Foxp3$^+$ regulatory T cells promote lung epithelial proliferation

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Acute respiratory distress syndrome (ARDS) causes significant morbidity and mortality each year. There is a paucity of information regarding the mechanisms necessary for ARDS resolution. Foxp3$^+$ regulatory T cells (Foxp3$^+$ Treg cells) have been shown to be an important determinant of resolution in an experimental model of lung injury. We demonstrate that intratracheal delivery of endotoxin (lipopolysaccharide) elicits alveolar epithelial damage from which the epithelium undergoes proliferation and repair. Epithelial proliferation coincided with an increase in Foxp3$^+$ Treg cells in the lung during the course of resolution. To dissect the role that Foxp3$^+$ Treg cells exert on epithelial proliferation, we depleted Foxp3$^+$ Treg cells, which led to decreased alveolar epithelial proliferation and delayed lung injury recovery. Furthermore, antibody-mediated blockade of CD103, an integrin, which binds to epithelial expressed E-cadherin decreased Foxp3$^+$ Treg numbers and decreased rates of epithelial proliferation after injury. In a non-inflammatory model of regenerative alveologenesis, left lung pneumonectomy, we found that Foxp3$^+$ Treg cells enhanced epithelial proliferation. Moreover, Foxp3$^+$ Treg cells co-cultured with primary type II alveolar cells (AT2) directly increased AT2 cell proliferation in a CD103-dependent manner. These studies provide evidence of a new and integral role for Foxp3$^+$ Treg cells in repair of the lung epithelium.

INTRODUCTION
Acute respiratory distress syndrome (ARDS) is characterized by rapid-onset bilateral pulmonary infiltrates hallmarked by an inflammatory response with neutrophil accumulation, increase in alveolar fluid, and pro-inflammatory cytokine release. This syndrome has significant morbidity and mortality, with in-hospital mortality as high as 44%, and accounts for nearly 200,000 hospitalizations and 75,000 deaths each year in the United States. Despite years of research, the only treatment for ARDS demonstrated to improve outcomes are supportive.

Repair of the alveolar epithelium after acute lung injury (ALI) is necessary to restore homeostasis, and current views have proposed that the immune system may have an important role in protecting epithelial surfaces by enhancing barrier function and promoting repair. In acute or chronic injury, the failure to regenerate the lung epithelium has a role in such processes as ALI, pneumonia, pulmonary fibrosis, chronic obstructive pulmonary disease, and aging. The underlying mechanisms involved in epithelial repair remain largely unknown. Previous work demonstrates a central role for Foxp3$^+$ regulatory T cells (Foxp3$^+$ Treg cells) in the resolution of experimental lung ALI by modulating pro-inflammatory alveolar macrophages and reducing fibroproliferation by decreasing fibrocyte recruitment. Moreover, Foxp3$^+$ Treg cells have been shown to increase in the bronchoalveolar lavage (BAL) fluid of patients with ARDS.

Foxp3$^+$ Treg cells are a distinct population of lymphocytes, which express the transcription factor forkhead homeobox protein-3 (Foxp3). This T-cell subset has been demonstrated to suppress or downregulate immune responses in allergic and autoimmune diseases, as well as in cancer biology. The mechanisms involved in Foxp3$^+$ Treg cell suppressor activity depend on the context of the response, and include...
contact-dependent inhibitory cell surface receptors (CTLA-4 and LAG-3), secretion of inhibitory cytokines (interleukin-10 and transforming growth factor-β), competition for growth factors (interleukin-2), and direct lysis (granzymes).12,13

Prior work has highlighted an important role for Foxp3+ Treg cells in the resolution of experimental lung injury;8,14 however, pro-resolution mechanisms still remain to be explored. In this study, multicolor flow cytometry was used to identify epithelial populations in the distal lung along with their rates of proliferation during resolution. Using an established model of experimental ALI, intratracheal lipopolysaccharide (IT LPS), we identified a function of Foxp3+ Treg cells in augmenting the proliferation of the epithelium during ALI resolution. In addition, CD103 (an integrin molecule that binds E-cadherin) blockade decreases Foxp3+ Treg cell abundance and alveolar epithelial proliferation during resolution from injury. To determine whether these findings extended to a non-ovet inflammatory model of lung growth, a left unilateral pneumonectomy (PNX) model in mice was employed. The left lung is surgically removed eliciting a compensatory response in the remaining right lung, which undergoes a process described as regenerative alveologenesis.15 Foxp3+ Treg cell numbers increased in the alveolar and total lung compartments 7 days post PNX, and mice lacking mature lymphocytes (recombinase-activating gene-1-deficient (Rag-1−/−)) or Foxp3+ Treg-depleted animals (Foxp3DTR) had decreased rates of epithelial proliferation. Furthermore, in vitro co-culture studies demonstrated that proliferation of primary type II alveolar epithelial (AT2) cells was enhanced when cultured with Foxp3+ Treg cells, suggesting a direct effect on lung epithelial proliferation. These studies provide evidence of a new and integral role for Foxp3+ Treg cells in repair of the epithelial cells during inflammatory and non-inflammatory models of lung injury and growth.

RESULTS

Flow cytometry method for identification of alveolar epithelial cells

Multicolor flow cytometry was used to identify specific subpopulations of the alveolar epithelium during and after experimental lung injury from live, single lung cell suspensions obtained similar to previous methods.16–18 To identify the epithelial cell population in the single-cell suspensions, surface markers CD31 (endothelial lineage) and CD45 (hematopoietic lineage) were used to exclude these two lineages (Lin−) when gated against a pan-epithelial surface marker CD326 (epithelial cell adhesion molecule) in a manner similar to previous reports6,19 (see Supplementary Figure E1A online). Type I alveolar epithelial (AT1) cells were further delineated in the Lin− CD326− subpopulation by employing an antibody against a well-characterized marker for AT1 cells, T1α (also known as RTI140 or podoplanin).20,21 Cells gated by this method also contained markers for airway cells such as club cells (formerly called Clara cells) and were identified through the expression of club cell secretory protein (CC10). AT2 cells were identified by surface expression of major histocompatibility class II (MHCII; anti-I-A/I-E antibody), which has previously been reported to be constitutively expressed on AT2 cells.22 Therefore, the Lin− CD326+ population could be further divided into Lin− CD326+ MHCII+ T1α− (AT2) or Lin− CD326− T1α+ (AT1/club cells) (Supplementary Figure E1A). Additional detail on the method for identification and confirmation of lineage is provided in an online data supplement (Supplementary Figure E1B). We used this scheme to identify changes in alveolar epithelial cell populations in response to experimental lung injury or PNX.

Lung epithelial cells proliferate after LPS-induced ALI

Following administration of IT LPS (3 mg kg−1) in wild-type (WT) (C57BL/6) mice, there is an influx of inflammatory cells and peak injury occurs at day 3 as measured by histology (Figure 1a), BAL protein (Figure 1b), and BAL cell count (Figure 1c). These parameters of lung injury return toward uninjured levels by days 7 to 10 (Figure 1a–c). The receptor for advanced glycation end products (RAGE) is expressed on the basolateral surface of AT1 cells and increased levels in the BAL fluid indicates direct alveolar epithelial damage in lung injury models.23 After IT LPS administration, BAL-soluble RAGE levels increased at day 1, with a return towards baseline levels by day 3 (Figure 1d).

IT LPS lead to more than a two-fold increase in the total number of lung cells at peak injury (day 3), with a return to baseline by days 7 and 10 (Figure 1e). Inflammatory cells such as neutrophils and macrophages made up a large portion of the influx of cells. The number of Lin− CD326+ epithelial cells significantly decreased at day 3 after IT LPS administration (Figure 1f). Labeling of E-cadherin (CD324), another pan-epithelial marker, demonstrated similar percentages and numbers of epithelial cells as compared with Lin− CD326+ cells, with significant overlap (Figure 1f), irrespective of injury phase (data not shown).24 The percentage of Lin− CD326+ or Lin− E-cadherin+ epithelial cells staining for ki-67, a marker of proliferation, increased from a baseline (for Lin− CD326+) of 1.37 ± 0.39% (mean ± s.d.; n = 11) at control conditions to an average peak proliferation rate of 15.90 ± 6.80% (mean ± s.d.; n = 10) 7 days after LPS-induced injury (Figure 1g). The predominant proliferating alveolar epithelial cell type was AT2 as measured by Lin− CD326+ MHCII+ T1α− ki-67+ cells (Supplementary Figure E2A–B). These studies indicate that IT LPS induces epithelial loss and damage at peak lung injury and subsequent proliferation of alveolar epithelial cells during time points of ALI resolution.

Alveolar and interstitial Foxp3+ Treg cells increase after ALI

The timing of peak alveolar Foxp3+ Treg cells correlates with maximal epithelial proliferation (Figure 1h). To further understand the potential contribution of Foxp3+ Treg cells in the epithelial response after injury, we performed flow cytometry on both the alveolar compartment (BAL) and single-cell lung suspensions (whole lung) to determine potential changes in Foxp3+ Treg cell numbers after injury. We used the Foxp3gfp reporter mouse that expresses a N-terminal green fluorescent protein (GFP)–Foxp3 fusion protein to identify...
Foxp3⁺ Treg cells in our ALI model had similar increases in the numbers of Foxp3⁺ Treg cells and rates of epithelial proliferation 7 days post IT LPS administration (Supplementary Figure E3A-D). We also detected a significant increase in Foxp3⁺ Treg cells in the lung compartment after LPS injury (Figure 1i) in Foxp3GFP mice despite no difference in total lung cell numbers between control and mice 7 days post injury. These observations show that Foxp3⁺ Treg cell numbers increase in the lung during experimental ALI resolution.
Epithelial proliferation during ALI resolution is impaired in Foxp3+ Treg cell-depleted mice

To determine whether Foxp3+ Treg cells affect epithelial proliferation during resolution from LPS-induced injury we used a transgenic Foxp3DTR mouse. Foxp3DTR mice express the human diphtheria toxin receptor (DTR) along with GFP, whose genes have been inserted into the 3′-untranslated region of the Foxp3 locus. These mice allow specific elimination of Foxp3+ Treg cells in vivo through intraperitoneal (i.p.) administration of diphtheria toxin (DT).

We examined the role of Foxp3+ Treg cells in lung cell numbers and rates of proliferation after LPS-induced injury and DT administration in Foxp3DTR mice (Figure 2a). Foxp3+ Treg-depleted mice have decreased total CD326+ cells compared with similarly treated Foxp3gfp mice (Figure 2d, P = 0.0535, column 3 versus column 4). Injured Foxp3+ Treg-depleted mice had lower absolute numbers of proliferating CD326+ cells (Figure 2e) along with a lower percentage of proliferating CD326+ cells compared with their controls (Foxp3gfp mice administered both LPS and DT) (Figure 2f). Similar results were detected with E-cadherin (data not shown). Greater than 90% Foxp3+ Treg-depleted mice have persistent histological lung injury 7 days after IT LPS administration (Figure 2b) with increased BAL total protein (Supplementary Figure E4A). The total number of lung cells was not statistically different 7 day post IT LPS administration (Figure 2c). Foxp3+ Treg-depleted mice had decreased total CD326+ cells compared with similarly treated Foxp3gfp mice (Figure 2d, P = 0.0535, column 3 versus column 4). Injured Foxp3+ Treg-depleted mice had lower absolute numbers of proliferating CD326+ cells (Figure 2e) along with a lower percentage of proliferating CD326+ cells compared with their controls (Foxp3gfp mice administered both LPS and DT) (Figure 2f). Similar results were detected with E-cadherin (data not shown). Greater than 90% Foxp3+ Treg-depleted mice have persistent histological lung injury 7 days after IT LPS administration (Figure 2b) with increased BAL total protein (Supplementary Figure E4A). The total number of lung cells was not statistically different 7 day post IT LPS administration (Figure 2c). Foxp3+ Treg-depleted mice had decreased total CD326+ cells compared with similarly treated Foxp3gfp mice (Figure 2d, P = 0.0535, column 3 versus column 4). Injured Foxp3+ Treg-depleted mice had lower absolute numbers of proliferating CD326+ cells (Figure 2e) along with a lower percentage of proliferating CD326+ cells compared with their controls (Foxp3gfp mice administered both LPS and DT) (Figure 2f). Similar results were detected with E-cadherin (data not shown). Greater than 90% Foxp3+ Treg-depleted mice have persistent histological lung injury 7 days after IT LPS administration (Figure 2b) with increased BAL total protein (Supplementary Figure E4A). The total number of lung cells was not statistically different 7 day post IT LPS administration (Figure 2c). Foxp3+ Treg-depleted mice had decreased total CD326+ cells compared with similarly treated Foxp3gfp mice (Figure 2d, P = 0.0535, column 3 versus column 4). Injured Foxp3+ Treg-depleted mice had lower absolute numbers of proliferating CD326+ cells (Figure 2e) along with a lower percentage of proliferating CD326+ cells compared with their controls (Foxp3gfp mice administered both LPS and DT) (Figure 2f). Similar results were detected with E-cadherin (data not shown). Greater than 90% Foxp3+ Treg-depleted mice have persistent histological lung injury 7 days after IT LPS administration (Figure 2b) with increased BAL total protein (Supplementary Figure E4A). The total number of lung cells was not statistically different 7 day post IT LPS administration (Figure 2c). Foxp3+ Treg-depleted mice had decreased total CD326+ cells compared with similarly treated Foxp3gfp mice (Figure 2d, P = 0.0535, column 3 versus column 4). Injured Foxp3+ Treg-depleted mice had lower absolute numbers of proliferating CD326+ cells (Figure 2e) along with a lower percentage of proliferating CD326+ cells compared with their controls (Foxp3gfp mice administered both LPS and DT) (Figure 2f). Similar results were detected with E-cadherin (data not shown). Greater than 90% Foxp3+ Treg-depleted mice have persistent histological lung injury 7 days after IT LPS administration (Figure 2b) with increased BAL total protein (Supplementary Figure E4A). The total number of lung cells was not statistically different 7 day post IT LPS administration (Figure 2c). Foxp3+ Treg-depleted mice had decreased total CD326+ cells compared with similarly treated Foxp3gfp mice (Figure 2d, P = 0.0535, column 3 versus column 4). Injured Foxp3+ Treg-depleted mice had lower absolute numbers of proliferating CD326+ cells (Figure 2e) along with a lower percentage of proliferating CD326+ cells compared with their controls (Foxp3gfp mice administered both LPS and DT) (Figure 2f). Similar results were detected with E-cadherin (data not shown).
T_{reg}-cell depletion was obtained as evaluated by flow cytometry of the spleen (Supplementary Figure E4B). I.p. administration of DT alone in the absence of IT LPS had no effect on the rate of epithelial proliferation (Figure 2e and f) or markers of lung inflammation as measured by BAL protein (Supplementary Figure E4A).\(^{26}\) In summary, injured mice lacking Foxp3\(^+\) T_{reg} cells have both impairment in resolution of lung injury and decreased rates of epithelial proliferation.

**Epithelial proliferation after ALI is abrogated with blockade of CD103**

One mechanism by which lymphocytes may localize and interact with epithelial surfaces is through their expression of the integrin \(\alpha_E\beta_7\). Integrin \(\alpha_E\) (CD103) binds integrin \(\beta_7\) to form a heterodimeric molecule whose only known ligand is E-cadherin expressed on epithelial cells.\(^{29,30}\) The percentage of Foxp3\(^+\) T_{reg} cells expressing CD103 increased 3 days after IT LPS administration when compared with baseline levels (Figure 3a). To examine the role of CD103, we use antibody-mediated blockade of CD103 delivered to WT mice (i.p.) on days 0, 1, 3, and 5 after IT LPS administration (Figure 3b) and assessed epithelial proliferation at day 7. CD103 blockade significantly decreased Foxp3\(^+\) T_{reg} cells and decreased both the percentage and number of proliferating epithelial cells (CD326\(^+\) ki67\(^+\)) in the lung at 7 days after injury when compared with isotype antibody-treated animals (Figure 3c–e).

**Lung Foxp3\(^+\) T_{reg} cell total numbers directly correlated with greater epithelial cell proliferation (Figure 3f).** There was no statistical difference in injury parameters in mice administered CD103 blockade versus antibody control (see Supplementary Figures E5A-E). These results support a role for Foxp3\(^+\) T_{reg} CD103 in modulating lung epithelial proliferation and further show a strong correlation between Foxp3\(^+\) T_{reg} numbers in the lung and increased rates of epithelial proliferation after injury.

**Transfer of CD103\(^-/\)-Foxp3\(^+\) T_{reg} cells into lymphopenic mice fails to augment epithelial proliferation**

CD103 expression is not limited to Foxp3\(^+\) T_{reg} cells but is also found on other lymphocyte subsets such as intraepithelial lymphocytes and Thy-1\(^+\) dendritic epidermal T cells along with subsets of dendritic cells.\(^{28,30}\) To determine the role of CD103 on Foxp3\(^+\) T_{reg} cells in mediating epithelial proliferation we...
isolated CD4+ CD25− or CD4+ CD25+ cells from the spleens of WT or CD103−/− mice and performed adoptive transfers (AT) into Rag-1−/− mice exposed to IT LPS, followed by collection at 7 days. We have shown that lymphocyte-deficient Rag-1−/− mice have impaired lung injury resolution despite similar initial and peak parameters of lung injury following administration of IT LPS compared with WT mice.8

Surviving Rag-1−/− mice had decreased numbers of CD326+ cells when compared with WT mice despite a similar number of total lung cells (Supplementary Figure E6A-B). AT of WT Treg cells (CD4+ CD25+ lymphocytes) into Rag-1−/− mice restored the percentage of proliferating CD326+ Ki67+ cells back to WT levels. In contrast, AT of WT CD4+ CD25− or CD103−/− CD4+ CD25+ cells back to Rag-1−/− mice did not increase CD326+ epithelial proliferation (Figure 4a). Among the AT groups, Rag-1−/− mice that received WT CD4+ CD25+ cells had significantly more Foxp3+ cells than Rag-1−/− mice that received CD103−/− CD4+ CD25+ cells (Figure 4b). Furthermore, AT of WT CD4+ CD25+ cells but not CD4+ CD25− or CD103−/− CD4+ CD25+ cells into Rag-1−/− mice led to restoration of barrier permeability as measured by BAL total protein levels (Supplementary Figure E6C). No significant difference was detected in BAL total cell count between AT of WT CD4+ CD25+ or CD4+ CD25+ CD103−/− cells into Rag-1−/− mice (Supplementary Figure E6D); however, AT of WT CD4+ CD25+ cells but not CD4+ CD25− or CD4+ CD25+ CD103−/− cells into Rag-1−/− mice led to restoration of histopathologic resolution 7 days after LPS-induced injury (Supplementary Figure E6E). These results reinforce the role for Foxp3+ Treg cells and its integrin CD103 in the proliferation of alveolar epithelial cells after injury and demonstrate a partial complementation in inflammatory resolution of the alveolar epithelium after acute injury.

Foxp3+ Treg cells increase in the right lung after left lung PNX

We sought to determine whether Foxp3+ Treg cells can induce augmentation of epithelial proliferation independent on their ability to dampen alveolar inflammation. We employed a left lung PNX murine model characterized by contralateral lung growth without significant inflammation.15,31

After PNX of the left lung, we observed an increase in the right lung cellularity by histology compared with sham surgery (L thoracotomy) (Figure 5a). Total Foxp3+ Treg cells increased in the BAL and in the right lung of contralateral PNX-treated mice compared with control or sham surgery of WT (Figure 5b and c). Peak levels of epithelial proliferation occurred at day 7 post PNX (data not shown). The increase in Foxp3+ Treg cells also correlated with the peak level of CD326+ epithelial proliferation (Figure 5f; column 1 and 2). There was no difference in markers of inflammation, including BAL protein or BAL cell count between WT mice undergoing left PNX versus sham surgery (data not shown). These observations indicate that Foxp3+ Treg cell numbers increase in the remaining lung post PNX with a concomitant increase in epithelial proliferation.

Foxp3+ Treg cells augment epithelial proliferation after PNX

To further examine whether Foxp3+ Treg cells can modulate epithelial proliferation in a non-inflammatory model of lung growth, Rag-1−/−, Foxp3GFP, and Foxp3DTR mice underwent left lung PNX. Subsets of Foxp3GFP and Foxp3DTR mice were also administered DT at the time of PNX and again at days 2, 4, and 6 post PNX. The increase in the number of cells seen by histology (Figure 5a) was also determined in the prepared single-cell lung suspensions. No statistical difference in total lung cells was detected between WT sham and WT PNX (Figure 5d; column 1 and 2). There was an increase in total lung cells between Rag-1−/− sham and Rag-1−/− PNX, and between Foxp3DTR and Foxp3GFP mice undergoing PNX (Figure 5d). There was a significant difference in total CD326+ cell number between Foxp3GFP and Foxp3DTR mice undergoing PNX and administered DT (Figure 5e; columns 5 and 6). Mice lacking lymphocytes (Rag-1−/−) or depleted of Foxp3+ Treg cells (Foxp3DTR) but not controls (Foxp3GFP) had a 50% reduction in epithelial proliferation when compared with the WT mice 7 days after PNX (Figure 5f). Similar to the role of

**Figure 4** Adoptive transfer (AT) of regulatory T cells (Treg) augments epithelial proliferation after acute lung injury (ALI) in Rag-1−/− mice. AT of wild-type (WT) Treg cells augments epithelial proliferation after ALI in Rag-1−/− mice. Rag-1−/− mice were challenged with intratracheal lipopolysaccharide (IT LPS) and 1 h afterward received 1 × 106 WT CD4+ CD25+ WT CD4+ CD25− or CD103−/− CD4+ CD25+ cells and lungs collected at day 7 post LPS administration. (a) Percentage of proliferating CD326+ Ki67+ epithelial cells at day 7 post LPS in WT mice or Rag-1−/− mice after infusion of designated lymphocytes subsets (n = 6–12 per group). (b) Total number of Foxp3+ cells in the lungs of WT mice or Rag-1−/− mice after infusion of designated lymphocytes subsets (n = 4–7 per group). P-values determined by Student’s t-test.
Foxp3\(^+\) T\(_{reg}\) in the IT LPS inflammatory model, Foxp3\(^+\) T\(_{reg}\) cells enhanced epithelial proliferation in a non-inflammatory model of alveolar growth.

**Foxp3\(^+\) T\(_{reg}\) cells enhance proliferation of AT2 cells in vitro**

To determine a potential direct effect of Foxp3\(^+\) T\(_{reg}\) cells in modifying epithelial proliferation, AT2 cells were grown in co-culture experiments with specific lymphocytes subsets (1:5 ratio of lymphocytes: AT2). This ratio was chosen as previous reports have estimated that there are \(\approx 10-20\) intraepithelial lymphocytes per 100 bronchial epithelial cells.\(^{32}\) AT2 cells co-cultured for 24 h with CD4\(^+\) CD25\(^-\) (Foxp3\(^+\) T\(_{reg}\)) cells had increased rates of proliferation when compared with those cultured with CD4\(^+\) CD25\(^-\) lymphocytes (Figure 6).

The augmentation of proliferation by CD4\(^+\) CD25\(^+\) cells continued when the two cell types were separated by a transwell insert, demonstrating that the effect has contact independence (Figure 6). When CD103-blocking antibody was added to the co-cultures of AT2 and Foxp3\(^+\) T\(_{reg}\) cells, epithelial proliferation rates decreased to control levels (Figure 6). When we extended the co-cultures out to 72–96 h, a greater number of AT2 cells cultured with CD4\(^+\) CD25\(^+\) cells had continued expression of surfactant protein-C as demonstrated by expression of GFP through a surfactant protein-C promoter, and decreased levels of T1\(\alpha\) expression by imaging (Supplementary Figure E7). These data demonstrate that Foxp3\(^+\) T\(_{reg}\) cells directly increase primary AT2 proliferation and this effect is in part CD103 dependent.
DISCUSSION

In this report we identify that Foxp3+/Treg cells augment the rate of epithelial proliferation in an experimental model of lung injury and also in a compensatory lung growth model (PNX). Lymphocyte and, specifically, Foxp3+/Treg cell effects on alveolar epithelial proliferation after ALI have not been previously described. Prior work has demonstrated an integral role for Foxp3+/Treg cells during LPS-induced lung inflammation resolution and that these cells exhibit suppressive function mainly on pro-inflammatory alveolar macrophages and neutrophils. In this study we use both a depletive method (Foxp3DTR mice) and an additive method (AT of lymphocyte subsets into Rag-1-/- mice) to determine the effect of Foxp3+/Treg cells on proliferation after injury. Furthermore, our PNX findings reinforce that Foxp3+/Treg cells exert an effect on the lung epithelium in a non-inflammatory context. Herein, we also show that the number of Foxp3+/Treg cells increases in the whole lung after both IT LPS and PNX. The extent to which the increase in total number represents proliferation, retention, recruitment, or a combination of all three is still unknown; however, results from this study suggest retention through Foxp3+/Treg cells expression of CD103 may have a role, and prior reports suggest proliferation and recruitment. Probably, all three mechanisms have a role and the interplay with other immune cells such as macrophages and dendritic cells promoting development of Foxp3+/Treg cells are likely involved.

The LPS model was chosen, as it has been widely characterized and elicits reproducible alveolar epithelial damage. One limitation of the LPS model is that in the absence of lymphocytes (Rag-1-/- mice) or specifically Foxp3+/Treg cells (Foxp3DTR mice depleted of Foxp3+ cells) there is a sustained inflammatory response that may directly or indirectly dampen the epithelial reparative process. Persistent injury in the absence of Foxp3+/Treg cells led us to explore our findings into the PNX model to better determine Foxp3+/Treg cell effects on lung epithelial proliferation independent of sustained inflammation.

We identified specific lung epithelial subpopulations during resolution of lung injury by using multicolor flow cytometry. The cytometric scheme presented allows for identification of alveolar epithelial cells and builds on the previous literature. In this report we use MHC II molecules to identify AT2 cells within the CD326+ population. MHC II has been previously described to be constitutively expressed on AT2 cells. Lee et al. published the construction of a transgenic mouse that expresses GFP through an SP-C promoter and demonstrated that CD74 can be used to identify AT2 cells. CD74 or invariant chain is part of the MHC II system. Additional work is needed to better identify subsets; however, this methodology allows for identification without the need for a transgenic report strain for identification of a specific epithelial population.

Through flow cytometry we detected a significant decrease in total CD326+ or E-cadherin+ epithelial cells at peak levels of injury after IT LPS administration. This decrease in cell number could be due to damage elicited by LPS as confirmed by elevated levels of soluble RAGE after injury. Other possibilities could include loss of cellular expression of these markers through enzymatic cleavage, degradation, proliferation, or expansion of a specific progenitor population with altered or lack of expression of these epithelial markers at specific times during the reparative process. Peak proliferation of the epithelium occurs 7 days after IT LPS administration, which also corresponded to the peaked increase in Foxp3+/Treg cells. Depletion of Foxp3+/Treg cells throughout the injury course correlated with lower numbers of total CD326+ epithelial cells, likely due to persistent inflammation and cellular damage and the remaining CD326+ epithelial cells were less proliferative when compared with controls.

The mechanisms underlying the pro-proliferative effects on epithelium regeneration are complex and involve progenitor populations, signaling pathways, and interaction with the extracellular matrix. The paradigm for alveolar epithelial repair has been that AT1 cells are susceptible to damage and AT2 cells migrate into and proliferate at sites of injury. AT2 cells undergo hyperplasia and then differentiate into AT1 cells to reform an intact functional epithelial barrier. This report supports that the presence of Foxp3+/Treg cells modulates proliferation of the epithelium after injury. Interestingly, earlier studies in cultured human fetal colon tissue demonstrated that...
pokeweed mitogen or anti-CD3+ activated lamina propria CD3+ cells underwent induced expression of CD25+, and this T-cell activation increased the rate of epithelial proliferation in the crypts of Lieberkühn. This study was performed before identification of the transcription factor Foxp3, but the results support that immunological factors can exert effects on epithelial turnover and differentiation. To our knowledge this is the first report demonstrating a role for Foxp3+ Treg cells exerting an effect on epithelial proliferation in general and specifically the lung. A previous study found that intraepithelial γδ T cells (dendritic epidermal T cells) induced epithelial cell proliferation in a murine keratinocyte cell line (Balb/MK) through production of keratinocyte growth factor. Foxp3+ Treg cells may also exhibit similar effects. The effects may be organ or tissue specific, and the increased rates of epithelial turnover in certain tissues such as the gastrointestinal tract or skin may not be as influenced by Foxp3+ Treg cells as the lung epithelium, and recent work demonstrate that Foxp3+CD4+ Treg cells promote muscle repair after injury. Future work examining Foxp3+ Treg cells’ effects in other models of injury (naphthalene, bleomycin, or live bacterial), and in respect to progenitor populations may provide a better understanding of the Foxp3+ Treg cell mechanisms involved in epithelial repair, and are the subject of ongoing investigation.

Foxp3+ Treg cells have been previously demonstrated to migrate to sites of infection, inflammation, and tumor microenvironments. The αE (CD103) chain is part of the integrin αEβ7, which is well described and may function to localize certain immune cells such as dendritic cells and lymphocyte subsets to epithelial surfaces through binding to E-cadherin. Little is known about the immune regulation of CD103 since it was first described, and CD103 expression is found largely on lymphocytes at epithelial sites. Furthermore, CD103 expression is induced by transforming growth factor-β1 and may induce integrin αEβ7 expression on T cells at epithelial surfaces along with inducing Foxp3+ Treg cell development. The increased percentage of CD103+ Foxp3+ Treg cells detected at days 3–4 days post IT LPS administration may be due in part to increased levels of transforming growth factor-β1 during injury.

We detected a decrease in Foxp3+ Treg cells in the lung interstitium in injured animals treated with anti-CD103 blockade, which in turn affected the rate of epithelial proliferation. Moreover, when correlating the number of Foxp3+ Treg cells to the number of proliferating CD326+ cells in these conditions we found a strong linear correlation. Moreover, in contrast to CD103+ Treg cells, adoptively transferring CD103−/− Treg cells into Rag−/− mice did not augment epithelial proliferation. CD103 blockade did not impact lung injury resolution. Several possibilities could explain these findings. First, it is possible that greater blockade is needed to see an effect on injury resolution as the number of lung Foxp3+ Treg cells still increased after CD103 blockade but not to the levels of isotype antibody control. The decrease Foxp3+ Treg cell number after LPS-induced injury by CD103 blockade may allow for changes in detection of epithelial proliferation but may be enough to adequately suppress the ability of Foxp3+ Treg cells to dampen alveolar inflammation. Furthermore, mechanisms underlying Foxp3+ Treg modulation of epithelial repair (e.g. CD103) may be distinct from pathways used by Foxp3+ Treg to modulate inflammation or abrogate macrophage/neutrophil responses. Nevertheless, the AT experiments with Rag−/− mice transferred CD103−/− Foxp3+ Treg cells, and our co-culture experiments with Foxp3+ Treg and AT2 cells in the presence of anti-CD103 antibodies support the a role for this integrin’s contribution in Foxp3+ Treg cell-mediated epithelial repair.

The in vitro co-culture studies demonstrate that there is a contact-independent effect along with the results demonstrating that blocking CD103 abrogates Foxp3+ Treg cell effects on AT2 epithelial cell proliferation in vitro. These findings strongly indicate an interaction between Foxp3+ Treg cells and AT2 cells in culture, and this interaction in part is driven by CD103 as demonstrated by the antibody-mediated blockade effects. Several potential mechanisms from these experiments can be considered and may not be mutually exclusive. First, CD103’s role could be ascribed to retain Foxp3+ Treg cells to dampen alveolar inflammation. Furthermore, Foxp3+ Treg cells to increase keratinocyte growth factor similar to that by Foxp3+ Treg cells specifically express CD103 or not. This potential mechanism is supported in part by the AT experiments where Rag−/− mice were transferred CD103−/− Foxp3+ Treg cells (Figure 4). This subset of ATs had a lower percentage of epithelial proliferation and a lower number of Foxp3+ cells in the lung compared to the WT Treg AT subset. However, the in vitro co-culture experiment where anti-CD103 antibodies were added to the co-culture of Foxp3+ Treg cells and AT2 cells already in direct contact demonstrated a lower level of AT2 proliferation compared with IgG isotype controls. This strongly supports a role for Foxp3+ Treg cell expression of pro-proliferative soluble mediator, which enhances epithelial proliferation (such as a cytokine or growth factor). Foxp3+ Treg cells may demonstrate similar ability to induce epithelial cell proliferation through production of growth factors such as keratinocyte growth factor similar to that shown by intraepithelial γδ T cells, and may help explain a contact-independent mechanism for epithelial proliferation. This is supported by the contact-independent experiment where Foxp3+ Treg cells still exert an effect on AT2 proliferation across a transwell membrane. A third possibility is that CD103 antibody-mediated blockade may downregulate or inactivate cell-to-cell CD103–E-cadherin interactions that may be important to enhance downstream pro-proliferative signaling in epithelial cells. This mechanism could be supported by the in vivo antibody-mediated blockade data (Figure 3), and also by the antibody-mediated blockade in vitro experiments...
In summary, alveolar epithelial repair is a dynamic process and further studies are needed to clarify the role of the cell types involved in the reparative process after ALI. Moreover, recent studies have demonstrated that Foxp3+ Treg cells may have additional functions in non-lymphoid tissues. These studies suggest an important role for Foxp3+ Treg cells in alveolar epithelial repair after LPS-induced ALI. Furthermore, Foxp3+ Treg cells also promote CD326+ epithelial cell proliferation in a non-inflammatory compensatory lung growth model. The integrin CD103 has a role in retaining Foxp3+ Treg cells in the lung after injury and blocking this interaction abrogates the Foxp3+ Treg cell proliferative effects on the lung epithelium. This work highlights an undescribed role Foxp3+ Treg cells in alveolar epithelial proliferation. A better understanding of the interactions of Foxp3+ Treg cells and alveolar epithelium during ALI resolution may provide valuable insight into the processes of resolution after injury and potentially uncover mechanisms that enhance endogenous lung repair.

METHODS

Mice. C57BL/6 WT, Rag-1−/−, and αε−/− (CD103−/−) mice (6–8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free facility. SP-C-GFP mice were a gift from Dr John K. Heath (University of Birmingham). Foxp3DTR and Foxp3DTR mice were gifts of Dr Alexander Y. Rudensky (Sloan-Kettering Institute). Procedures were approved and conducted under protocols by the Johns Hopkins Animal Care and Use Committee.

LPS administration. Mice were anesthetized and LPS instilled as previously described. Additional detail is provided in an online data supplement.

Unilateral left lung PNX. Nine- to 12-week-old C57BL/6, Rag-1−/−, Foxp3DTR, and Foxp3DTR mice underwent left PNX, and lung tissue obtained at 3, 7, or 14 day post procedure for endpoints as previously described. Diphtheria toxin administration. Diphtheria toxin (List Biological Laboratories, Campbell, CA) was suspended in phosphate-buffered saline. Stock solutions were thawed once and mice were injected i.p. with 50 or 10 μg kg−1 of diphtheria toxin similar as previously described.

In vivo blockade of CD103. WT animals were given 0.15 mg/dose/mouse of i.p. injections of a blocking CD103 rat polyclonal antibody (M290; BioXcell, West Lebanon, NH) or isotype (rat IgG, Sigma-Aldrich, St Louis, MO) on days 0, 1, 3 and 5 after IT LPS challenge.

Analysis of BAL fluid. BAL was obtained by cannulating the trachea with a 20-gauge catheter, and lungs lavaged and fluid obtained as previously described. Additional detail is provided in an online data supplement.

Lung morphology. Lungs from animals were inflated to 25 cm H2O with 1% of low-melting agarose (Invitrogen, Carlsbad, CA) for histological evaluation by hematoxylin and eosin staining.

Preparation of lung single-cell suspensions for multi-color flow cytometry. After obtaining the BAL fluid for experiments the lungs were infused with 1 ml dispase (BD Bioscience, East Rutherford, NJ) and elastase 3 U ml−1 (Worthington Biochemical, Lakewood, NJ) before 1% (v/v) low-melting agarose (Invitrogen) infusion similar to previous descriptions. Lungs were minced and filtered as described and additional detail is provided in an online data supplement.
Isolation of CD4<sup>+</sup> CD25<sup>+</sup> T cells and CD4<sup>-</sup> CD25<sup>-</sup> T cells, and AT. Spleens were removed and prepared for single-cell suspensions. CD4<sup>+</sup> T cells were isolated from the resulting splenocytes using magnetic bead separation as previously described.<ref>5</ref>

**BAL RAGE levels.** BAL fluid was used in enzyme-linked immuno-sorbent assays to determine levels of soluble RAGE (R&D Systems, Minneapolis, MN) as previously described.<ref>23</ref>

**Isolation of AT2 cells and co-culture experiments.** Single-cell suspension was obtained as per the flow cytometry method above and labeled for surface stains before sorting performed with FACS<sup>Aria</sup> and FACS<sup>Diva</sup> software (Becton Dickinson, BD Bioscience) as previously described.<ref>7</ref> Additional detail is provided in an online data supplement.

**Immunoblot analysis.** Whole lung samples were prepared and used for immunoblot analysis as previously described.<ref>56</ref> Additional detail is provided in an online data supplement.

**Immunofluorescence.** AT2 cell were grown on chamber slides (Lab-Tek, Waltham, MA) and at time points of interest the media was incubated at 37 °C for 30 min. The slides were then washed with phosphate-buffered saline and then slides covered with Fluoromount (Sigma-Aldrich), sealed, and visualized by immunofluorescence with a Leica SP2 Confocal Microscope (Buckalo Grove, IL).

**Statistical analysis.** Markers of injury were compared using Mann–Whitney rank sum test. Pair-wise comparisons were made by using either Student’s two-tailed unpaired t-test or Mann–Whitney rank sum test. Data are expressed as the mean ± s.e.m. where applicable. Statistical analysis was performed using GraphPad Prism 5 software (La Jolla, CA). Statistical difference was accepted at P<0.05.

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at http://www.nature.com/mi

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**AUTHOR CONTRIBUTIONS**

J.R.M., B.T.G., N.R.A., B.D.S., L.S.K., and F.R.D.: conceived and designed experiments; J.R.M., B.T.G., J.J., N.L., B.D.S., E.C., R.R., and J.R.M., B.T.G., N.R.A., B.D.S., V.S., L.S.K., and F.R.D.: performed experiments and analysis; B.T.G. and B.D.S.: provided assistance with statistical analysis; J.R.M., N.R.A., B.D.S., W.M., E.M.W., L.S.K., and F.R.D.: wrote the manuscript and provided creative input.

**DISCLOSURE**

The authors declared no conflict of interest.

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