Airway epithelial cells suppress T cell proliferation by an IFNγ/STAT1/TGFβ-dependent mechanism

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Deppong CM, Xu J, Brody SL, Green JM. Airway epithelial cells suppress T cell proliferation by an IFNγ/STAT1/TGFβ-dependent mechanism. Am J Physiol Lung Cell Mol Physiol 302: L167–L173, 2012. First published October 14, 2011; doi:10.1152/ajplung.00188.2011.—Organ-specific regulation of immune responses allows inflammation to be localized in a manner appropriate for each tissue compartment. Thus, individual organs may have fundamentally different responses to the same antigenic challenge (13, 19). For example, the large bacterial burden present in the bowel or constant exposure of the airways to inhaled antigen is tolerated in those tissues but would cause marked inflammation in the normally sterile compartment of other tissues. How such organ specificity is achieved is not entirely clear but includes participation of nonhematopoietic parenchymal cells. Although traditionally considered nonimmune, these cells are able to actively regulate inflammatory responses and, thereby, shape the outcome of local immune reactions.

During respiratory tract infections, airway epithelial cells (ECs) secrete chemokines and cytokines that recruit and/or activate effector cells, as well as display antigens in the context of major histocompatibility complex, resulting in direct activation of T cells (23). Airway ECs also express ligands for important regulatory receptors on T cells that further modify the T cell’s response to antigen (5, 7, 8, 20, 27). Thus, ECs have the potential to directly deliver to T cells a number of signals that profoundly influence the outcome of an inflammatory response.

While the role of ECs during infection has been extensively studied, less is known about how these cells might influence immune responses in a resting, noninfected state. In the lung, the constant exposure to environmental antigen has the potential to directly deliver to T cells a number of signals that profoundly influence the outcome of an inflammatory response in the lung than in other organs. Recent data have suggested that airway and alveolar ECs dampen inflammatory responses, primarily through mechanisms that alter the activity of antigen-presenting cells (APCs) (21, 27). Here, we explored whether and how primary airway ECs influence the activation of naive T cells. Using an in vitro coculture system, we demonstrate that airway ECs potently inhibit naive T cell proliferation. This is mediated by an IFNγ/STAT1/transforming growth factor-β (TGFβ)-dependent mechanism that arrests the activated T cell prior to cell division. Thus, in the absence of pathogen, resting airway cells effectively restrain T cell proliferation, preventing unwanted inflammation.

MATERIALS AND METHODS

Mice. C57Bl/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). STAT1-deficient mice were a generous gift of M. Holtzman and Y. Zhang (Washington University). All mice were housed in pathogen-free facilities at Washington University School of Medicine, and all animal studies were approved by the Washington University Animal Studies Committee.

Antibodies. Antibodies included α-CD3 (clone 145-2C11), α-CD28 (0.1 μg/ml, clone 37.51), and α-STAT1 (1/Stat1), all from BD Biosciences (San Jose, CA), and α-CD4 (clone GK1.5, Biologend, San Diego, CA), α-IFNγ (XMC1.2, eBioscience, San Diego, CA), α-TGFβ (clone 1D11, R & D Systems, Minneapolis, MN), and α-pSTAT1 (Cell Signaling, Danvers, MA). Flow cytometric analysis was performed on a FACSCalibur using CellQuest software (BD Biosciences). Analysis was performed using Winlist software (Verity Software, Topsham, ME).

Isolation and culture of murine tracheal ECs. Primary culture mouse tracheal ECs (mTECs) were cultured and differentiated using air-liquid interface (ALI) conditions, as described previously (28). Briefly, cells were released from tracheas of C57Bl/6J mice (≥8 wk old) by pronase digestion, and ECs were isolated. Cells were seeded on supported membranes (0.33 cm², Transwell, Costar-Corning, Corning, NY) that were coated with type I rat tail collagen and allowed to proliferate in growth factor-enriched medium until they were confluent. The ALI condition was then established for induction of differentiation by aspirating apical chamber medium and changing the medium in the lower compartment to medium containing NuSerum (BD Biosciences), a low-serum supplement. Cells used in studies were cultured at ALI conditions for ≥7 days, at which time ciliated (30–40%) and secretory cells were present. For coculture with splenocytes, medium was changed to DMEM supplemented with 10% FCS at the time splenocytes were added.

Proliferation assays and cytokine ELISA. Bulk splenocytes were isolated from 6- to 8-wk-old mice by density gradient centrifugation using Lympholyte-M (Cedarlane Labs, Burlington, ON, Canada) and then labeled with carboxyfluorescein diacetate succinimidy l-ester (CFSE), as previously described (6). The CFSE-labeled splenocytes (2 × 10⁶) were layered on top of the mature mTECs in the apical compartment of the Transwell apparatus and left unstimulated or stimulated with α-CD3 (0.01–0.1 μg/ml) and α-CD28 (0.1 μg/ml) for 96 h. In some experiments, a second set of splenocytes was cultured in the lower chamber, below the membrane. The cells were then
differentiated at ALI. The cultures were not treated or were freshly isolated splenocytes with primary mTECs that had been
response of naive T cells to antigen activation, we cocultured immune cells to antigen. To test if resting ECs affect the
have the capacity to express a variety of cell surface proteins, as well as secrete cytokines that may alter the response of
incubated with α-CD3 alone or in combination with α-CD28; then proliferation was determined by CFSE dye dilution. Representa
tive fluorescent-activated cell sorting plots are shown in Fig. 1A, and combined data from four independent replicates are
markedly reduced when the splenocytes were cocultured with mTECs. CD4+ and non-CD4+ cells were affected, although
viability was similar between cells cultured in the presence and absence of mTECs (data not shown). Suppression of T cell
for all subsequent experiments, cells cultured at ALI were used as a more authentic model of the normal airway.
To determine whether the T cells were blocked early or late following initial T cell activation, we assayed expression of
activation markers on the T cells. Expression of CD69 or CD25 was not detectable in unstimulated cells (Fig. 2) or in
cultured with mTECs, despite the profound reduction in proliferation when T cells were cocultured with mTECs. Consistent
that initial receptor engagement and signaling have occurred and, furthermore, that the failure to proliferate is not
due to a failure to express the IL-2 receptor (CD25).
We also directly assayed levels of multiple cytokines in the
culture supernatants (Table 1). Most cytokine levels were considerably higher in cultures of splenocytes that included
mTECs. Notably, IL-2 was increased ~200-fold, IFNγ was increased ~10-fold, and IL-10, which can inhibit cell prolif-
eration, was increased ~4-fold. The large increase in IL-2 likely reflects production in the absence of consumption. These
findings further support the idea that the cells received an effective activating stimulus and that the block to proliferation

fig. 1. Airway epithelial cells (ECs) inhibit T cell proliferation. Freshly isolated, carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled splenocytes were cultured alone or cocultured with primary murine tracheal ECs (mTECs) for 96 h. Splenocytes were recovered, stained with α-CD4, and analyzed by flow cytometry. A: representative fluorescent-activated cell sorting plots. B: combined data from 4 independent experiments. Values are means ± SD. *P < 0.05 vs. stimulation in the absence of mTECs (by 2-tailed Student’s t-test).
Table 1. Levels of multiple cytokines in culture supernatants

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<tr>
<th>Cytokine</th>
<th>+mTEC (Unstimulated splenocytes)</th>
<th>+mTEC (Stimulated splenocytes)</th>
<th>-mTEC (Unstimulated splenocytes)</th>
<th>-mTEC (Stimulated splenocytes)</th>
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Values are expressed in ng/ml. Splenocytes were cultured alone or with murine tracheal epithelial cells (mTECs) and left unstimulated or stimulated with α-CD3/α-CD28. Culture supernatants were collected at 96 h and assayed for cytokines by multiplex ELISA. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemotactatant protein-1; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted.

is downstream of initial T cell activation and, perhaps, is mediated by secreted cytokines.

Inhibition of proliferation requires contact between ECs and T cells. Soluble factors such as IL-10 or other inhibitory cytokines might mediate the inhibition of T cell proliferation, or alternatively inhibition of T cell proliferation might require a cognate interaction of the T cell with the EC layer. To test this, we activated freshly isolated splenocytes in the presence of mTECs, whether or not splenocytes were also present in the upper chamber (Fig. 4). Unstimulated cells from either compartment did not proliferate. Stimulated cells from the lower chamber proliferated to an extent similar to that of those cultured in the absence of mTECs, whether or not splenocytes were also present in the upper chamber. In contrast, proliferation of the CD4+ cells in the upper chamber with mTECs was markedly reduced, demonstrating that direct contact between the ECs and T cells is required and that soluble factors alone are not sufficient to mediate the observed antiproliferative effect.

B- and T-lymphocyte attenuator (BTLA) and programmed death 1 (PD-1) are inhibitory receptors expressed on the surface of activated T cells (3). In a related system, regulation of these receptors has been implicated in inhibition of T cell proliferation by an airway cell line (14). Given that inhibition was contact-dependent and that ECs can be induced to express the ligands for these regulatory receptors (4, 18, 22), we tested whether T cell expression of BTLA or PD-1 was required. Splenocytes from mice deficient in BTLA or PD-1 cocultured with wild-type mTECs were as profoundly inhibited in their proliferative response as wild-type T cells (Fig. 5). Thus, while contact is necessary, engagement of PD-1 or BTLA is not required.

Inhibition requires IFNγ, STAT1, and TGFβ. A number of potentially inhibitory cytokines, including IFNγ, were detected in the culture supernatant of mTEC-splenocyte cocultures. Neutralization of IFNγ resulted in a partial restoration of proliferation, suggesting a role for this cytokine in inhibiting cell division (Fig. 6). Given that soluble factors alone were not sufficient, we reasoned that IFNγ might be acting as an intermediate to induce expression of another factor on the EC. The transcription factor STAT1 is required for signaling through the IFNγ receptor; therefore, we tested whether mTEC expression of STAT1 was required. As shown in Fig. 7, STAT1-deficient mTECs were markedly less effective at inhibiting T cell proliferation than mTECs isolated from wild-type mice, and coculture induced IFNγ-dependent phosphorylation of STAT1 in the ECs. Together, these data suggest that IFNγ resulted in STAT1-dependent expression by EC of a factor that is required for inhibition of T cell proliferation.

The expression of CD69 and CD25, as well as the high levels of cytokine secretion detected in the supernatant of cocultured T cells, suggested a late cell cycle blockade, a
phenotype consistent with inhibition of T cell proliferation by TGFβ (9, 25). Thus we tested whether neutralization of TGFβ would restore proliferation. Inclusion of TGFβ resulted in proliferation in the cocultures to levels comparable to splenocytes cultured in the absence of mTECs (Fig. 8). These data demonstrate that TGFβ is required for the antiproliferative effect of mTECs on T cells.

**DISCUSSION**

The importance of nonhematopoietic cells in modulating inflammatory responses has been increasingly appreciated. Parenchymal cells provide contextual information to the immune cells and, in doing so, create a local environment that ensures an appropriate response for that site (13, 19). We have examined how airway ECs might influence the response of naive T cells to activation. The conducting airways of the lung are continuously exposed to inhaled antigens, many of which might provoke a T cell-mediated immune response. Without tight regulation, unchecked inflammation would likely lead to organ dysfunction and compromise the health of the host. We postulated that cells from the airway might raise the threshold for T cell activation or proliferation, thereby limiting airway inflammation.

**Fig. 4.** Inhibition requires EC-T cell contact. Splenocytes were added to top chamber, bottom chamber, or both chambers of the coculture system and activated with α-CD3/α-CD28, and proliferation of cells from each compartment was measured independently. Ovals in conditions 3, 4, 7, and 8 indicate the cell culture compartment that was analyzed. Only cells cultured in the top chamber in contact with ECs were inhibited in their proliferative response. A: representative data from 1 of 3 independent experiments. B: combined data from 3 replicate experiments. *P < 0.05 vs. stimulation in the absence of mTECs (by 2-tailed Student’s t-test).

**Fig. 5.** Inhibition is not mediated by programmed death 1 (PD-1) or B- and T-lymphocyte attenuator (BTLA). Splenocytes from mice deficient in PD-1 (PD-1/) or BTLA (BTLA/) were cocultured with wild-type (WT) airway ECs and activated with α-CD3/α-CD28, and proliferation was measured. All genotypes were equally inhibited. A: representative data from 1 of 3 independent experiments. B: combined data from 3 replicate experiments. *P < 0.005 vs. stimulation in the absence of mTECs (by 2-tailed Student’s t-test).
To experimentally model the interaction of airway cells with T cells, we employed a coculture system using primary cells. mTECs differentiated at ALI provided a high-fidelity model of the airway. This methodology has previously been well characterized and shown to result in a columnar epithelium containing ciliated and nonciliated cells (28). These cells are morphologically and functionally similar to airway epithelium in vivo.

We found that when primary splenocytes were cultured with the airway cells and treated with a potent activating stimulus, there was a profound reduction in the percentage of proliferating CD4+ cells. Neutralization of IFNγ partially restores proliferation. Splenocytes were cocultured with airway ECs and activated with α-CD3/α-CD28, and α-IFNγ MAb was added to the cultures. Cultures in which IFNγ was neutralized proliferated more than cultures in control conditions but were still partially inhibited relative to splenocytes cultured in the absence of ECs. A: representative data from 1 of 3 independent experiments. B: combined data from 3 replicate experiments. *P < 0.01 vs. −α-IFNγ (by 2-tailed Student’s t-test).

Epithelial expression of STAT1 is required to inhibit T cell proliferation. A: airway ECs from wild-type (STAT1+/+) or STAT1-deficient (STAT1−/−) mice were cocultured with wild-type splenocytes, and proliferation was measured. STAT1-deficient mTECs were much less effective at inhibiting T cell proliferation than mTECs from wild-type mice. B: combined data from 3 replicate experiments. *P < 0.05 vs. STAT1+/+ (by 2-tailed Student’s t-test). C: expression of phosphorylated STAT1 (pSTAT1) and total STAT1, measured by Western blot of EC lysates from cocultures of wild-type mTECs with unstimulated or stimulated splenocytes alone or in the presence of α-IFNγ antibody, demonstrating IFNγ-dependent phosphorylation of STAT1 in the cocultures. Western blot is representative of 2 independent experiments.

Inhibition of T cell proliferation is mediated by TGFβ. Cocultures of activated splenocytes and mTECs were treated with α-TGFβ antibody, and proliferation was measured. Neutralization of TGFβ restored proliferation to levels close to cultures of splenocytes in the absence of mTECs. A: representative data from 1 of 3 independent experiments. B: combined data from 3 replicate experiments. *P < 0.02 vs. −α-TGFβ (by 2-tailed Student’s t-test).
cells that underwent cell division. However, these splenocytes did express activation markers and secreted higher levels of most cytokines than activated cells that were not cultured with airway cells. Thus the block to proliferation appears to be downstream of initial events of T cell activation. While this system most closely mimics an in vivo situation in which T cells in the airway lumen contact the apical aspect of the epithelium, it is possible that similar effects might be observed on T cells that are in contact with basal or lateral aspects of the airway EC. Nonetheless, particularly under inflammatory conditions, lymphocytes do pass through to the lumen of the airway, as evidenced by their recovery in bronchoalveolar lavage specimens. Regulation of lymphocyte proliferation in this compartment may be especially important, given the potential deleterious consequences of unchecked inflammation in the lung.

In further characterizing this effect, we determined that contact or, at the minimum, close proximity of the T cell and EC is required. This was supported by the observation that suppressive activity was not transferred in CM and that only T cells layered directly on the ECs were inhibited in the coculture. However, this was not mediated by engagement of the major inhibitory receptors BTLA and PD-1. In seeming contradiction to the requirement for cell contact, neutralization of the cytokine IFNγ partially reversed the suppressive effect of mTECs on T cells. However, the requirement for STAT1 in the ECs suggests that IFNγ is not acting directly on the T cell but, instead, on the EC, which, in turn, mediates the suppressive effect.

The apparent late cell cycle blockade, suggested by the preservation of activation marker expression and cytokine secretion, was consistent with inhibition by TGFβ (25). This effect was supported by demonstrating that neutralization of TGFβ reversed the suppressive effect of mTECs. TGFβ is well known to inhibit lymphocyte proliferation and is highly expressed by lung and immune cells. A critical role for this cytokine in regulating lung development, growth, and repair has been established using a variety of models. Mice globally lacking TGFβ1 develop diffuse inflammation in multiple organs, including the lung (24). In the lung, TGFβ has been implicated in the pathogenesis of fibrosis and emphysema (11, 26, 27). Mice generated to lack T cell production of TGFβ also developed a diffuse inflammatory disorder, with infiltration of colon, liver, and lung, leading to wasting and lethality as early as 4 mo of age (12). Thus, T cell-derived TGFβ appears to be critical for maintaining homeostasis and limiting inflammation in the absence of specific pathogen exposure.

Mayer et al. (14) demonstrated that secretion of TGFβ by the BEAS-2B EC line was one mechanism by which these cells altered T cell responses. In contrast to our results, this group found that CM did inhibit T cell proliferation, an effect that was partially reversed by blocking TGFβ. It is possible that differences in the amount of TGFβ secreted by BEAS-2B vs. primary ECs account for this discrepancy or that, in our system, the TGFβ requires cell-cell contact for activation and is therefore unable to be transferred with CM. Alternatively, the TGFβ could be bound to the T cell and/or consumed during the culture and, therefore, would not be transferred in the culture supernatant.

TGFβ is secreted in an inactive form, bound to a latency-associated protein and to latent TGFβ-binding proteins, which inactivate and localize it to the extracellular matrix (1). To exert its biological effect, activation of latent TGFβ is required. Activation can occur by proteolytic and nonproteolytic mechanisms (1). In the lung, activation by αvβ6-integrin is particularly important, as mice lacking the β6-subunit develop exaggerated pulmonary inflammation and emphysema (10, 15–17). Expression of αvβ6-integrin is restricted primarily to ECs and is increased in areas of inflammation (2). Thus, in the lung, a feedback loop may exist in which lung inflammation induces β6-subunit expression, which activates TGFβ, thereby damping local inflammation.

Mayer et al. (14) and others (7, 20, 21) also demonstrated that airway ECs can indirectly influence T cell activation by altering the function of APCs or by inducing Treg cells. While we did not directly test the function or phenotype of APCs in our system, it is unlikely that this is the primary mechanism by which T cell proliferation is inhibited in our system. Stimulation with a combination of α-CD3 and α-CD28 is perhaps the most potent direct receptor-mediated agonist of T cell activation and is largely independent of a requirement for APCs. In addition, we did not detect an expansion of Treg cells as defined by CD4/CD25/FoxP3+ cells (data not shown). However, this does not exclude the possibility that the activity or function of Treg cells or other cell types could be enhanced or altered and that this indirectly is modulating the proliferative response of the T cell. In addition, the cellular source of the TGFβ may be EC, hematopoietic cell, or both.

The data presented here support a model in which the EC has the ability to locally limit airway inflammation. Activated inflammatory cells secrete cytokines, including IFNγ, which alone or in combination with other inflammatory mediators may regulate TGFβ expression or activity, perhaps by inducing expression of αvβ6-integrin. This may result in integrin-mediated activation of latent TGFβ most likely produced by the T cell itself, or perhaps by the lung EC. Once activated, the TGFβ can act locally to restrain T cell proliferation and limit inflammation. While this mechanism may be operative to maintain homeostasis in the absence of infection, when exposed to potential pathogens, additional signals must exist that override the TGFβ signal, removing the block from proliferation and allowing an appropriate immune response to occur.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.M.D., S.L.B., and J.M.G. are responsible for conception and design of the research; C.M.D. and J.X. performed the experiments; C.M.D., S.L.B., and J.M.G. analyzed the data; C.M.D., S.L.B., and J.M.G. interpreted the results of the experiments; C.M.D., S.L.B., and J.M.G. prepared the figures; C.M.D., J.X., S.L.B., and J.M.G. edited and revised the manuscript; C.M.D., J.X., S.L.B., and J.M.G. approved the final version of the manuscript; J.M.G. drafted the manuscript.
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