Costimulation through B7-2 (CD86) Is Required for the Induction of a Lung Mucosal T Helper Cell 2 (TH2) Immune Response and Altered Airway Responsiveness

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Summary

The recruitment of eosinophils into the airways after allergen exposure is dependent on interleukin (IL) 5 secreted from antigen-specific CD4\(^+\) T cells of the T helper cell (Th) 2 subset. However, while it is established that costimulation through CD28 is required for TCR-mediated activation and IL-2 production, the importance of this mechanism for the induction of a Th2 immune response is less clear. In the present study, we administered the fusion protein CTLA-4 immunoglobulin (Ig) into the lungs before allergen provocation to determine whether CD28/CTLA-4 ligands are required for allergen-induced eosinophil accumulation and the production of Th2 cytokines. Administration of CTLA-4 Ig inhibited the recruitment of eosinophils into the lungs by 75% and suppressed IgE in the bronchoalveolar lavage fluid. CTLA-4 Ig also inhibited the production of IL-4, IL-5, and IL-10 by 70–80% and enhanced interferon-γ production from CD3–T cell receptor–activated lung Thy1.2 cells. Allergen exposure upregulated expression of B7-2, but not B7-1, on B cells from the lung within 24 h. Moreover, airway administration of an anti-B7-2 monoclonal antibody (mAb) inhibited eosinophil infiltration, IgE production, and Th2 cytokine secretion comparable in magnitude to that observed with CTLA-4 Ig. Treatment with an anti-B7-1 mAb had a small, but significant effect on eosinophil accumulation, although was less effective in inhibiting Th2 cytokine production. The anti-B7-2, but not anti-B7-1, mAb also inhibited antigen-induced airway hyper-responsiveness in vivo. In all of the parameters assessed, the combination of both the anti-B7-1 and anti-B7-2 mAb was no more effective than anti-B7-2 mAb treatment alone. We propose that strategies aimed at inhibition of CD28 interactions with B7-2 molecules may represent a novel therapeutic target for the treatment of lung mucosal allergic inflammation.

Bronchial asthma is characterized by the infiltration of eosinophils into the airway submucosa (1). Activation of eosinophils with the subsequent secretion of highly charged granular cationic proteins such as major basic protein is believed to be important in the pathogenesis of bronchial asthma (2–4). While the precise mechanisms by which eosinophils are recruited into the lungs are not fully understood, there is increasing evidence suggesting that activation of antigen-specific CD4\(^+\) T cells of the Th2 subset in the lungs resulting in IL-5 secretion plays a major role in allergic inflammation of the airways (5–7).

T cell activation requires interaction between the TCR and specific antigen, presented in the form of processed peptides in association with MHC class II molecules. However, for complete T cell activation, a second signal termed costimulation is required (8, 9). The most widely studied costimulatory molecule is CD28, which is constitutively expressed on the surface of both CD4\(^+\) and CD8\(^+\) T cells. Activation of CD28 in conjunction with the CD3–TCR complex is required for T cell proliferation and IL-2 production (10–12). In the absence of CD28-mediated signaling, a state of unresponsiveness or anergy develops (9–11). The natural ligands for CD28 have more recently been identified as B7-1 or CD80 (13–15), and B7-2 or CD86 (16, 17), and have been demonstrated to be present on the surface of antigen-pulsed B cells (13–15) and dendritic cells (18) and may account for the efficacy of these cells as APCs.

B7-1 and B7-2 also bind to CTLA-4, a homologue of CD28 (16, 19). A soluble chimeric fusion protein consisting of the extracellular domain of CTLA-4 and a human γ-1 constant region, CTLA-4 Ig (20), binds to both B7-1 and B7-2 with an affinity 20 times greater than CD28, and inhibits CD28- and CTLA-4-mediated signaling (19–22). In...
vitro studies using murine and human cells have demonstrated that CTLA-4 Ig inhibits T cell proliferation and IL-2 production, and induces a state of T cell unresponsiveness (19–22). However, while it is clearly established that CD28-mediated signaling is required for IL-2 production, the requirement of costimulation through CD28 for the induction of a Th2 immune response is less clear. In the present series of experiments, we have administered CTLA-4 Ig into the airways before aerosol antigen challenge to determine whether costimulation through these molecules is required for the recruitment of eosinophils into the lungs and the production of Th2 cytokines. To further delineate the involvement of B7-1 and/or B7-2, the expression of these molecules on lung B cells after allergen provocation was assessed and neutralizing mAbs to either B7-1 and/or B7-2 were administered into the lung before allergen provocation. The results of this study indicate that allergen exposure selectively upregulates B7-2 expression and that costimulation through this molecule is required for the induction of a lung mucosal Th2 immune response and for allergen-induced altered airway responsiveness.

Materials and Methods

Antibodies and Cytokines

The CTLA-4 Ig used is a fusion protein between the extracellular domain of murine CTLA-4 and human γ-1 obtained from a CTLA-4 Ig-producing hybridoma (20) provided by Dr. Peter Lane (Basel Institute of Immunology, Basel, Switzerland). Supernatants were then concentrated and purified over a protein A column. PE-labeled Thy1.2 (30-H12), FITC-labeled B7-1 (16-10A1), FITC-labeled and purified B7-2 (GL-2), and CD16/CD32 (2.4G2, Fc Block™) were purchased from Pharmingen, (San Diego, CA). Purified B7-1 (16-10A1; reference 23) was obtained from Dr. Hans Reiser (Dana Farber Cancer Institute, Boston, MA). Human recombinant IL-2 and anti-CD3 (2C11) was obtained from Ciba-Geigy AG. (Basel, Switzerland).

In Vivo Experiments

Quantification of Eosinophil Recruitment to the Lung

Sv129 mice (20–25 g) of either sex were immunized intraperitoneally with 10 μg of OVA (grade V; Sigma Chemical Co., St. Louis, MO) in 0.2 ml of alum (Serva, Heidelberg, Germany). 10 d later, animals were anesthetized by inhaling 2% Forene with CD16/CD32 mAb (Beckton Dickinson, San Jose, CA). After incubation with dilutions of BAL fluid, sample-bound mouse IgE was detected with biotinylated rat anti-murine IgE 3-11. Data were expressed as total IgE per ml of BAL fluid. The limit of detection of the assay was 2 ng/ml of IgE.

Purification of Lung T Cells

To analyze the lung T cell cytokine profile, experiments were performed 72 h after the last antigen challenge. After the four repeated lavages for assessment of the inflammatory cell infiltrate, the lungs were perfused via the right ventricle with 5 ml of PBS containing 100 U/ml of heparin to remove any blood and intravascular leukocytes. The lungs were then removed and placed into DMEM containing 10% FCS, 2-mercaptoethanol (50 μM), sodium pyruvate (1 mM), Hepes (10 mM), and gentamycin (50 μg/ml). The lungs were then gently homogenized, the cell suspension was filtered through a 70-μM filter, and lymphocytes enriched over a single step Ficoll gradient. B cells were depleted from the cell suspension using magnetic sheep anti-mouse Ig beads (Dynabeads, Nycor, Norwayne). Cells were then labeled with Thy1.2-PE and purified by flow cytometry (Beckton Dickinson, San Jose, CA) as described previously (5). In all experiments, purity was >99% Thy1.2+.

Cell Culture

Lung Thy1.2+ cells were then plated at a concentration of 2 × 10^5 in 96-well microtiter plates coated with an anti-CD3 antibody (2C11, 50 μg/ml). Cells were cultured for 72 h in the presence of human IL-2 (200 U/ml). Supernatants were harvested and cytokine production determined by ELISA as described elsewhere (5). The limits of detection were IL-4, 0.5 U/ml; IL-5, 100 U/ml; IL-10, 10 U/ml; IL-2, 0.2 U/ml; IFN-γ, 50 U/ml.

Immunofluorescence and Flow Cytometry Analysis

To determine the expression of CD28 ligands on lung B lymphocytes 24 h after the first, third, or fifth aeroallergen challenge, lymphocytes were enriched from the lung as described above and stained with anti-CD28-PE. Nonspecific binding to Fc receptors was then blocked by incubating cells at 4°C for 30 min with CD16/CD32 mAb (2.4G2). Cells were then washed twice and incubated for a further 30 min with FITC anti-B7-1 or FITC anti-B7-2. Expression of B7 molecules after allergen exposure was compared to either before challenge or after repeated challenge with PBS. Fluorescence was analyzed by flow cytometry, gated on the region of B20+ cells (FACScan™) using consort 30 software, and 10,000 events acquired.

1Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; MCH, methacholine.  

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**Results**

**CTLA-4 Ig Inhibits Antigen-induced Eosinophil Recruitment to the Lungs.** Exposure to five repeated aerosol provocations of OVA induced a selective infiltration of eosinophils into the airways as assessed by BAL (40.0 ± 11.0 eosinophils/ml × 10^5, n = 6). Administration of the human γ-1 as the isotype-matched control antibody 4 h before the first allergen provocation had no significant effect on antigen-induced eosinophil recruitment (38.9 ± 6 eosinophils/ml × 10^5, P > 0.1, n = 6). Pretreatment with 100 μg of CTLA-4 Ig induced a marked attenuation in the recruitment of eosinophils into the airways (7.9 ± 2.4 eosinophils/ml × 10^5, n = 6, P < 0.05, Fig. 1). Administration of CTLA-4 Ig before PBS provocation of immunized animals failed to induce any inflammatory changes in the airways (Fig. 1).

**CTLA-4 Ig Inhibits the Local IgE Production in the Lungs.** Repeated allergen provocation increased levels of IgE in the BAL fluid as compared to PBS-exposed animals (PBS challenged, <2 ng/ml, n = 3; antigen challenged, 72 ± 14 ng/ml, n = 8, P < 0.01). Local airway administration of CTLA-4 Ig inhibited the production of IgE in the BAL fluid (9.7 ± 2.9 ng/ml, n = 5, P < 0.001; Fig. 2). In contrast, CTLA-4 Ig failed to suppress total serum IgE (data not shown).

**CTLA-4 Ig Inhibits Th2 Cytokine Production and Enhances IFN-γ Production.** Activation of FACS-purified Thy1.2+ cells from the lung via the CD3-TCR complex from immunized, PBS-challenged mice produced IL-2 and IFN-γ, with no detectable IL-4, IL-5, or IL-10. In contrast, repeated allergen provocation of immunized mice treated with...
human γ-1 resulted in the secretion of high amounts of IL-4, IL-5, and IL-10, elevated levels of IL-2, and reduced amounts of IFN-γ (Fig. 3). Pretreatment with CTLA-4 Ig inhibited the production of Th2 cytokines by 70–80%, enhanced the production of IFN-γ by threefold, but had no significant effect on IL-2 production compared to control Ig-treated mice.

B7-2, but Not B7-1, Is Upregulated on Lung B Cells after Allergen Provocation. We next investigated whether antigen provocation leads to the expression of the CD28 counterligands B7-1 and B7-2. B cells obtained from the lungs of immunized mice before challenge expressed low levels of B7-1 and B7-2 (Fig. 4, a and b). However, B7-2 expression was markedly upregulated within 24 h after the first allergen exposure and was maintained after three or five allergen provocations (data not shown). In contrast, B7-1 was not upregulated on lung B cells at any time point after antigen challenge (Fig. 4, c and d).

B7-2 Is the Primary CD28 Counterligand Required for the Induction of a Th2 Immune Response. It then became important to determine whether B7-1, B7-2, or both molecules are required for the induction of a lung Th2 immune response. Anti-B7-2 mAb treatment inhibited the recruitment of eosinophils in the lung by >85%, compared to control Ig-treated mice (control Ig, 24.6 ± 3.6 eosinophils/ml × 10⁶, n = 10; anti-B7-2 mAb, 3.07 ± 1.1 eosinophils/ml × 10⁶, n = 5, P < 0.005; Fig. 5). In contrast, the anti-B7-1 mAb had a minor, although significant, inhibitory effect on eosinophil recruitment into the lung (anti-B7-1 mAb, 16.8 ± 3.8 eosinophils/ml × 10⁶, n = 9, P < 0.05). However, the combination of both mAbs was no more effective than anti-B7-2 mAb alone (2.4 ± 0.80 eosinophils/ml × 10⁶, n = 10, P > 0.05, compared to anti-B7-2 mAb alone). In addition, in vivo treatment with anti-B7-2 mAb inhibited the production of IL-4 and IL-5 and enhanced production of IFN-γ from purified lung T cells activated through the CD3-TCR complex, whereas treatment with anti-B7-1 mAb had a minor effect on Th2 cytokine secretion (Fig. 6). The combination of both mAbs was no more effective than anti-B7-2 mAb in inhibiting Th2 cytokine production. Finally, measurement of IgE levels in the BAL supported the principle role for costimulation through B7-2, as IgE production was suppressed by the anti-B7-2 mAb (control Ig, 65 ± 9 ng/ml; anti-B7-2 mAb, 12 ± 5 ng/ml, P < 0.05), but not by the anti-B7-1 mAb (57 ± 12 ng/ml, P > 0.05). The combination of both mAbs was no more ef-

Figure 4. Representative FACS® analysis of expression of B7-1 and B7-2 on lung B220+ cells before (a and b) and 24 h after antigen challenge in immunized mice. Cells were stained with PE-B220 alone (solid lines) or PE-B220 and FITC-B7-1 or B7-2 (dotted lines). Data are expressed as histograms of B7 expression after gating on B220 positive cells. Data are representative of three separate experiments.

Figure 5. Effect of mAbs to B7-1 (open bar), B7-2 (striped bar), or B7-1 and B7-2 (shaded bar) on antigen-induced eosinophil infiltration. Control mice were treated with rat IgG (filled bar). Data are expressed as mean ± SEM of eosinophils/ml × 10⁶ for n = 9–10 mice. Statistical significance (*) was determined by a Student's t test and a value of P < 0.05 was considered to be significant.
B7-2, but Not B7-1, Is Required for the Induction of Airway Hyperresponsiveness in Vivo. We next investigated whether B7-1 and/or B7-2 contribute to the development of airway hyperresponsiveness (AHR) to methacholine (MCh). Allergen provocation of immunized mice treated with control rat Ig resulted in the development of AHS (baseline Penh, 0.37 ± 0.04; after MCh, 2.8 ± 0.27, n = 5), as compared to immunized mice treated with control rat Ig and exposed to PBS (baseline Penh, 0.39 ± 0.01; after MCh 0.56 ± 0.03, n = 4, P <0.05; Fig. 8A). There was no change in AHR in PBS-exposed mice that were treated with either B7-1 and/or B7-2 mAb (data not shown). Pretreatment with anti-B7-2 mAb abrogated the development of allergen-induced AHR (baseline Penh, 0.39 ± 0.04; after MCh, 0.90 ± 0.33, P <0.05). In contrast, anti-B7-1 mAb had no significant effect, and the MCh response was comparable to that observed in IgG-treated, antigen-exposed mice. (baseline Penh, 0.35 ± 0.03; after MCh, 3.38 ± 0.85, P = 0.48). Pretreatment with the combination of anti-B7-1 and B7-2 mAbs was no more effective than anti-B7-2 mAb treatment alone AHR (baseline Penh, 0.36 ± 0.02; after MCh, 0.70 ± 0.16, P >0.05), compared to anti-B7-2 mAb treatment alone (Fig. 8B).

Discussion

Activation of antigen-naive CD4+ T cells produce predominately IL-2. However, under the influence of IL-4, these cells differentiate into a phenotype that produces high levels of Th2 cytokines (29–31). In recent years, evidence has increased to suggest that activation of antigen-specific CD4+ Th2 cells in the lungs of allergic individuals plays a central role in the pathogenesis of bronchial asthma by providing IL-4, which is essential for B cell switching to IgE to occur (32), and IL-5, which is required for eosinophil mobilization and/or accumulation (5–7). It is now clearly established that in addition to interactions between peptides bound to MHC class II molecules and the CD3-TCR-α/β complex, a second signal is required. CD28-mediated signaling via its natural ligands, B7-1 and B7-2, provides the signal necessary for T cell proliferation and IL-2 gene transcription (11–18). However, the costimulatory signals that are required for the induction of a Th2 immune response are less clear. In the present study, we use the fusion pro-
IgE production at least in part by the upregulation of IFN-γ, whether CTLA-4 Ig suppresses eosinophil infiltration and recruitment of eosinophils into the lungs. These observations are supported by the demonstration that CTLA-4 Ig treatment suppresses the production of IL-4, IL-5, and IL-10 from lung T cells. In contrast, pretreatment with CTLA-4 Ig enhanced the production of IFN-γ from lung T cells by threefold. Thus, in the absence of costimulation through CD28 ligands, cells can default to a phenotype that produces less Th2 cytokines and more IFN-γ. As IFN-γ has been shown to inhibit the proliferation of Th2 cells (33), reciprocally regulate IL-4-dependent IgE production (34), and can inhibit the accumulation of eosinophils into the lung in vivo (35), it remains to be clarified, using antibodies to IFN-γ or mice with a disrupted IFN-γ system, whether CTLA-4 Ig suppresses eosinophil infiltration and IgE production at least in part by the upregulation of IFN-γ production.

The requirement of CD28 as a costimulatory molecule for activation of Th2 cells is controversial at present. In vitro studies have demonstrated that Th2 cells can use other costimulatory signals besides CD28, and attention has focused on an important role for IL-1α. More recently, IL-4 production has been shown to be independent of CD28-mediated stimulation, although CD28 signaling was required for inducing responsiveness to IL-4 (36). CTLA-4 Ig has also been demonstrated to inhibit alloantigen-specific responses, and IL-2 and IFN-γ, but not IL-4 secretion (38). In addition, antigen-specific stimulation of CD4+ T cells from TCR transgenic mice have shown that IL-4 secretion from Th2 cells is relatively independent of costimulation through CD28 (38). These in vitro observations are also supported by in vivo experiments showing that CTLA-4 Ig specifically inhibits the production of Th1 cytokines, but spares the production of Th2 cytokines in a rat renal allograft model (39). In contrast, after infection with Heligmosomoides polygyrus, CD28-mediated stimulation is required for IL-4 production and B cell switch to IgE production, although the development of a peripheral blood eosinophilia was largely independent of CD28-mediated costimulation (40). Likewise, after infection with L. major (41) or in a primary immune response induced by anti-IgD (42), CTLA-4 Ig inhibits the appearance of IL-4-producing cells. However, in these two models, the production of IL-5 and IL-10 was independent of costimulation through CD28. Interestingly, administration of CTLA-4 Ig treatment several days after treatment with either anti-IgD or H. polygyrus failed to inhibit the development of IgE-producing cells (40, 42). These results suggest that in vivo priming of naive T cells generally requires B7-CD28-mediated costimulation, whereas activation of differentiated Th2 cells is largely B7 independent.

If CD28 is required for differentiation towards a Th2 immune response, it still remains unclear whether activation of CD28 is mediated by either B7-1 and/or B7-2. B7 molecules have been shown to be expressed on activated B cells (14–17), functional mature dendritic cells (18), and more recently, on activated CD4+ and CD8+ T cells (43). However, the expression of these molecules is differentially controlled in that B7-2 expression is upregulated within 6 h of B cell activation after LPS, Con A, or antigen-specific stimulation, whereas B7-1 was not increased until 72 h later. Moreover, while LPS and Con A induced B7-1 expression, cross-linking of the Ig receptor failed to upregulate B7-1 expression (44, 45). These in vitro studies are supported by the present in vivo observations that 24 h after aeroantigen exposure, B7-2, but not B7-1, expression was increased on lung B cells and expression was maintained after the repeated antigen provocations (data not shown). However, it is important to note that despite the lower level of expression of B7-1, the slower off-rate of B7-1, compared to B7-2, may allow this molecule to exert potent costimulatory functions (46). In addition, the ability of B7-1 and B7-2 to bind to distinct CD28 determinants with different affinities (47) raises the possibility that these molecules play distinct functions (48). Freeman et al. have suggested that repeated alloantigen stimulation by B7-2, but not B7-1, transfected cells selectively primes naive cells for IL-4 production (49). Likewise, in a recent study using 1-Aβ-restricted, antigen-specific CD4+ T cells obtained from transgenic mice, it was shown that anti-B7-2 mAb markedly suppressed the development of IL-4- and IL-10-producing cells, whereas blockade of B7-1 facilitated differentiation towards IL-4 production and inhibited IFN-γ secretion (50). To investigate the role of these two molecules in the induction of the allergic inflammatory response, we administered neutralizing mAbs to B7-1 and/or B7-2 directly into the lungs. Our observations suggest that costimulation through B7-2 is the primary CD28 counterreceptor involved in the induction of a lung mucosal Th2 immune response, since administration of the anti-B7-2 mAb inhibited the recruitment of eosinophils into the lungs and Th2 cytokine production. However, we cannot rule out a small contribution of B7-1-mediated costimulation, since eosinophil accumulation was reduced by 25%. However, coadministration of both mAbs was no more effective than treatment with anti-B7-2 alone, supporting the evidence that B7-2 is the principal CD28 ligand required in this response. Distinct roles for B7 ligands have also been suggested in another in vivo study where neutralization of B7-2 inhibited the production of Th2 cytokines, enhanced the production of IFN-γ, and was associated with enhanced disease severity in a model of experimental allergic encephalomyelitis (51). Likewise, differential functions for B7-1 and B7-2 have also been suggested in the development of diabetes in nonobese diabetic mice whereby anti-B7-2 mAb inhibited the development of diabetes, compared to anti-B7-1 mAb, which accelerated the disease development (52). It therefore became important to determine whether B7-2-mediated costimulation was not only associated with the inflammatory response in the lungs, but was also implicated in a pathophysiological-relevant response. Our results show that the induction of airway hyperresponsiveness, which is a characteristic feature of bronchial asthma, was abrogated by treatment with the anti-B7-2 mAb. In contrast, administration of the anti-B7-1 mAb provided no protection, despite...
the small reduction in the number of eosinophils in the lungs after allergen provocation. Taken together, we suggest that interaction of B7-2 with CD28 is important in the development of a lung inflammatory response and is essential for the induction of airway hyperresponsiveness. Nevertheless, further studies are required to elucidate whether B7-1 and B7-2 also provide distinct costimulatory functions during different immune responses (soluble protein antigens compared to intact pathogens), or whether both molecules can elicit similar responses with the appropriate cytokine secretion.

In conclusion, repeated allergen provocation results in the commitment of cells to the Th2 phenotype, the selective recruitment of eosinophils to the lung, and the production of IgE, which is dependent on the activation of CD28 ligands. In particular, we suggest an important role primarily for B7-2, based on the selective upregulation of this molecule, the marked suppression of lung Th2 mucosal immune responses, and abrogation of airway hyperresponsiveness after allergen exposure by the anti-B7-2 mAb. These observations open up the attractive therapeutic potential that inhibiting B7-2–CD28 interactions in the lungs of allergic individuals may lead to the development of novel therapies for the treatment of allergic lung disease.

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