B and T Lymphocyte Attenuator Regulates T Cell Survival in the Lung

Christine Deppong, Jessica M. Degnan, Theresa L. Murphy, Kenneth M. Murphy and Jonathan M. Green

*J Immunol* 2008; 181:2973-2979; doi: 10.4049/jimmunol.181.5.2973

http://www.jimmunol.org/content/181/5/2973

References

This article cites 12 articles, 5 of which you can access for free at: http://www.jimmunol.org/content/181/5/2973.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

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
B and T Lymphocyte Attenuator Regulates T Cell Survival in the Lung

Christine Deppong,*‡ Jessica M. Degnan,* Theresa L. Murphy,† Kenneth M. Murphy,†§ and Jonathan M. Green2**†

The initiation, intensity, and duration of T cell-directed inflammatory responses are dependent upon the coordination of both activating and inhibitory signals mediated by specific receptors on the T lymphocyte. The recently described receptor, B and T lymphocyte attenuator (BTLA), has been demonstrated to have an important role in limiting the duration of inflammation in a murine model of allergic asthma. In this study, we have examined the role of BTLA on the proliferation, recruitment, and survival of T cells in response to inhaled allergen. We find that there is decreased cell death in T cells from BTLA-deficient mice, whereas proliferation and recruitment to the lungs are unchanged. Thus, the regulation of cell death through BTLA signaling is a key determinant of the inflammatory response in the lung. The Journal of Immunology, 2008, 181: 2973–2979.

The lung faces unique challenges, in that it is continuously exposed to a variety of potentially noxious agents from the environment, yet must maintain a delicate architecture to allow for efficient gas exchange. Thus, immune responses in the lung are tightly regulated to protect the host without interfering with lung function. Individuals with allergic asthma respond to inhalation of allergens with the development of chronic inflammation. This inappropriate immune response results in the phenotype of airway hyperresponsiveness, excessive mucus production, and, in some instances, airway remodeling that may lead to long-term structural change.

To study the pathophysiology of this disease, we and others have used a murine model of experimental allergic airway inflammation (1). This model recapitulates aspects of the human disease and has been used to demonstrate the importance of costimulatory pathways, including CD28 and ICOS (2, 3). Recently, we have shown the importance of the receptors B and T lymphocyte attenuator (BTLA) and programmed death receptor-1 (PD-1) in regulating airway inflammation (4). Depending on the system, both PD-1 and BTLA have been observed to have either stimulatory or inhibitory effects on T cell function (5, 6). However, mice deficient in either of these receptors have a prolonged duration of inflammation following a single allergen challenge, suggesting primarily an inhibitory role in this setting (4). The role of these receptors in settings of chronic exposure and the mechanism by which they inhibit inflammation have not been fully defined. In this manuscript, we demonstrate that despite the hyperproliferative nature of BTLA-deficient cells in vitro, there is no evidence for increased proliferation in vivo. Adoptive transfer studies show that the phenotype is T cell intrinsic and that there is similar recruitment of Ag-specific T cells to the lung in both BTLA−/− and BTLA+/− backgrounds. Measurements of cell death demonstrate a decrease in the number and percentage of apoptotic T cells in the lungs of BTLA-deficient mice. Thus, the prolonged duration of inflammation in BTLA-deficient mice appears to be mediated primarily by increased T cell survival.

Materials and Methods

Mice

BTLA-deficient mice in the C57BL/6 background were generated, as previously described (6). The PD-1-deficient mice in the C57BL/6 background were originally obtained from T. Honjo (Kyoto University, Kyoto, Japan) (7) and bred at our facility. Mice deficient in BTLA and PD-1 on the C57BL/6 background were generated by a simple cross of the two strains and bred in our facility. DO11.10 and BTLA-deficient DO11.10 mice were bred, as previously described (8). C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory. All mice were bred and housed in specific pathogen-free facilities at Washington University School of Medicine. All animal studies have been approved by the Washington University Animal Studies Committee.

Antibodies

All Abs were purchased from eBiosciences, unless otherwise specified.

Allergic airway inflammation

Mice were primed and challenged with OVA, as previously described (4). Briefly, mice received i.p. injections of OVA adsorbed to alum on days 0 and 7. On day 14, the mice were intranasally challenged with 50 μl of 2% OVA in PBS in both the morning and afternoon. In some experiments, the mice received intranasal challenges on multiple days, as described in Results, and samples were harvested on the indicated days subsequent to challenge. Lung and bronchoalveolar lavage (BAL) specimens were harvested and analyzed, as previously described (9). All experiments were performed a minimum of three times, and a representative experiment is shown.
BrdU incorporation and measurement of in vivo proliferation and cell death

To measure proliferation during the effector phase, BrdU (1 mg/ml; Sigma-Aldrich) was added to the drinking water for 24 h before collection of tissue specimens. BrdU incorporation was determined by flow cytometry using the FITC BrdU Flow Kit (BD Pharmingen). All samples were costained with 7-aminoactinomycin D (7-AAD) to determine DNA content. Cells with a sub-G1 DNA content were identified as apoptotic.

For measurement of proliferation during priming, splenocytes were isolated from wild-type or BTLA-/- DO11.10 mice, and labeled with CFSE and 1 x 10^7 cells injected i.v. into naive BALB/c mice. The following day, the mice were injected i.p. with OVA/alum, and mesenteric lymph nodes were harvested 72 h later. The CFSE dye dilution profile was determined by flow cytometry, gating on CD4+ T cells.

Cell death was also assayed by annexin V staining. Cells recovered in the BAL from wild-type or BTLA-deficient mice were stained with allophycocyanin-conjugated annexin V, CD4-FITC, and 7-AAD and analyzed by flow cytometry. The annexin V and 7-AAD staining of the CD4-positive cell population is shown.

Immunohistochemistry

Lungs were harvested and frozen in OCT compound on dry ice and kept at -80°C until use. Frozen sections were prepared (6 μm) and fixed in acetone, and endogenous peroxidase was quenched using a mixture of methanol and hydrogen peroxide. Sections were blocked with 5% goat serum and primary Ab, or isotype controls were added and incubated overnight at 4°C. The HRP-conjugated secondary Ab was then added and incubated at room temperature. Detection was completed through the use of the HistoMark ORANGE Substrate System (Kirkegaard & Perry Laboratories). The sections were counterstained with Methyl Green.

Adoptive transfer

Splenocytes from DO11.10 TCR transgenic BALB/c BTLA-/- and BTLA-/- mice were isolated and activated in vitro with 0.3 μM OVA peptide in the presence of rIL-4 (R&D Systems) and anti-IL-12 to promote Th2 differentiation. Seven days after the initial stimulation, the Th2 profile was confirmed by measuring intracellular staining and FACS analysis for IL-4 and IFN-γ. A total of 2 x 10^6 wild-type or BTLA-/- Th2 cells, labeled with CFSE, was then transferred by i.v. injection to naive wild-type or BTLA-/- BALB/c mice. One day later, the mice were challenged with OVA. Specimens were collected for analysis 3 and 10 days following challenge. All experiments were performed a minimum of three times, and a representative experiment is shown.

FIGURE 1. Prolonged inflammation in the lungs of BTLA-/- mice. C57BL/6 BTLA+/- or BTLA-/- mice were sensitized and challenged, as described. On the indicated days, mice were euthanized and specimens were collected (n = 5 mice per group). The mean number of BAL cells recovered at each time point is shown. * p < 0.05, compared with wild-type cell counts on the same day by two-tailed Student’s t test.

FIGURE 2. Absence of BTLA leads to increased airway inflammation in a repeated Ag exposure model. A, Timeline of chronic allergic airway model. B, Representative H&E-stained sections of lung tissue obtained on days 42 and 50 (magnification, ×40). Cell counts (C) and cell differentials (D) from the BAL of wild-type or BTLA-/- mice (n = 4 or 5 per group; shown is mean ± SD). * p < 0.05; ** p < 0.005; *** p < 0.0005, by two-tailed Student’s t test compared with wild-type mice on the same day.
Results

**BTLA limits the duration of airway inflammation following both single and repeated allergen challenges**

In studying the regulation of acute allergic airway inflammation, we had previously noted that mice deficient in BTLA had persistent inflammation at time points that wild-type mice had successful resolution (4). Consistent with this, we found an increased number of total inflammatory cells in the BAL from BTLA-deficient mice at days 10 and 15 following inhaled allergen challenge (Fig. 1). Histologic examination confirmed the presence of peribronchial and perivascular inflammation in the lungs from the BTLA-deficient mice at all time points examined, whereas by day 7 the lungs from wild-type mice had no evidence of inflammation (data not shown).

Because most individuals with asthma are subject to repetitive allergen exposure, we tested whether BTLA was also important under such experimental conditions or if other factors served to limit inflammation following multiple challenges (Fig. 2). Similar to our observations following a single challenge, BTLA-deficient mice had persistent inflammation as far out as 15 days following the final challenge, whereas wild-type mice resolved the inflammation. As expected, the inflammatory cell infiltrate consisted of multiple cell types, including lymphocytes and eosinophils. Thus, BTLA is important in terminating inflammation following both acute and repeated administration of allergen.

**Expression of costimulatory and inhibitory receptor ligands in the lung**

In addition to BTLA, PD-1 also has an important role in terminating lung inflammation. Because both receptors require engagement by specific ligands to signal, we determined the expression of the ligands on cells isolated by BAL as well as on lung tissue at various time points following allergen challenge (Fig. 3). Flow cytometric analysis demonstrated that expression of the ligands for CD28 (CD80 and CD86) was differentially regulated. CD80 expression peaked on day 3 and then returned to baseline, whereas CD86 expression was induced by day 3 and remained elevated throughout. PDL1 expression on the non-CD4 population of cells peaked at day 3 and then declined, although expression did not return to baseline by day 15. PDL2 expression was not detected (data not shown). Herpes virus entry mediator (HVEM) expression, as detected by staining with BTLA tetramers, was induced on day 3 and remained elevated throughout. Staining of lung tissue revealed expression of both CD86 and PDL1 in challenged, but not unchallenged lungs (Fig. 3B). CD86 expression peaked on day 3 and then returned to baseline, whereas CD86 expression was induced by day 3 and remained elevated throughout.
was greatest at day 3, with some staining visible on days 5 and 7. In contrast, PDL1 staining was detected at a low level throughout the time course. We were unable to detect expression of CD80, PDL2, or HVEM by immunohistochemistry.

**BTLA and PD-1 serve redundant roles in terminating airway inflammation**

In a murine cardiac transplantation model, the function of BTLA was dependent on induction of PD-1. To determine whether this was the case in our model, and also to ascertain whether the two receptors function in distinct or overlapping roles, we bred mice deficient in both receptors. These mice were then sensitized and challenged with OVA, and specimens were collected 3 and 10 days following challenge (Fig. 4). At either time point, we saw no difference in the number or type of inflammatory cells recovered in the BAL than was observed in the BTLA- or PD-1-deficient mice, although all were greater than wild-type controls. The lack of an additive or synergistic effect suggests that in this circumstance these receptors function in a redundant manner, although in other conditions it remains possible that these receptors function nonredundantly. Similarly, these data demonstrate that the function of BTLA in this model is not dependent on PD-1 expression and vice versa.

**BTLA-deficient T cells do not have increased proliferation in vivo**

Increased clonal expansion either during the initial priming or subsequent effector response might account for the prolonged inflammation observed in the BTLA-deficient mice. To examine the

![FIGURE 4. PD-1 and BTLA have redundant functions in the acute allergic airway model. Wild-type C57BL/6 mice or mice lacking either BTLA, PD-1, or both BTLA and PD-1 (n = 4 or 5 mice per group) were immunized and challenged with OVA, and specimens were collected 10 days following challenge. Cell counts (A), differentials (B), and representative sections (C) of lung tissue from challenged mice stained with H&E (magnification, ×40) are shown.](http://www.jimmunol.org/Downloadedfrom)

![FIGURE 5. Proliferation of lymphocytes during the priming and effector phase of allergic airway inflammation. Naive BALB/c mice were injected with CFSE-labeled wild-type DO11.10 (A) or BTLA−/− DO11.10 (B) splenocytes and immunized with OVA/alum, and the CFSE profile was determined on CD4+ cells from mesenteric lymph. Data shown are representative of three independent experiments. Wild-type or BTLA−/− mice that had been immunized and challenged with OVA were fed BrdU in the drinking water for 24 h before harvest, and the incorporation of BrdU was determined in CD4+ cells from the BAL cells using flow cytometry. C, BrdU incorporation was determined on day 3 after inhaled challenge. D, BrdU incorporation of CD4+ cells in the BAL on days 7, 10, and 15 following challenge. Shown are means ± SD of five mice per group. *, p < 0.05 in comparison with day 7 time point for both wild type and BTLA−/−.](http://www.jimmunol.org/Downloadedfrom)
former possibility, we isolated T cells from BTLA\(^{+/+}\) or BTLA\(^{-/-}\) DO11.10 mice and labeled them with CFSE. The cells were then injected into naive BALB/c mice, which were then immunized i.p. with OVA/alum. Mesenteric lymph nodes were harvested 72 h following immunization, and proliferation was determined by analysis of the CFSE dilution profile. As shown in Fig. 5, A and B, BTLA-sufficient and BTLA-deficient T cells proliferated to a similar extent. To examine proliferation during the effector response, mice that had been immunized and challenged were fed BrdU in their drinking water for 24 h before specimen collection (Fig. 5, C and D). Incorporation of BrdU in lung CD4\(^{+}\) and CD4\(^{-}\) lymphocytes was similar between wild-type and BTLA-deficient T cells (Fig. 5, C and D, and data not shown). Thus, within the sensitivity limits of these assays, we were unable to detect a difference in vivo proliferation.

**BTLA-deficient T cells are both necessary and sufficient to mediate prolonged lung inflammation**

Although we were unable to detect a difference in the proliferation of lymphocytes between BTLA\(^{+/+}\) and BTLA\(^{-/-}\) recipients, it remained possible that undetected differences in the expansion of lymphocytes following priming or alterations in cell migration accounted for the increased duration of inflammation. In addition, because BTLA is expressed on both T and non-T cells, the phenotype might be intrinsic to the T cell or, alternatively, due to BTLA expression on a non-T cell. To address these questions, we adoptively transferred Th2-skewed wild-type or BTLA-deficient OVA-specific DO11.10 TCR transgenic CD4\(^{+}\) T cells into naive wild-type or BTLA-deficient BALB/c mice (Fig. 6). This approach allowed us to study the effector phase in isolation from priming. Normalization of the number of cells transferred into each mouse eliminated any differences in cell expansion. Before transfer, intracellular cytokine staining confirmed that wild-type and BTLA-deficient T cells expressed similar amounts of IL-4 and negligible IFN-\(\gamma\) (data not shown). One day following transfer, recipient mice were challenged intranasally with OVA, and lungs were harvested 3 and 10 days following challenge. Both wild-type and BTLA-deficient T cells mediated airway inflammation when examined at day 3 following challenge. However, only mice receiving BTLA-deficient T cells had inflammation at day 10 following transfer. This was true in both wild-type and BTLA-deficient recipients. Thus, these data demonstrate that the phenotype is T cell intrinsic and does not depend on generating an increased number of Ag-specific T cells during priming.

Staining for the clonotypic TCR revealed that similar numbers of transferred T cells were present in the BAL on day 3, suggesting that increased recruitment of Ag-specific T cells is unlikely to account for our findings (Fig. 6B). Examination of the CFSE profile of the transferred cells was no different between groups, consistent with the BrdU data (data not shown). Thus, the prolonged duration of inflammation in mice receiving BTLA-deficient T cells is due neither to increased recruitment nor proliferation of lung lymphocytes, suggesting a mechanism that involves alterations in either cell retention or survival.

**BTLA-deficient T cells have enhanced survival in the lung**

Because no differences in the proliferation or recruitment of T cells to the lung were detected, we next examined cell survival. Mice were administered BrdU in their drinking water for 24 h before specimen collection, and the BAL cells were stained with anti-BrdU Ab and 7-AAD. Apoptotic CD4\(^{+}\) T cells were identified as those with sub-G\(_1\) DNA content as determined by 7-AAD staining. This analysis revealed that fewer CD4\(^{+}\) T cells from the lungs of BTLA-deficient mice undergo apoptosis than those from wild-type mice (Fig. 7A). To confirm this finding, we also stained cells recovered in the BAL with annexin V and 7-AAD. At day 3 following inhaled challenge, there was a greater percentage of viable CD4\(^{+}\) T cells (defined as negative for both annexin V and 7-AAD staining) recovered from the BTLA-deficient mice than wild type (Fig. 7B). This trend was also apparent at later time points, supporting the findings by BrdU and 7-AAD staining (data not shown). Similarly, the percentage of cells that were both annexin V and 7-AAD positive is consistent with the findings shown in Fig. 7A. Thus, these data suggest that BTLA-deficient T cells have a
survival advantage, and this may be in part the mechanism leading to prolonged allergic airway inflammation.

**Discussion**

Chronic lung inflammation in allergic asthma can result in both significant symptoms and long-term damage to the lung. Over the past several years, there has been an increased appreciation that the primary defect of this disorder may lie within the immune system, rather than be intrinsic to the lung itself.

Several studies have affirmed the importance of T cells in the pathophysiology of airway inflammation (10). Although much is understood about the positive regulation of T cell function, considerably less is known about how immune responses are terminated in vivo. Previously, we have shown that the inhibitory receptors BTLA and PD-1 are integral to the termination of acute allergic airway inflammation (4). Our previous work examined only an acute model, in which the mice were exposed to allergen only on a single day. However, this is not reflective of the normal disease process, because asthmas are repetitively exposed to allergen. To mimic this more closely, we exposed mice to multiple inhaled challenges. As in the acute model, airway inflammation persisted for a significantly greater duration in the BTLA-deficient mice.

Given the similarity of the phenotype of the PD-1 and BTLA-deficient mice in the acute allergic airway inflammation model, we examined whether mice deficient in both receptors might have a more profound phenotype. However, we did not detect any difference in the double-deficient mice as compared with either of the singly deficient strains, suggesting functional redundancy in these circumstances. In a model of cardiac transplantation, BTLA deficiency resulted in prolonged graft survival (5). In this instance, it was associated with an induction of PD-1 expression, and BTLA/PD-1 double knockouts rejected the allograft in a time frame similar to wild type. However, we find that the prolonged airway inflammation in BTLA-deficient mice is not mediated by PD-1.

The increased duration of inflammation in the BTLA-deficient mice might reflect either alteration in the migration of cells to and from the lung, increased proliferation of the cells, increased cell survival, or some combination of these factors. Given that in vitro studies had demonstrated that BTLA could inhibit T cell proliferation, we first tested whether we could detect increased proliferation in vivo (8). Our studies failed to demonstrate any difference in the initial expansion of BTLA-deficient T cells following priming or in the subsequent proliferation at the site of inflammation. To further test this, as well as examine the recruitment of Ag-specific T cells to the lung, we adoptively transferred either Th2-skewed wild-type or BTLA-deficient DO11.10 TCR transgenic T cells into wild-type or BTLA-deficient naive BALB/c mice. These cells are specific for the OVA323–339 peptide and are recognized by the clonotypic Ab KJ1-26. This approach allowed us to assure that equal numbers of effector T cells were present in each genotype and to determine the number of Ag-specific T cells recruited to the lung. Transfer of BTLA-deficient cells into either wild-type or BTLA-deficient BALB/c mice recapitulated the phenotype of prolonged inflammation. Thus, augmented expansion of effector cells during priming is unlikely to be responsible for the increased duration of airway inflammation. We also detected similar numbers of transferred T cells in the BAL of mice receiving either wild-type or BTLA-deficient cells, suggesting that increased recruitment is not the mechanism.

In addition to being expressed on T cells, BTLA is also found on B cells and on some dendritic cells. Given this, the phenotype we observed may be due to defects in the T or non-T cell population of BTLA-expressing cells. Adoptive transfer of BTLA-deficient T cells into either wild-type or BTLA-deficient recipient mice recapitulated the phenotype of prolonged airway inflammation, whereas wild-type T cells did not; thus, the phenotype is T cell intrinsic.

Given that we were unable to detect any differences in T cell proliferation or recruitment, we examined cell survival. Staining of the cells recovered in the BAL with anti-BrdU and 7-AAD allowed us to identify the percentage of apoptotic cells in the lung. We found less cell death in BTLA-deficient cells at days 10 and 15. Similarly, staining with annexin V and 7-AAD revealed a ~2-fold increase in the percentage of viable cells recovered from BTLA-deficient mice at day 3, the time of peak inflammation. This trend was also apparent at later time points (data not shown). Apoptosis is a highly dynamic process, and apoptotic cells are typically rapidly cleared. Thus, at any given moment, the number of apoptotic cells detected is only a snapshot of the in vivo environment. However, the difference between wild-type and BTLA-deficient mice was only on the order of 2-fold; improved cell survival would appear to contribute to the prolonged duration of airway inflammation observed in the BTLA-deficient mice. However, it remains possible that undetected differences in proliferation coupled with...
the enhanced cell survival together lead to our findings. The molecular mechanism by which BTLA impacts cell survival is not clear. We detected no difference in expression of the antiapoptotic protein Bcl-xL in CD4 cells from BTLA-deficient mice (data not shown). Given that the change in cell survival is not absolute, biochemical differences may be difficult to detect.

BTLA has been shown to have disparate effects on immune responses depending on the system examined. The absence of BTLA led to an accelerated rejection of partially MHC-mismatched cardiac transplants, whereas it resulted in delayed rejection of fully MHC-mismatched allografts (5). In experimental autoimmune encephalomyelitis, BTLA-deficient mice have exacerbated disease, suggesting a predominantly inhibitory role for the receptor (6). However, in a model of graft-vs-host disease, BTLA-deficient cells had poorer survival in vivo, suggesting that BTLA functioned to enhance T cell survival in vivo, unlike the findings seen in experimental autoimmune encephalomyelitis (11). Although the basis for these seemingly contradictory results is not yet clear, it is evident that the biology of this receptor is complex and its function is dependent upon the context in which it is engaged.

Appropriate regulation of the immune system requires that a proper balance of activating and inhibitory signaling be maintained. During initiation and effector phases of inflammation, activating signals predominate, driving the initial expansion and effector response of the T cell. Inhibitory signaling by CD152 appears to be most important in the early phases of the T cell response, because engagement during initial activation can prevent the generation of an inflammatory response (12). Later, the immune response, inhibitory signaling through PD-1 and BTLA, as well as other receptors, may dominate over activating signals, leading to a waning of the inflammatory response. The importance of this aspect of immune regulation has gained increasing recognition. Further study may reveal that defects in inhibitory pathways form the biologic basis for some chronic inflammatory diseases.

Acknowledgments
We thank Traci Bricker and Lindzy Friend for expert technical assistance and helpful discussion, as well as Jonathan Boomer and Andrew Gelman for critical review of the manuscript.

Disclosures
The authors have no financial conflict of interest.

References