involvement of autoreactive T cells in the pathogenesis of human PBC (2-4). Using various animal models, we have demonstrated that CD8 T cells play a critical role in the pathogenesis of PBC (5-7). Therefore we submit that successful therapy of PBC utilizing immunological approaches requires control of T cell activation and proliferation, their recruitment to the liver and the secretion of pro-inflammatory cytokines by these cellular infiltrates (8-12).

Under physiological conditions, T cell activation involves two critical signals, one being the presentation of a peptide epitope in the context of class I or class II MHC by an antigen-presenting cell (APC) to the T cell receptor (13-15). The second signal is delivered by the co-stimulators CD80/CD86 on the APC to their receptor CD28 on the T cell (16, 17) which is required to sustain the APC-T cell formation of an immunological synapse leading to significant enhancement of T cell activation. In contrast, cytotoxic T lymphocyte antigen 4 (CTLA4), another receptor expressed by T cells is associated with inhibitory properties (18, 19). CTLA4 binds to CD80/CD86 with a much higher affinity than CD28 (20), and functions to attenuate T cell activation by inhibiting co-stimulation and transmitting inhibitory signals to T cells. This results in decreased cytokine production, inhibition of cell cycle progression, down-modulation of TCR signaling, and decreased activation of B cells and macrophages (21-26).

These properties of CTLA-4 prompted us to explore the potential of CTLA4-based therapy for PBC utilizing our murine model in which mice immunized with 2-octynoic acid conjugated BSA (2OA-BSA) develop a PBC-like cholangitis (7, 27, 28). CTLA4-Ig is a soluble recombinant human fusion protein comprised of the extracellular domain of human CTLA4 linked to a modified portion of the Fc domain of human IgG-1 in which the sequences involved in FcR binding and complement activation have been eliminated preventing ADCC and complement mediated activity (Abatacept, Bristol-Myers Squibb, Clinton, NY)(29-31). CTLA4-Ig reversibly binds to both human and murine CD80/86 via its CTLA4 portion, thereby preventing CD80/86 from interacting with CD28 and thereby inhibiting the delivery of the second signal required for full T-cell activation. We herein demonstrate that CTLA4-Ig treatment started one day before 2OA-BSA immunization prevents the clinical and histological manifestations of cholangitis including the inhibition of serum anti-mitochondrial antibody (AMA), intrahepatic inflammatory change and bile duct damage. Of importance is our finding that CTLA4-Ig treatment initiated after the onset of established disease in previously 2OA-BSA-immunized mice led to a significant reduction in levels of intrahepatic infiltrating pathogenic effector CD4 and CD8 T cells as well as the severity of biliary cell damage. Our data suggests that an optimized regimen with CTLA4-Ig commercially known as “Abatacept” is a potential therapeutic candidate for patients with PBC.
Materials and Methods

Mice

Female C57BL/6J (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in ventilated cages under specific pathogen-free conditions at the animal facilities of the University of California at Davis. All studies conducted with the use of these animals were approved by the Animal Care and Use Committee at the University of California at Davis.

Induction of cholangitis

Two-octynoic acid (2OA, a synthetic chemical mimic of lipoic acid-lysine located within the inner domain of PDC-E2) was coupled to bovine serum albumin (2OA-BSA) as previously described (7). For the induction of autoimmune cholangitis, 100 μg 2OA-BSA conjugate (in 50 μl PBS) were emulsified with 50 μl of Complete Freund's Adjuvant (CFA; containing 1mg/mL of Mycobacterium tuberculosis strain H37RA, Sigma-Aldrich, St. Louis, MO) and injected intraperitoneally (I.P.) into 6 week-old female B6 mice. Additionally, mice received 100 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) in 100 μl PBS by I.P. at the time of and two days after the initial immunization with 2OA-BSA. After 2 weeks, the mice were re-boosted with 100 μg 2OA-BSA in 50 μl PBS emulsified with 50 μl of Incomplete Freund's Adjuvant (IFA; Sigma-Aldrich) administered intraperitoneally.

CTLA4-Ig treatment

Abatacept (BMS-188667, CTLA4-Ig), utilized for the studies reported herein, was a gift from Bristol Meyers-Squibb (New York, NY). Each vial of CTLA4-Ig (250 mg/vial) for injection was reconstituted with 10 ml of sterile water to yield a concentration of 25 mg/ml, and further diluted with PBS to 4 mg/ml. Mice were injected I.P. 3 times a week at a dosage of 20 mg/kg body weight. Preventative treatment was started one day before the first immunization with 2OA-BSA and continued 3× weekly for 8 weeks. Therapeutic treatment was started 8 weeks after the first immunization with 2OA-BSA and continued 3× weekly for 4 weeks.

Detection of anti-PDC-E2 antibodies

Serum was collected every two weeks after 2OA-immunization and stored at −80°C. Levels of anti-PDC-E2 autoantibodies in serum were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (7, 32), using purified recombinant PDC-E2 to coat the ELISA plates. The serum samples were tested at a dilution of 1:250.

Cell Preparation and Flow Cytometry Analysis

Livers and spleens were harvested immediately following sacrifice. Livers were first perfused with PBS containing 0.2% BSA (0.2% BSA/PBS), passed through a 100-μm nylon cell strainer (BD Bioscience) and re-suspended in 0.2% BSA/PBS. Hepatocytes were removed as pellets after centrifugation at 75 g for 1 min and the remaining suspended cells were collected. Spleens were disrupted between two glass slides and suspended in 0.2% BSA/PBS. Mononuclear cells (MNCs) from the liver and spleen were isolated by gradient centrifugation using Histopaque-1.077 (Sigma-Aldrich, St Louis, MO, USA). For flow cytometry, cells were first incubated with mAb against Fc receptor for blocking non-specific binding (BioLegend, San Diego, CA, USA) for 10 min at 4°C and then stained with combinations of fluorochrome-conjugated antibodies, including fluorescein isothiocyanate (FITC)-conjugated anti-CD44 (BioLegend), phycoerythrin (PE)-conjugated anti-CD62L (BioLegend), allopheocyanin (APC)-conjugated anti-T cell receptor (TCR)-β (eBioscience, San Diego, CA, USA), PerCP-conjugated anti-CD8α (Biolegend) and APC-Cy7-conjugated...
anti-CD4 (BioLegend) for phenotypic analysis of T cell subsets. Multicolor flow cytometric analyses were performed using a FACScan flow cytometer (BD Biosciences) upgraded by Cytec Development (Fremont, CA, USA) to allow for five-color analysis. Acquired data were analyzed with CellQuest software (BD Biosciences).

Histopathologic staining

Livers and spleens were harvested immediately after sacrifice and aliquots fixed in 10% buffered formalin at room temperature for 2 days, embedded in paraffin, and cut into 4 μm sections for routine hematoxylin (DakoCytomation, Carpinteria, CA) and eosin (American Master Tech Scientific, Lodi, CA) (H&E) staining. Standard pathologic evaluation utilizing light microscopy was performed and the relative levels of biliary cell damage were recorded on coded H&E-stained sections. Each section was scored as either 0 = no pathologic change, 1 = minimal, 2 = mild, 3 = moderate, or 4 = severe pathology by a pathologist as previously described (7, 33, 34).

Statistical Analysis

Statistical differences between groups were determined using a two-tail unpaired t test and a Chi-squared test for independence. All results were expressed as mean ± standard error (SEM). The Prism statistical package (GraphPad Software Inc, La Jolla, CA) was used. A p-value of < 0.05 was considered statistically significant.

Results

CTLA4-Ig treatment prior to immunization with 2OA-BSA

In efforts to investigate the effects of CTLA4-Ig treatment on preventing 2OA-BSA-induced autoimmune cholangitis, groups of B6 mice were untreated (controls) or treated with CTLA4-Ig prior to immunization with 2OA-BSA. As expected, control mice developed significant levels of serum autoantibodies to PDC-E2. In contrast, anti-PDC-E2 autoantibodies were not detected in serum from mice treated with CTLA4-Ig (Figure 1), indicating that the administration of CTLA4-Ig before 2OA-BSA immunization prevented production of AMAs. Coincidentally, while cholangitis exemplified by lympho-plasmacytic infiltration and bile duct damage was readily found in the liver of control mice at eight weeks following 2OA immunization, liver tissues from mice pre-treated with CTLA4-Ig showed a significant decrease in the extent of liver inflammation (Figure 2A). Furthermore, interlobular bile duct damage was not observed in any of the treated mice (Figure 2B). Of note, administration of CTLA4-Ig alone without immunization with 2OA-BSA did not induce any detectable pathologic changes in mouse liver (data not shown). Finally, flow cytometric assisted analysis was also performed on intra-hepatic MNCs isolated from the liver of control and CTLA4-Ig treated mice at 8 weeks post 2OA-BSA immunization. Results of such analysis revealed that while the former showed marked increases (p < 0.001) in the frequency and absolute numbers of effector (CD44⁺CD62L⁻) CD4 and CD8 T cells, the latter demonstrated marked decreases in such infiltrates (see Figure 3). These data indicate that CTLA4-Ig treatment started prior to 2OA-BSA immunization completely prevents loss of tolerance and subsequent autoimmune cholangitis.

Therapeutic efficacy of CTLA4-Ig in 2OA-BSA-induced cholangitis in mice

In efforts to determine the therapeutic efficacy of CTLA4-Ig, we utilized our standard protocol to induce cholangitis in groups of mice by 2OA-BSA immunization. As seen in Figure 2, since the disease onset was detected at 8 weeks after the initial immunization, consistent with our previously data (7, 27, 28), we started treatment with CTLA4-Ig at that time point. After 4 weeks of treatment, mice were sacrificed and examined for liver...
pathology. As shown in Figure 4A, CTLA4-Ig treatment significantly reduced the extent and severity of liver inflammation and the extent of biliary cell damage (Fig. 4B) as compared to untreated mice. Flow cytometric analysis of the intra-hepatic cellular infiltrates reflect a marked diminution in the frequency and absolute numbers of CD44^+CD62L^− effector CD4 and CD8 T cells in CTLA4-Ig treated mice as compared with control mice (Fig. 5). Analysis of serum collected pre- and post CTLA4-Ig treatment from these mice for levels of PDC-E2-specific autoantibodies did not show any detectable difference in titers (Fig. 6).

**Discussion**

CTLA4-Ig (Abatacept) is a soluble recombinant human fusion protein comprised of the extracellular domain of human CTLA4, linked to a modified portion of the Fc domain of human IgG-1 which is devoid of FcR binding and complement activation activity (29-31). There is considerable conservation between murine and human CTLA4, including in studies of the effects of graft-versus-host disease across the major histocompatibility complex barrier in mice (35).

This agent mimics the action of native CTLA4 by binding to CD80/CD86 on APCs and competitively inhibits the essential CD28:CD80/CD86 co-stimulatory signal required for T cell activation resulting in down regulation of subsequent immune effector mechanisms (31, 36, 37). CTLA4-Ig similarly inhibits the interaction between murine CD28 and its ligand CD80/CD86. CTLA4-Ig is an FDA approved drug for patients with rheumatoid arthritis and juvenile idiopathic arthritis (38, 39). CTLA4-Ig has also been shown to ameliorate autoimmunity in vivo during collage-induced arthritis (40), experimental autoimmune encephalomyelitis (41), psoriatic arthritis (42), systemic lupus erythematosus (43), ankylosing spondylitis (44) and shown to be effective in the prolongation of allograft and xenograft survival (45-48).

In the current study we used our animal model of xenobiotic-induced cholangitis to investigate the potential effects of CTLA4-Ig both as a preventative and as a therapeutic agent. Our data demonstrated that the development of both anti-PDC-E2 and liver pathology was successfully prevented in mice treated with CTLA4-Ig one day before immunization with 2OA. Since CTLA4 inhibits co-stimulation of T cells required for T cell activation, our results demonstrate the critical role of T cell activation in the development of 2OA-induced autoimmune cholangitis in which T cells serve as a pathogenic effector, as well as in the autoreactive B cell response in which the T cell help is also essential. We thus proceeded to treat mice with established cholangitis at 8 weeks following 2OA-BSA immunization. Treatment with CTLA4-Ig in this group not only resulted in reduced histological inflammatory changes in liver and biliary cell damage but also reduced levels of intrahepatic pathogenic effector CD4 and CD8 T cells, indicating the potential therapeutic effect of CTLA4-Ig.

Although CTLA4-Ig treatment did not result in complete resolution of established cholangitis in our mouse model, the reduced frequency and absolute numbers of liver infiltrating pathogenic T cells and improved liver histology are important initial therapeutically beneficial findings. These data are consistent with the view that CTLA4-Ig treatment inhibits activation of naïve T cells but not previously primed autoreactive memory T cells since the later have a lower threshold of activation and are less dependent on co-stimulatory signals for activation. However, it is important to keep in mind that activated T cells also need interaction with co-stimulatory molecules to provide survival signals for sustained effector T cell responses. Thus, while the results of the current studies show significant therapeutic benefit, the studies only address the acute effects of CTLA4-Ig therapy. Studies to determine if discontinuing treatment will result in relapse of cholangitis,
and if a prolonged CTLA4-Ig regimen (>4 weeks) will improve its therapeutic efficacy need to be performed.

Although treatment with CTLA4-Ig post immunization reduced intra-hepatic effector T cell infiltration and biliary cell damage, these mice continued to manifest AMAs at levels comparable with control mice. The serum AMA is produced by long-lived plasma cells, which can be replenished from previously primed memory B cells with a lower threshold of activation (49, 50). This would explain the lack of effect of CTLA4-Ig treatment on serum AMA levels in this group of mice. The differential response to CTLA4-Ig treatment on T cells versus AMAs, as well as its impact on the therapeutic efficacy for PBC, should also be evaluated in future studies.

It is becoming increasingly clear that besides CTLA4 there are a number of other inhibitory receptors that function to control immune responses which include TIGIT (T cell immunoreceptor with immunoglobulin and ITIM domains), PD-1 (programmed death-1), and TIM-3 (T cell immunoglobulin and mucin domain containing molecule-3). It is not clear at present whether there is a hierarchy amongst these molecules in terms of an ordered sequence by which these molecules act to regulate immune responses. In light of our previous studies on the role of inflammatory cytokines, such as IFNγ, IL-6, IL-12 and IL-23 (51-53), in the pathogenesis of PBC (including autoantibody production and biliary cell damages), it is reasonable to assume that CTLA4 could influence the effect of these downstream inflammatory cytokines. However, it is clear that they do serve at different check points along the T cell activation pathway and it is likely that an optimized therapeutic approach may require a combination of therapeutic inhibitors to maintain long term inhibition of autoimmune effector mechanisms (54) without compromising anti-viral and tumor surveillance mechanisms.

With regards to CTLA4, despite its extensive experimental and clinical use for therapy of autoimmune disease and transplant rejection, there is remarkably a paucity of data on precisely how and when CTLA4-Ig mediates its effect on T cells in vivo. In addition, the effect of CTLA4-Ig on T-dependent B cell responses are poorly understood and/or characterized. Although Abatacept has been demonstrated to have a significant therapeutic benefit in patients with rheumatoid arthritis (55, 56), and increased risk of infection, malignancy and autoimmune events have been reported (57, 58). We reason that elucidation of the precise mechanisms by which CTLA4-Ig mediate its effect will aid in defining optimum therapeutic application of this unique immunomodulatory drug while minimizing in vivo toxicity and clinically non-beneficial effects.

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References


**Abbreviations**

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<tr>
<td>PBC</td>
<td>Primary biliary cirrhosis</td>
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<tr>
<td>CTLA4</td>
<td>cytotoxic T lymphocyte antigen 4</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>2OA-BSA</td>
<td>2-octynoic acid conjugated BSA</td>
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<tr>
<td>CFA</td>
<td>Complete Freund's Adjuvant</td>
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<tr>
<td>IFA</td>
<td>Incomplete Freund's Adjuvant</td>
</tr>
<tr>
<td>MNCs</td>
<td>Mononuclear cells</td>
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<tr>
<td>OD</td>
<td>optical value</td>
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<td>AMA</td>
<td>anti-mitochondrial antibody</td>
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Figure 1.
CTLA4-Ig treatment inhibited production of PDC-E2-specific autoantibodies induced by 2OA-BSA immunization. CTLA4-Ig treatment was started 1 day before initial 2OA-BSA immunization and continued for 8 weeks (2OA+CTLA). Untreated mice (2OA) were utilized as a control group. Serum samples were collected at different time points, diluted 1:250 and tested for anti-PDC-E2 reactivity by ELISA assay. OD, optical value. Each group included 8 mice.
Figure 2.
CTLA4-Ig treatment protected mice against 2OA-BSA-induced cholangitis. CTLA4-Ig treatment was started 1 day before initial 2OA-BSA immunization and continued for 8 weeks. (A) H&E-stained representative liver sections from 2OA-BSA-immunized mice treated with CTLA4-Ig compared to controls. Note, epithelioid granuloma (red arrowhead) and interlobular bile duct damage (red arrow). (B) Bile duct damage was evaluated as described on H&E stained liver sections. Each group included 8 mice. *, p < 0.05 (Chi-squared test for independence).
Figure 3.
CTLA4-Ig treatment prevented intrahepatic infiltration of effector T cells. CTLA4-Ig treatment was started 1 day before initial 2OA-BSA immunization. The frequency of the effector (CD44+CD62L−) population in CD4 and CD8 T cells as well as number of these populations in liver were analyzed by flow cytometry. Each group included 8 mice. *, p < 0.05; **, p < 0.001; ***, p < 0.001 (two-tailed unpaired t test).
Figure 4.
Effects of CTLA4-Ig treatment on 2OA-BSA-induced cholangitis. 2OA-BSA immunized mice were treated with CTLA4-Ig for 4 weeks, started 8 weeks after initial immunization (2OA+CTLA). 2OA-BSA immunized mice without CTLA treatment were utilized as control group (2OA). (A) H&E-stained representative liver sections. (B) Scores of intrahepatic bile duct damage in 2OA-immunized mice with or without CTLA4-Ig treatment. Each group included 12 mice. *, p < 0.05 (Chi-squared test for independence).
Figure 5.
CTLA4-Ig treatment reduced intrahepatic infiltration of effector T cells. 2OA-immunized mice were treated with CTLA4-Ig for 4 weeks, started 8 weeks after initial immunization. Frequency of the effector (CD44^{+}CD62L^{-}) population in CD4^{+} and CD8^{+} T cells as well as the number of these populations in liver were analyzed by flow cytometry. Each group included 8 to 12 mice. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (unpaired t-test).
Figure 6.
CTLA4-Ig treatment started after onset of cholangitis did not affect serum levels of PDC-E2-specific autoantibodies. CTLA4-Ig treatment was started 8 weeks after initial 2OA-BSA immunization and continued for 4 weeks. Each group included 6 to 12 mice.