Blockade of T Cell Costimulation by CTLA4-Ig Inhibits Lung Inflammation in Murine Hypersensitivity Pneumonitis

Evelyne Isaël-Assayag, Marcien Fournier and Yvon Cormier

*J Immunol* 1999; 163:6794-6799;
http://www.jimmunol.org/content/163/12/6794

References
This article cites 28 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/163/12/6794.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Hypersensitivity pneumonitis (HP) is characterized by an influx of activated T cells in the lungs. The CD28/B7 system provides costimulatory signals essential for complete T cell activation and differentiation. We have previously demonstrated that alveolar macrophages from patients with HP have an up-regulated expression of B7 molecules. In the present study, we investigated the effect of i.p. administration of CTLA4-Ig, a CD28/B7 antagonist, on the lung inflammation of mice inoculated with Saccharopolyspora rectivirgula (SR), a major causative agent of HP. Five groups of C57BL/6 mice were intranasally instilled with SR or saline for 3 consecutive days per wk during 3 wk. CTLA4-Ig was administered starting either after 1 wk of SR challenge or 6 h before the first antigenic exposure and continued during the whole period of sensitization. A control-IgG was given similarly during the 3 wk of SR exposure. The groups included: 1, saline; 2, SR; 3, SR + control-Ig; 4, SR + CTLA4-Ig for the last 2 wk; and 5, SR + CTLA4-Ig for 3 wk. CTLA4-Ig treatment markedly decreased lung inflammation as shown by significantly fewer inflammatory cells in the bronchoalveolar lavage and in lung tissue and reduced SR-specific serum and bronchoalveolar lavage Ig levels. Production of IL-4, IL-10, and IFN-γ by IL-2-stimulated pulmonary T cells was also decreased by CTLA4-Ig. Administration of CTLA4-Ig did not affect the SR-induced up-regulation of B7-2 expression. These results show that blockade of CD28/B7 interactions by CTLA4-Ig inhibits SR-induced lung inflammation and immune response to SR Ag in mice and may provide a novel approach in the treatment of HP.


Blockade of T Cell Costimulation by CTLA4-Ig Inhibits Lung Inflammation in Murine Hypersensitivity Pneumonitis

Evelyne Israël-Assayag, Marcien Fournier, and Yvon Cormier

Hypersensitivity pneumonitis (HP) is an inflammatory lung disease caused by an exacerbated response to repeated inhalations of organic dust or small m.w. molecules. The condition is characterized by a massive infiltration of lymphoid cells in the lungs. Well-known causative agents include bacterial (farmer’s lung), fungal (humidifier lung, peat moss-induced HP), and animal (bird fancier’s disease) proteins. T lymphocytes play a pivotal role in the pathogenesis of HP: their number and percentage are increased in the bronchoalveolar lavage (BAL) of patients with HP and may account for 60–80% of the recovered cells (3), and they present an activated phenotype (4), react specifically to the causative Ags (5), and, in animal models, are able to adoptively transfer sensitization to naive animals (6). The role of Ags in determining and maintaining the lymphocytic alveolitis in HP has been further demonstrated by studies showing the specific expansion of BAL T cells bearing restricted TCR-Vβ regions (7, 8). These expansions occur mainly in the CD8 subset and subside following removal from the antigenic exposure.

Following cellular activation, lymphocytes can differentiate into Th1 or Th2 cells according to the type of cytokine they produce, the balance of which will ultimately determine the outcome of the cellular response. Typically, production of IFN-γ and IL-12 will favor a Th1 response, whereas presence of IL-4 and IL-10 will determine a Th2 pattern. In HP no clear pattern of polarization has been defined. In experimental models, Th1 responses may be important since Th1 CD4+ cells can adoptively transfer the disease to healthy animals (9); IFN-γ and IL-12 may also play a role in the pathogenesis of HP in mice (10, 11), but in human HP, recent studies suggest that a Th2-type response is predominant (12–14).

T cell activation requires at least two distinct signals (15); the first is Ag-specific and is delivered through the engagement of TCRs. The second signal is mediated by the interaction of costimulatory molecules present on APCs with their ligands on T cells. The B7/CD28/ACTL4 is a major pathway which provides these potent signals, crucial for complete T cell activation. CD28 and CTLA4 are ligands for B7-1 and B7-2. These ligands bind to both B7 but with different avidities, CTLA4 binding is 20- to 100-fold higher than CD28 (16). This difference in avidity has been exploited to block B7-CD28 interactions by the use of CTLA4-Ig, a soluble fusion protein made from the extracellular portion of CTLA4 linked to the Fc portion of IgG (17). Furthermore, B7/CD28 costimulatory pathway may influence not only the extent of T cell activation but also the regulation of T cell differentiation (18). Depending on the system studied, B7 costimulation has been shown to influence both Th1 and Th2 cytokine production (19).

In the normal lung, alveolar macrophages (AM) have a low expression of B7 molecules and a poor capacity to function as APCs (20). In diseases with lymphocytic alveolitis such as HP and sarcoidosis, these cells are activated and show an increased Ag-presenting capacity compared with AM from normal subjects (21, 22). We previously reported a marked up-regulation of B7 molecule expression on AM from patients with active HP and asymptomatic Ag-exposed subjects compared with normal nonsmokers and smokers (23). In a pilot study, we found a similar increase of B7 expression on AM in a mouse model of HP (our unpublished observations). This up-regulation may increase the costimulatory signals necessary for T cell activation and differentiation. The aim of the present study was to verify the role of this up-regulation on...
the immune response to HP Ags, both in terms of intensity of the response and type of T cell response (Th1 vs Th2) involved. For this purpose, we investigated the effect of CTLA4-Ig on the proliferation of lymphocytes, Ab production, and cytokine expression by these lymphocytes. To verify whether any effect could be observed on an already ongoing response or whether blockade had to be present before sensitization, CTLA4-Ig was administered starting either 1 wk after the beginning of sensitization with an antigenic preparation of *Saccharopolyspora rectivirgula* (SR), the bacteria most frequently responsible for farmer’s lung, and given for 2 wk, or starting before and given throughout the whole period of sensitization.

**Materials and Methods**

**Animals**

CS7BL/6 female mice (Charles River, St. Constant, Quebec, Canada), a SR-sensitive strain of mice, weighing 18–22 g were used in this study. The animals were kept in pathogen-free conditions and cared for according to the guidelines of the Canadian Council on Animal Care.

**Antigens**

Lyophilized Ag was produced from a live culture of SR as described previously (24), reconstituted with pyrogen-free saline at a concentration of 5 mg/ml, aliquoted, and stored at ~70°C. A sample was tested in a *Limulus* amebocyte lysate assay (Sigma, St. Louis, MO) and was shown to be endotoxin free.

**Recombinant proteins and Abs**

CTLA4-Ig, a soluble chimeric fusion protein consisting of the extracellular domain of murine CTLA4 and a mouse IgG2a constant region, was kindly provided by the Genetics Institute (Cambridge, MA). Mouse IgG2a (Sigma) was used as a control protein. Abs to mouse B7-1 and B7-2, the isotypic controls and anti-CD3, were obtained from PharMingen Canada (Mississauga, Ontario, Canada). Recombinant mouse IL-2 was purchased from PeproTech (Rocky Hill, NJ). Determination of IL-4, IL-10, and IFN-γ was performed using ELISA kits from Endogen (Woburn, MA).

**Experimental design**

Five groups of 20 or 10 mice were studied. Group 1 (n = 20) received saline only and groups 2–5 were sensitized to SR. The animals were lightly anesthetized with isoflurane and instilled by nasal route with 50 μl of saline or with the antigenic solution of SR for 3 consecutive days/week for 3 wk. Group 2 (n = 20) had no further treatment. For group 3 (n = 20), mice were injected i.p. with 50 μg of mouse IgG2a every 2 days, during 3 wk. Mice of group 4 (n = 10) were injected i.p. with CTLA4-Ig (50 μg in 200 μl saline/mouse) every other day during 2 wk, beginning after 1 wk of SR exposure. Group 5 (n = 10) was treated with CTLA4-Ig every other day for the 3 wk of SR exposure, starting 6 h before the first Ag instillation on day 1.

**BAL**

Four days after the last instillation of saline or Ag, the animals were anesthetized with isoflurane and blood was collected via the orbital sinus for cascade of 0.5 μl/mouse) was used as a control protein. Abs to mouse B7-1 and B7-2, the isotypic controls and anti-CD3, were obtained from PharMingen Canada (Mississauga, Ontario, Canada). Recombinat mouse IL-2 was purchased from PeproTech (Rocky Hill, NJ). Determination of IL-4, IL-10, and IFN-γ was performed using ELISA kits from Endogen (Woburn, MA).

**Flow Cytometry Analysis of B7 Molecule Expression**

BAL cells from 3 to 10 mice were pooled and washed in PBS with 1% BSA. Fc receptors were blocked by incubation with an excess of mouse IgG (10 μg/106 cells) for 20 min at 4°C. Cells were then washed and further incubated with PE-labeled anti-B7-1 or B7-2 (PharMingen Canada) for 45 min at 4°C. PE-labeled isotype mouse Igs were used as negative controls. Macrophages were gated according to size and granularity on a log scale. Analysis was performed on a Coulter EPICS Elite ESP flow cytometer equipped with a 488-nm argon laser (Coulter, Hialeah, FL).

**Histopathology**

Separate groups of mice were killed 4 days after the last instillation for pathologic evaluation. Lung sections were fixed in Bouin’s solution, embedded in paraffin, cut in 0.5-μm sections, and stained with hematoxylin and eosin. The slides were blindly examined by a pathologist (M.F.). The degree of peribronchial and perivascular infiltration of inflammatory cells was evaluated by light microscopy and graded as a histology score on an arbitrary scale from 0 to 4.

**BAL and serum Ig determination**

The blood collected from the orbital sinus was centrifuged, and the sera were analyzed for the presence of specific Abs. SR-specific serum IgG (dilution 1/500) and BALF IgG and IgA levels (dilution 1/5) were measured by ELISA as described previously (24).

**Isolation and stimulation of lung T cells**

Pulmonary lymphocytes from three mice in groups 1, 2, 3, and 5 were isolated according to a previously described method (25). To this end, the lung vascular bed was flushed via the right ventricle with 5 ml cold PBS to remove any blood. The lungs were then removed, placed in RPMI 1640 medium supplemented with 10% FCS, 20 U/ml collagenase (Sigma), and 2 μg/ml DNase. Lungs were gently minced, and the mixture was incubated for 1 h at 37°C. Lung tissues were disrupted with a Pasteur pipette, and cell debris were removed by filtration through a 70-μm nylon cell strainer. The cellular suspension was centrifuged, and cell pellet was resuspended in 1 ml RPMI 1640 medium. Lymphocytes were isolated on a Ficoll-Hypaque gradient. Pulmonary lymphocytes were plated onto 24-well microplates, previously coated with mouse anti-CD3 Ab (25 μg/ml), at a concentration of 0.5 × 105 cells/ml and stimulated for 72 h with recombinant mouse IL-2 (10 ng/ml). Supernatants were collected and stored at ~70°C until the determination of IL-4, IL-10, and IFN-γ cytokine production by ELISA.

**Statistical analysis**

Comparisons between groups were performed using a one-way ANOVA. A p value of <0.05 was considered significant.

**Results**

**Effect of CTLA4-Ig on cellular infiltration**

As expected, the total number of cells (Fig. 1) in BAL of SR-sensitized untreated mice was significantly increased compared with saline-instilled animals (491 × 103 vs 40 × 103 ± 6 cells/ml recovered BALF, p < 0.0001). This increase was due mainly to an
increase in lymphocyte number. The total number of cells in SR-sensitized mice treated with the control IgG2a was not significantly different from that of SR-sensitized untreated mice ($446 \times 10^3 \pm 38$ cells/ml). Mice treated with CTLA4-Ig for the final 2 wk of Ag exposure had significantly fewer BAL cells compared with the nontreated mice: $197 \times 10^3 \pm 28 \ (p = 0.001)$. The number of lymphocytes was also lower in the CTLA4-Ig-treated group: $49 \times 10^3 \pm 9 \ (p < 0.0001) \ vs \ 219 \times 10^3 \pm 19$ for the control IgG-treated mice. Administration of CTLA4-Ig throughout Ag exposure had an even greater inhibitory effect on the cellular response to SR, with the number of total cells being $81 \times 10^3 \pm 5$, and the number of lymphocytes only $26 \times 10^3 \pm 3$ cells/ml in this group.

**Histopathology**

Histopathology examination (Fig. 2) showed a marked peribronchial and perivascular infiltration of inflammatory cells, mainly mononuclear cells, in SR and SR + control (cont)-IgG-treated mice. Lung sections from the CTLA4-Ig-treated groups, after 2 or 3 wk of treatment, had a normal bronchial epithelium similar to the one from control mice instilled with saline (A).

**FIGURE 2.** Lung section micrographs for the different groups. Histopathology showing a marked peribronchial and perivascular infiltration of inflammatory cells in SR (B) and SR + cont-IgG2a-treated cells (C). Lung sections from CTLA4-Ig treated mice after 2 (D) or 3 wk (E) of treatment had a normal bronchial epithelium similar to those from control mice instilled with saline (A).
treated groups and the saline control group (p = 0.142 for SR + CTLA4-Ig treated for 2 wk and 0.640 for SR + CTLA4-Ig treated for 3 wk).

**Expression of B7 molecules**

As for our pilot study, repeated exposure of mice to SR resulted in a marked increase of B7 receptors expression on AM. A higher percentage of cells express B7-2 compared with B7-1 (33.3% vs 0.5%), whereas no B7 expression at all is detected in saline-instilled mice (Fig. 4). A total of 35.1% of AM from mice injected with the control IgG were positive for B7-2. CTLA4-Ig treatment had no effect on SR-induced expression of B7-2 on AM, (37.6% positive cells).

---

**FIGURE 3.** Histology score. The degree of inflammatory cell infiltration was evaluated by light microscopy and graded on an arbitrary scale from 0 to 4. (0, no inflammatory cells; 1, <10%; 2, 10–25%; 3, 25–50%; 4, >50%). Data are expressed as mean ± SEM for 10 animals in each group. For each group, columns with different letters are significantly different.

**FIGURE 4.** Effect of CTLA4-Ig on AM B7 expression. SR-induced B7-2 expression on AM was not affected by CTLA4-Ig administration. Results are obtained from BAL cells pooled from 4 to 10 mice.

**FIGURE 5.** Effect of CTLA4-Ig on Ab production. SR-specific serum IgG (dilution 1/500) and BALF IgG and IgA levels (dilution 1/5) measured by ELISA according to a previously described method (16). CTLA4-Ig administration significantly reduced serum and BAL SR-specific Ig levels. Data are expressed as mean OD ± SEM for 10 animals in each group. For each group, columns with different letters are significantly different.

---

**Ab response**

CTLA4-Ig-treated mice developed much lower levels of SR-specific Abs in serum and BAL (Fig. 5) compared with SR alone or SR + cont-Ig groups (p < 0.0001). Specific IgA Abs in the lavage
fluid were also markedly lower after treatment with CTLA4-Ig ($p = 0.0002$).

Cytokine production

Cells stimulated with immobilized CD3 and recombinant murine IL-2 from groups 2 and 3 of SR-sensitized mice (Fig. 6) produced elevated levels of IFN-γ, a Th1-type cytokine (18 and 20 ng/ml, respectively), compared with cells from mice instilled with saline only (0.4 ng/ml).

IL-10 (a Th2 cytokine) levels were also high (565 and 581 pg/ml, respectively) in these groups, whereas saline-instilled mice had undetectable levels. IL-4 levels were also increased to 31 and 42 pg/ml compared with 11 pg/ml in the saline control group. CTLA4-Ig administration, given before and throughout SR exposure, resulted in a much lower production of IFN-γ, IL-10, and IL-4 (3.7 ng/ml, 45 pg/ml, and 16 pg/ml respectively) compared to the untreated groups.

Discussion

A mechanism to down-regulate T cell-mediated immune activation seen in HP has been addressed in this study. The ability of CTLA4-Ig fusion protein to bind B7 molecules with high avidity and to prevent the binding of costimulatory molecules in vivo has been used successfully in other experimental models. For example, CTLA4-Ig has beneficial effects on graft survival (26), improves autoimmune diseases (27), and decreases airway eosinophilia and hyperresponsiveness in asthma (28). In a phase 1 clinical trial in patients with psoriasis (29), CTLA4-Ig treatment resulted in a marked improvement of clinical disease activity. CTLA4-Ig reduces the humoral immune response to T cell-dependent Ags in experimental models (17).

Results from the current study show that blockade of T cell costimulation by in vivo administration of CTLA4-Ig does not affect their B7 expression, but inhibits the recruitment of lymphocytes in the BAL, cellular infiltration in lung tissue, and reduces SR-specific Ig levels in serum and BALF in response to SR Ag stimulation. This inhibitory effect was observed when CTLA4-Ig was given either throughout the period of sensitization or starting 1 wk after Ag exposure. The blunting effect was however somewhat less complete when CTLA4-Ig was administered after 1 wk of sensitization. To verify the effect of the treatment on the type of response produced, we tested the production of cytokines by lung lymphocytes. CTLA4-Ig inhibited the Ag-induced increase of both Th1-type response (IFN-γ) and Th2-type response (IL-10 and IL-4) by pulmonary T cells. In experimental models of OVA-induced asthma, CTLA4-Ig administration before Ag immunization (25) or after sensitization (30) resulted in a decreased Th2 cytokine production and an increase (25) or no changes (30) in IFN-γ secretion. However, another study (31) using CTLA4-Ig blockade at the time of sensitization showed an inhibition of both Th1- and Th2-type cytokines. The asthma and HP mouse models differ in the type of response produced after Ag sensitization. Sensitization with OVA induces a Th2-type response whereas sensitization with SR in mice produces a Th-1 pattern as seen by the ability of CD4+ Th1 cells to adoptively transfer the disease to healthy animals (9), and the involvement of IFN-γ and IL-12 in the pathogenesis of HP in mice (10, 11). However, the type of response obtained in experimental HP seems to be different from the one observed in human HP where a Th2 response seems to be more important (12–14).

Two possible mechanisms could explain the inhibitory effect of CTLA4-Ig: either an immune deviation toward a Th1 response as seen in asthma, or a global inhibition of T cell activation and differentiation as seen in our study. The observed reduction in T cell numbers in BAL and in lung tissue may be attributed to an altered T cell recruitment into the inflammatory sites probably by inhibiting expression of adhesion molecules, inhibition of local expansion of Ag-specific T cells, or increased apoptosis of these
Ag-specific T cells. The simultaneous decrease in AM could be explained by the inhibitory effect of CTLA4-Ig on lymphocyte activation and proliferation via a decrease in the production of IFN-γ. This could lead to an inhibition of AM priming to produce TNF-α and IL-1, decreasing the release of monokines such as monocyte chemotactic protein-1 and therefore attracting fewer monocytes into the lung.

This study therefore further demonstrates the importance of ongoing T cell costimulation in the maintenance of the lymphocytic alveolitis that characterizes HP. We could hypothesize that during chronic exposure to harmful environmental Ags, the use of agents that block the costimulatory pathways could provide a useful therapeutic approach for the treatment of HP.

References

nol. 144:2147.
amisms accounting for granulomatous responses in hypersensitivity pneumonitis. Sarcoi-
11. Gudmundsson, G., M. M. Monick, and G. W. Humphinghake. 1998. IL-12 mod-
19. Schwarz, J. M., and A. H. Sharpe. 1998. Studies using antigen-presenting cells lacking expression of both B7-1 (CD80) and B7-2 (CD86) show distinct require-
22. Dakhama, A., E. Israel-Assayag, and Y. Cormier. 1996. Altered immun-sup-
27. Finck, B. K., P. S. Linsky, and D. Wofsky. 1994. Treatment of murine lupus with 
CTLA4-Ig. Science 265:1225.
B7-CD28/CTLA4 costimulatory pathways are required for the development of T 