

CUTTING EDGE

Cutting Edge: B and T Lymphocyte Attenuator and Programmed Death Receptor-1 Inhibitory Receptors Are Required for Termination of Acute Allergic Airway Inflammation¹

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T cell activation is regulated by coordinate interaction of the T cell Ag receptor and costimulatory signals. Although there is considerable insight into processes that regulate the initiation of inflammation, less is known about the signals that terminate immune responses. We have examined the role of the inhibitory receptors programmed death receptor-1 and B and T lymphocyte attenuator in the regulation of allergic airway inflammation. Our results demonstrate that there is a temporally regulated expression of both the receptors and their ligands during the course of allergic airway inflammation. Following a single inhaled challenge, sensitized wild-type mice exhibit peak inflammation on day 3, which resolves by day 10. In contrast, mice deficient in the expression of programmed death receptor-1 or B and T lymphocyte attenuator have persistent inflammation out to 15 days following challenge. Thus, these receptors are critical determinants of the duration of allergic airway inflammation. The Journal of Immunology, 2006, 176: 3909–3913.

T cell activation requires the integration of signals derived from the TCR in combination with costimulatory and/or inhibitory receptors. Positive costimulatory receptors expressed by T cells include CD28 and ICOS, which augment both the priming and effector activity of T lymphocytes (1). In contrast, CD152 (CTLA-4), B and T lymphocyte attenuator (BTLA),³ and programmed death receptor-1 (PD-1) are inhibitory receptors that dampen the T cell response (2, 3). CTLA-4 has been thought to be important in regulating the early activation of T lymphocytes. The point of regulation at which PD-1 and BTLA influence immune responses is less

clear. Both are expressed primarily on activated T cells, suggesting that they may regulate effector function (3–5). Deficiency in PD-1 results in a loss of cell tolerance characterized by the gradual acquisition of autoimmune disease (6, 7). BTLA deficiency has not yet been reported to cause spontaneous autoimmunity, but did lead to an enhanced susceptibility to experimental allergic encephalomyelitis (3).

Allergic airway inflammation is a Th2-mediated immune response in the lung. Following a single allergen challenge, sensitized mice develop an eosinophilic inflammatory cell infiltrate around airways and blood vessels, which spontaneously resolves (8). Although CD28 and ICOS mediate the initiation and maintenance of airway inflammation, the basis for resolution has not been fully explored (9–12). In this study, we examined the role of BTLA and PD-1 in acute allergic airway inflammation by analysis of mice deficient in these inhibitory receptors. Although there was a minor role for these in regulating the intensity of acute airway inflammation, the receptors were crucial in limiting the duration of inflammation. This result correlated with the relatively late induction of the ligand for BTLA, herpes virus entry mediator (HVEM), and enhanced expression of the ligands for PD-1 at later times. These results suggest that the inhibitory receptors BTLA and PD-1 act as terminators of an established immune response, and may be important for limiting the extent and duration of inflammation at peripheral sites.

Materials and Methods

Mice

BTLA-deficient mice (strain Btla^{tm1kmm}) in the C57BL/6 background were generated as described previously (3). PD-1-deficient mice (strain Pdcd1^{tm1hon}) in the C57BL/6 background were obtained from Tasuka Honjo (Kyoto University, Kyoto, Japan). C57BL/6 mice were purchased from The Jackson Laboratory. All mice were housed in specific pathogen-free facilities at Washington

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Received for publication November 30, 2005. Accepted for publication January 27, 2006.

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¹ This work was supported in part by Grant HL062683 (to J.M.G.) from the National Institutes of Health.

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³ Abbreviations used in this paper: BTLA, B and T lymphocyte attenuator; PD-1, programmed death receptor-1; HVEM, herpes virus entry mediator; RPA, RNase protection assay; mTEC, murine tracheal epithelial cell; BAL, bronchoalveolar lavage.

University School of Medicine (St. Louis, MO). All animal studies have been approved by the Washington University Animal Studies Committee.

Antibodies

Anti-BTLA Ab (Clone 6F7, mouse IgG1) was generated as described previously (13). Anti-PD-1 and anti-CD4 Abs were purchased from eBioscience. Flow cytometric analysis was performed on a FACSCalibur cytometer using CellQuest software (BD Biosciences). Analysis was performed using FlowJo software.

Muc5AC staining

For Mucin 5AC immunostaining, Ag retrieval was performed using Ag Unmasking solution (Vector Laboratories). Lung sections were blocked with 2% fish gel in PBS and then incubated with biotinylated anti-mucin 5AC mAb (Neomarkers) at a final concentration of 2 μ g/ml. Ab binding was visualized with Vectastain ABC Elite kit (Vector Laboratories), diaminobenzidine substrate, and hematoxylin counterstain.

RT-PCR and RNase protection assay (RPA)

Total RNA was extracted from lung tissue of control or allergen-challenged mice using TRIzol (Invitrogen Life Technologies). Random primed cDNA was prepared using the Retroscript kit (Ambion). Specific primers for PDL1, PDL2, and HVEM were designed that spanned intronic sequences. Control primers amplify ribosomal S15 RNA and are provided with the Retroscript kit. Multiprobe RPA was performed on total lung RNA using the mCK-1 probe set per the manufacturer's directions (BD Pharmingen).

Experimental allergic airway inflammation

Mice were sensitized and challenged with OVA as described previously (11). Briefly, mice received injections i.p. with OVA adsorbed to alum on days 0 and 7. On day 14, they received an intranasal challenge of 50 μ l of 2% OVA in the morning and afternoon. Samples were collected as previously described on the indicated days following inhaled challenge (11).

Preparation of murine tracheal epithelial cells (mTEC)

Primary mouse airway epithelial cells were cultured and differentiated using an established model of the mouse airway (14, 15). Briefly, epithelial cells were harvested from mouse tracheas of C57BL/6 mice (5- to 6-wk-old) using pronase digestion and differential adherence to yield a preparation composed of >99% epithelial cells. Mouse tracheal epithelial cells were cultured in the presence of growth factor-supplemented medium on semipermeable membranes (Transwell; Corning-Costar) and then cultured at air-liquid interface to generate a multilayer model of the airway composed of ciliated, secretory, and basal airway epithelial cells. RNA was prepared from day 7 ALI cultures using TRIzol reagent.

Results and Discussion

Regulated expression of PD-1 and BTLA during acute allergic airway inflammation

We first determined the kinetics of lymphocyte accumulation and receptor expression *in vivo* by examining the cells recovered

in the bronchoalveolar lavage (BAL) fluid at days 1, 3, 4, or 7 following challenge. CD4⁺ T cells appeared in the BAL by day 3 and peaked on day 7 (Fig. 1). Staining for PD-1 and BTLA revealed that PD-1 expression gradually increased, being detectable on day 3 and reaching its maximum on day 7 following challenge. BTLA expression exhibited a reciprocal pattern with expression being greatest on day 3 and nearly undetectable by day 7 (Fig. 1).

We next examined the allergic response of mice deficient in either BTLA or PD-1 at 3 days following allergen challenge (Fig. 2). Both BTLA-deficient and PD-1-deficient mice showed an increase in inflammatory cell recruitment compared with wild-type mice (Fig. 2, *A* and *B*). All genotypes had a mixed inflammatory cell infiltrate; however, there was an increased percentage of neutrophils and eosinophils in the BTLA-deficient mice (Fig. 2, *A* and *B*). Examination of the lung tissues revealed an increase in the intensity of inflammatory infiltrates in PD-1 and BTLA-deficient animals compared with wild-type controls (Fig. 2*C*). Goblet cell metaplasia was apparent in all genotypes as determined by immunostaining for Muc5AC (Fig. 2*D*). Thus, consistent with PD-1 and BTLA functioning as inhibitory receptors in other settings, we found that loss of their function caused a small increase in the initial inflammatory response to allergen.

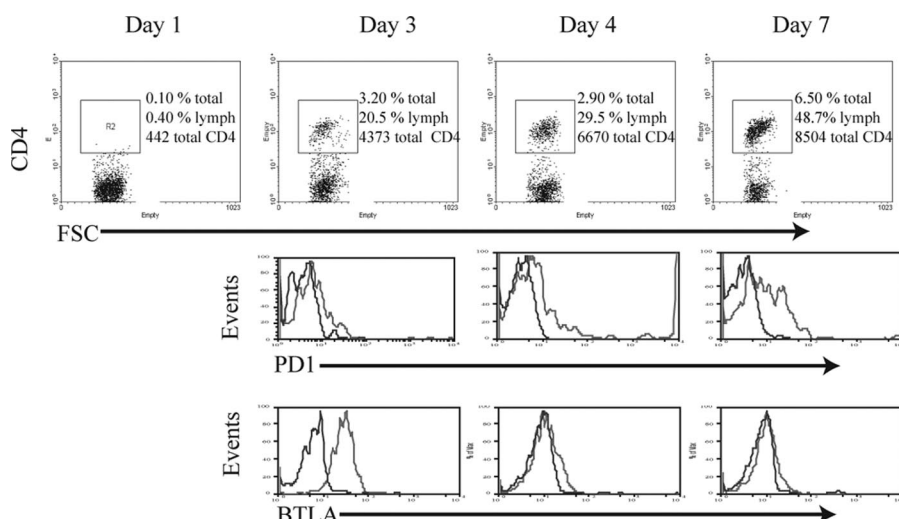
Delayed expression of ligands for BTLA and PD-1 in acute allergic airway inflammation

We next examined the kinetics of expression of the ligands for each receptor. HVEM, the ligand for BTLA, was nearly undetectable in the first 4 days, but became detectable by day 7 and was maximal by day 10 and 15 (Fig. 3, *upper panels*). PDL1 expression was detectable early and increased over time, reaching a maximum between days 10 and 15 postchallenge. Expression of PD-L2, a second ligand for PD-1, was maximal at day 4 following intranasal challenge, and declined subsequently (Fig. 3). Both HVEM and PDL1 were detectable in RNA samples obtained from cultured mTEC, suggesting that the source of ligand may be nonimmune cells of the lung (Fig. 3, *lane 8*).

BTLA and PD-1 limit the duration of allergic airway inflammation

Given the observed kinetics of ligand expression, we next examined the allergic response at day 10 and day 15 following intranasal challenge (Fig. 4). Wild-type mice completely resolved the inflammation by day 10, as evidenced by a low number of cells recovered

FIGURE 1. PD-1 and BTLA are expressed on BAL CD4⁺ T cells. C57BL/6 mice were sensitized and challenged with OVA. On days 1, 3, 4, and 7 following challenge, groups of mice were euthanized and the cells recovered in the BAL analyzed for expression of CD4 and PD-1 or BTLA by two-color flow cytometry. The percentage of cells positive for CD4 as a fraction of either the total sample or of the lymphocyte gate as well as the total number of CD4⁺ cells recovered is indicated in each box. Histograms of PD-1 or BTLA expression on the CD4⁺ cells are shown for days 3, 4, and 7. Isotype staining is shown in black, and PD-1 or BTLA is illustrated in gray. Representative data of three independent experiments are presented.



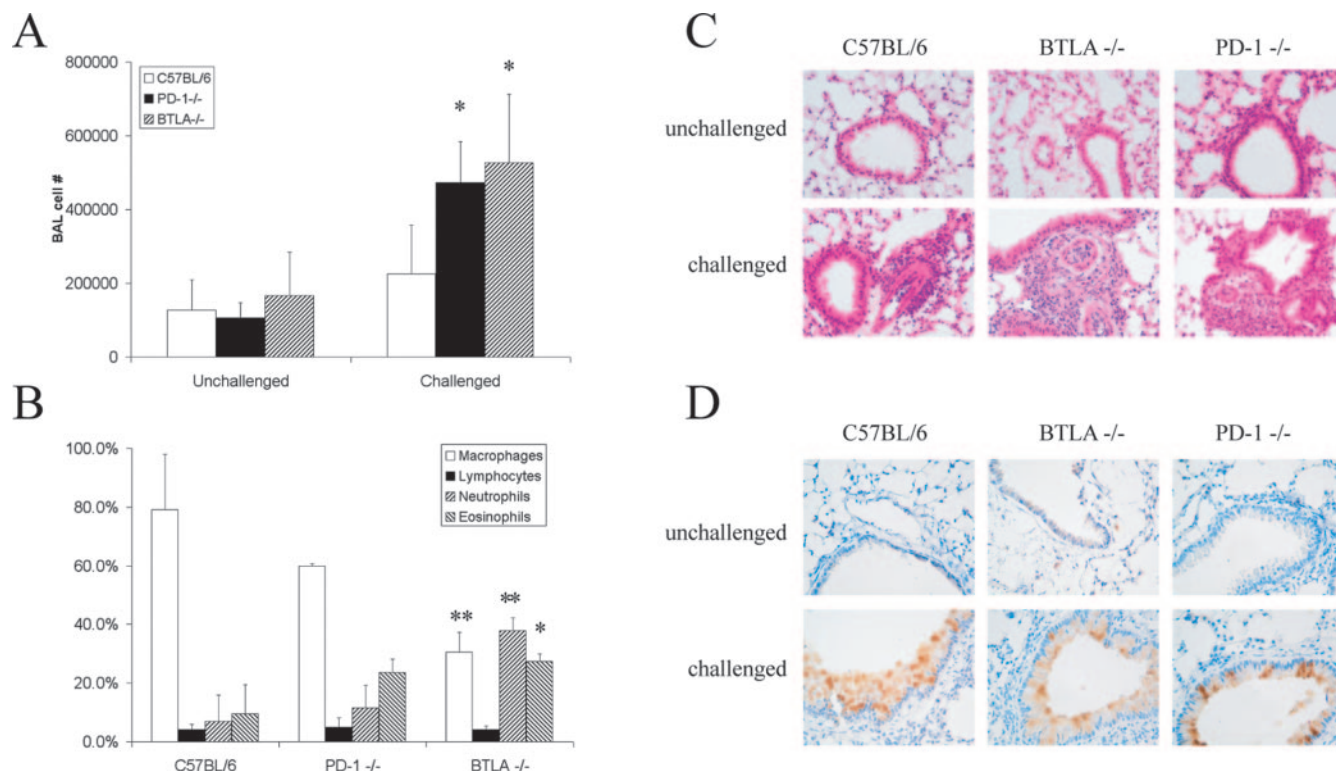


FIGURE 2. PD-1 and BTLA have a minor effect on acute allergic airway inflammation. C57BL/6, PD-1^{-/-}, and BTLA^{-/-} mice ($n = 5$ per group) were sensitized and challenged with OVA. Three days following challenge, the mice were euthanized and samples collected for analysis. *A*, Total cell counts in the BAL fluid. *B*, Differential analysis of the cell types present in the BAL. *C*, Representative fields of H&E-stained sections or (*D*) stained with anti-Muc5AC Ab (magnification, $\times 40$). *, $p < 0.05$; **, $p < 0.005$ compared with C57BL/6 $\times$ two-tailed t test. Representative data from five independent experiments are shown.

in the BAL fluid and histology (Fig. 4*A*). By contrast, mice deficient in BTLA and PD-1 showed a persistent increase in BAL cells on day 10 following intranasal challenge. Furthermore, the composition of these cells in this fluid revealed a greater proportion of lymphocytes and eosinophils in comparison to the few cells in the wild-type mice, which consisted predominantly of macrophages (Fig. 4*A*). Even on day 15, examination of BTLA-deficient mice revealed the continued presence of increased numbers of lymphocytes and eosinophils. Direct histological examination of H&E-stained sections also demonstrated persistent inflammation and mucus cell metaplasia in the lungs of both PD-1 and BTLA-deficient mice at days 10 and 15, whereas the wild-type mice had complete resolution in this time frame (Fig. 4*B*). Thus, these inhibitory receptors are critical for resolving airway inflammation.

To determine whether there were qualitative differences in the cytokine profiles, we performed a multiprobe RPA on total lung RNA isolated at day 3, 10, and 15 after challenge. Samples obtained on day 3 postchallenge revealed expression of IL-4, IL-5, IL-10, IL-13, and γ -IFN in all mice with no difference between strains (data not shown). Overall, cytokine mRNA levels were decreased on days 10 and 15; however, there was a persistence of mRNA for IL-10 and γ -IFN in the PD-1-deficient mice (Fig. 4*C* and data not shown). Of the four BTLA-deficient mice, one demonstrated persistence of cytokine message, whereas none of the wild-type mice had detectable message. Similar results were seen in samples collected on day 10 following challenge (data not shown). As we have previously observed, IL-15 was detected in all samples including unchallenged mice (Ref. 10 and data not shown).

T cell-dependent immune responses are determined by the integration of signals derived from both cell:cell interactions

and soluble mediators. We have recently described a novel role for CD28 signaling not only in the early priming phase, but also in maintenance of the effector phase of allergic airway inflammation (11). These studies focused on an acute model, acting between days 1 and 3. By contrast, the present results show that the inhibitory receptors BTLA and PD-1 exert only a slight effect in attenuating the degree of acute inflammation but instead exert a profound effect on the duration of inflammation, suggesting they act to terminate the immune response. We also observed a temporal regulation of expression of the ligands for these receptors during the course of the inflammatory response. The differences in the kinetics of receptor and ligand expression for PD-1 and BTLA suggest that they may act at different times in the immune response. Alternatively, it remains possible that signaling through one regulates the expression or function of

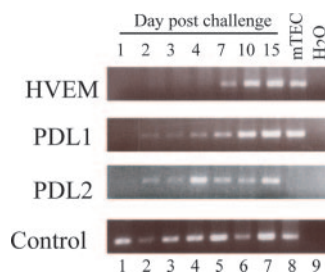


FIGURE 3. Expression of the ligands for PD-1 and BTLA during allergic airway inflammation. Total RNA was isolated from whole lungs of allergen-challenged mice on the indicated days postchallenge or from primary cultured mTEC. RT-PCR was performed using specific primers that spanned intronic sequences of each gene. Shown are representative data from two independent experiments.

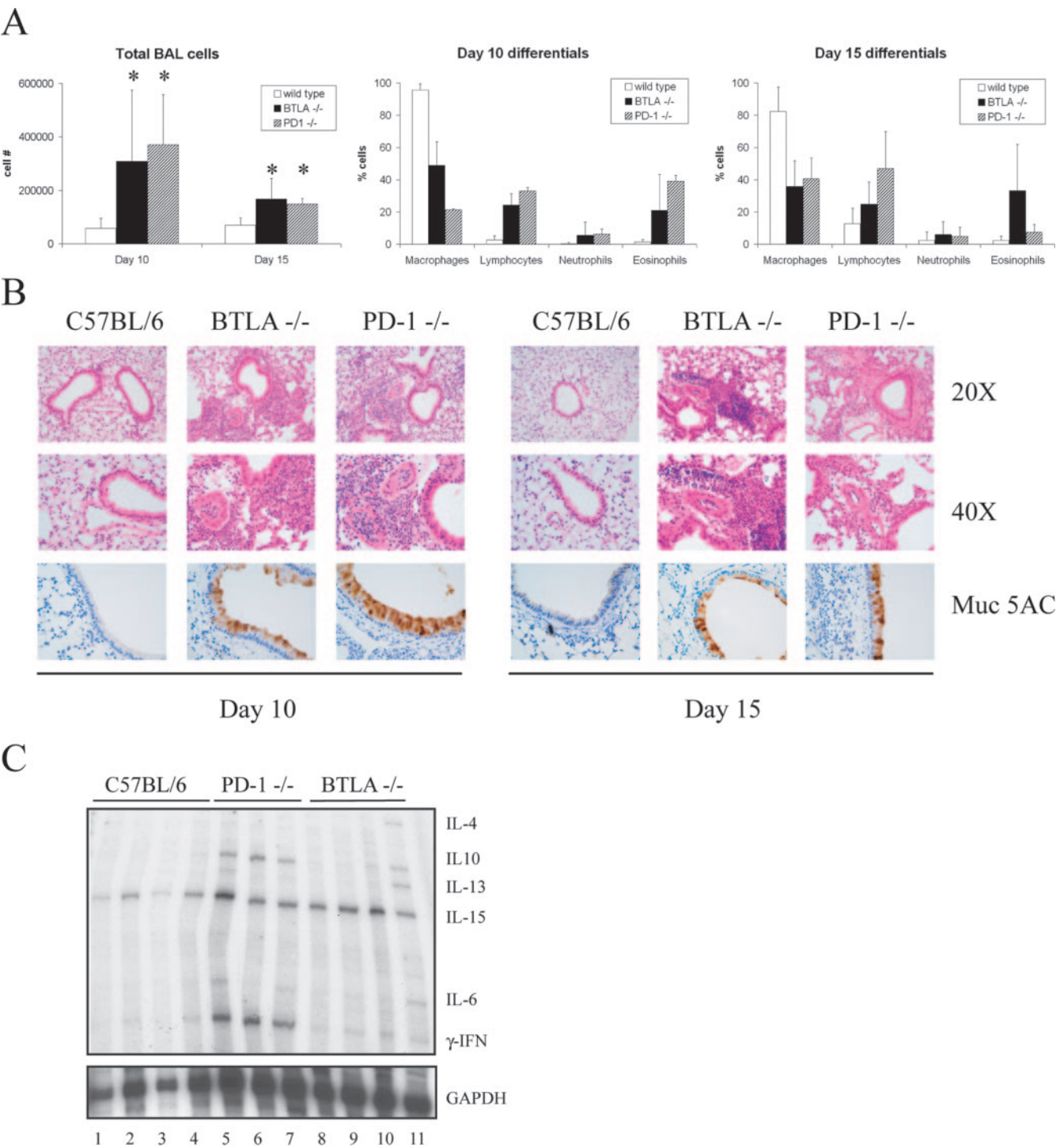


FIGURE 4. PD-1 and BTLA-deficient mice have a prolonged duration of airway inflammation. C57BL/6, PD-1 $^{-/-}$, and BTLA $^{-/-}$ mice were sensitized and challenged with OVA. On days 10 and 15 cohorts of mice ($n = 5$ /group) were euthanized and samples collected for analysis of the BAL (*A*) and histology and Muc5AC staining (*B*). *C*, Cytokine mRNA was assayed by multiprobe RPA on total lung RNA isolated 15 days following challenge. Representative data from three independent experiments are presented. *, $p < 0.05$ compared with C57BL/6 using a two-tailed t test.

the other. Nonetheless, these data support that the regulated expression of inhibitory receptors on lymphocytes and their ligands in the lung are critical for the proper termination of the acute inflammatory response. We propose, based on these findings, that abnormalities in this immune axis could play a role in pathologic situations such as chronic persistent asthma and may represent novel targets for therapeutic intervention.

Acknowledgments

We thank Dr. Steven L. Brody (Washington University, St. Louis, MO) for providing mTEC RNA for analysis, and Dr. T. Honjo for providing the PD-1-deficient mice.

Disclosures

The authors have no financial conflict of interest.

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