HHS Public Access

Author manuscript

Mucosal Immunol. Author manuscript; available in PMC 2015 May 01.

Published in final edited form as:

Mucosal Immunol. 2014 November ; 7(6): 1440–1451. doi:10.1038/mi.2014.33.

Foxp3+ Regulatory T Cells Promote Lung Epithelial Proliferation

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Abstract

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 (T_{regs}) have been shown to be an important determinant of resolution in an experimental model of lung injury. We demonstrate that intratracheal delivery of endotoxin (LPS) elicits alveolar epithelial damage from which the epithelium undergoes proliferation and repair. Epithelial proliferation coincided with an increase in Foxp3$^+$ T_{reg} cells in the lung during the course of resolution. To dissect the role that Foxp3$^+$ T_{reg} cells exert on epithelial proliferation, we depleted Foxp3$^+$ T_{reg} 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$^+$ T_{reg} numbers and decreased rates of epithelial proliferation after injury. In a non-inflammatory model of regenerative alveologenesis, left lung pneumonectomy (PNX), we found that Foxp3$^+$ T_{reg} cells enhanced epithelial proliferation. Moreover, Foxp3$^+$ T_{reg} 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$^+$ T_{reg} cells in repair of the lung epithelium.

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Author Contributions: JRM, BTG, NRA, BDS, VS, LSK, FRD: conceived and designed experiments; JRM, BTG, JJ, NL, BDS, EC, RR, DCF: performed experiments and analysis; BTG, BDS: provided assistance with statistical analysis; JRM, NRA, BDS, WM, EMW, LSK, FRD: Wrote the manuscript and provided creative input

Supplementary Material

Supplementary material is linked to the online version of the paper at http://www.nature.com/mi.

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 [1]. 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 [2]. Despite years of research the only treatments for ARDS demonstrated to improve outcomes are supportive [3,4].

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 play an important role in protecting epithelial surfaces by enhancing barrier function and promoting repair [5,6]. In acute or chronic injury the failure to regenerate the lung epithelium plays a role in such processes as ALI, pneumonia, pulmonary fibrosis, COPD, and aging [5]. Underlying mechanisms involved in epithelial repair remain largely unknown. Previous work demonstrates a central role for $Foxp3^+$ regulatory T cells ($Foxp3^+$ T_{reg} cells) in the resolution of experimental lung ALI by modulating pro-inflammatory alveolar macrophages and reducing fibroproliferation by decreasing fibrocyte recruitment [7,8]. Moreover, $Foxp3^+$ T_{reg} cells have been shown to increase in the bronchoalveolar lavage (BAL) fluid of patients with ARDS [8].

$Foxp3^+$ T_{reg} cells are a distinct population of lymphocytes which express the transcription factor forkhead homeobox protein-3 (Foxp3) [9,10]. This T cell subset has been demonstrated to suppress or down-regulate immune responses in allergic and autoimmune diseases, as well as in cancer biology [11]. The mechanisms involved in $Foxp3^+$ T_{reg} cell suppressor activity depend on the context of the response, and include contact-dependent inhibitory cell surface receptors (CTLA-4, LAG-3), secretion of inhibitory cytokines (IL-10 and TGF-β), competition for growth factors (IL-2), and direct lysis (granzymes) [12,13].

Prior work has highlighted an important role for $Foxp3^+$ T_{reg} 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 $Foxp3^+$ T_{reg} cells in augmenting the proliferation of the epithelium during ALI resolution. Additionally, CD103 (an integrin molecule which binds E-cadherin) blockade decreases $Foxp3^+$ T_{reg} cell abundance and alveolar epithelial proliferation during resolution from injury. To determine if these findings extended to a non-overt 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]. $Foxp3^+$ T_{reg} cell numbers increased in the alveolar and total lung compartments 7 days post-PNX, and mice lacking mature lymphocytes (*Rag-1*$^{-/-}$) or $Foxp3^+$ T_{reg} depleted animals (*Foxp3*^{DTR}) 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$^+$ T_{reg} cells—suggesting a direct effect on lung epithelial proliferation. These studies provide evidence of a new and integral role for Foxp3$^+$ T_{reg} cells in repair of the epithelial 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 utilized 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 (EpCAM) in a manner similar to previous reports [16,19] (see Figure E1A in the online data supplement). 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 RT140 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 expression of club cell secretory protein (CC10). AT2 cells were identified by surface expression of major histocompatibility class II (MHC II; 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) (Figure E1A). Additional detail on the method for identification and confirmation of lineage is provided in an online data supplement (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) in WT (C57BL/6) mice there is an influx of inflammatory cells and peak injury occurs at day 3 as measured by histology (Figure 1A), bronchoalveolar lavage (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, 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 to 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 ± SD; n = 11) at control conditions to an average peak proliferation rate of 15.90% ± 6.80% (mean ± SD; n = 10) 7 days after LPS-induced injury (Figure 1G). The predominate proliferating alveolar epithelial cell type was AT2 as measured by $Lin^{-}CD326^{+}MHCII^{+}T1\alpha^{-}$ ki-67^{+} cells (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^{+} T_{reg} cells increase after ALI

The timing of peak alveolar Foxp3^{+} T_{reg} cells correlates with maximal epithelial proliferation (Figure 1H). To further understand the potential contribution of Foxp3^{+} T_{reg} 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^{+} T_{reg} cells numbers after injury. We utilized the *Foxp3^{gfp}* reporter mouse that expresses a N-terminal GFP-Foxp3 fusion protein to identify Foxp3^{+} T_{reg} cells in our ALI model [25]. *Foxp3^{gfp}* and WT mice had similar increases in the numbers of Foxp3^{+} T_{reg} cells and rates of epithelial proliferation 7 days post IT LPS (Figure E3A–D). We also detected a significant increase in Foxp3^{+} T_{reg} cells in the lung compartment after LPS injury (Figure 1I) in *Foxp3^{gfp}* mice despite no difference in total lung cell numbers between control and 7 days post injury. (Figure E3C). These observations show that Foxp3^{+} T_{reg} cell numbers increase in the lung during experimental ALI resolution.

Epithelial proliferation during ALI resolution is impaired in Foxp3^{+} T_{reg} cell-depleted mice

To determine whether Foxp3^{+} T_{reg} cells affect epithelial proliferation during resolution from LPS-induced injury we utilized a transgenic *Foxp3^{DTR}* mouse. *Foxp3^{DTR}* 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 [26]. These mice allow specific elimination of Foxp3^{+} T_{reg} cells *in vivo* through intraperitoneal (i.p.) administration of diphtheria toxin (DT) [26,27].

We examined the role of Foxp3^{+} T_{reg} cells in lung cell numbers and rates of proliferation after LPS-induced injury and DT administration in *Foxp3^{DTR}* mice (Figure 2A). Foxp3^{+} T_{reg} depleted mice have persistent histological lung injury 7 days after IT LPS (Figure 2B) with increased BAL total protein (Figure E4A). The total number of lung cells was not statistically different 7 day post IT LPS (Figure 2C). Foxp3^{+} T_{reg} depleted mice had decreased total CD326^{+} cells compared to similarly treated *Foxp3^{gfp}* mice (Figure 2D, p = 0.0535, column 3 versus column 4). Injured Foxp3^{+} T_{reg} depleted mice had lower absolute numbers of proliferating CD326^{+} cells (Figure 2E) along with a lower percentage of proliferating CD326^{+} cells compared to their controls (*Foxp3^{gfp}* mice administered both LPS and DT) (Figure 2F). Similar results were detected with E-cadherin (data not shown). Greater than 90% Foxp3^{+} T_{reg} cell depletion was obtained as evaluated by flow cytometry of the spleen (Figure E4B). Intraperitoneal (i.p.) administration of DT alone in the absence of IT LPS had no effect on the rate of epithelial proliferation (Figure 2E–F) or markers of lung inflammation as measured by BAL protein (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 $\alpha_E\beta7$ [28–30]. Integrin α_E (CD103) binds integrin β7 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 when compared to 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 (Figure 3B) and assessed epithelial proliferation at day 7. CD103 blockade significantly decreased Foxp3$^+$ T_{reg} cells and decreased both percentage and number of proliferating epithelial cells (CD326$^+$ ki67$^+$) in the lung at 7 days after injury when compared to 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 Figures E5A–E in the online data supplement). 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 (IEL) and Thy-1$^+$ dendritic epidermal T cells (DETC) 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 spleens of WT or *CD103*$^{-/-}$ mice and performed adoptive transfers (AT) into *Rag-1*$^{-/-}$ mice exposed to IT LPS, followed by harvest at 7 days [8]. 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 to WT mice [8].

Surviving *Rag-1*$^{-/-}$ mice had decreased numbers of CD326$^+$ cells when compared to WT mice despite a similar number of total lung cells (Figure E6A–B). AT of WT T_{reg} (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 (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 (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-induce injury (Figure E6E). These results reinforce the role for Foxp3$^+$ T_{reg} 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$^+$ T_{reg} cells increase in the right lung after left lung PNX

We sought to determine whether Foxp3$^+$ T_{reg} cells can induce augmentation of epithelial proliferation independent on their ability to dampen alveolar inflammation. We employed a left lung pneumonectomy (PNX) murine model characterized by contralateral lung growth without significant inflammation [15,31].

After left PNX, we observed an increase in right lung cellularity by histology compared to sham surgery (L thoracotomy) (Figure 5A). Total Foxp3$^+$ T_{reg} cells increased in the BAL and in the right lung of contralateral PNX treated mice compared to control or sham surgery of WT (Figure 5B–C). Peak levels of epithelial proliferation occurred at day 7 post PNX (data not shown). The increase in Foxp3$^+$ T_{reg} 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$^+$ T_{reg} cell numbers increase in the remaining lung post PNX with a concomitant increase in epithelial proliferation.

Foxp3$^+$ T_{reg} cells augment epithelial proliferation after PNX

To further examine if Foxp3$^+$ T_{reg} cells can modulate epithelial proliferation in a non-inflammatory model of lung growth, *Rag-1*$^{-/-}$, *Foxp3*^{gfp}, and *Foxp3*^{DTR} mice underwent left lung PNX. Subsets of *Foxp3*^{gfp}, and *Foxp3*^{DTR} 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 *Foxp3*^{DTR} compared to *Foxp3*^{gfp} mice undergoing PNX (Figure 5D). There was a significant difference in total $CD326^+$ cell number between *Foxp3*^{gfp} and *Foxp3*^{DTR} mice undergoing PNX and administered DT (Figure 5E; columns 5 and 6). Mice lacking lymphocytes (*Rag-1*$^{-/-}$) or depleted of Foxp3$^+$ T_{reg} cells (*Foxp3*^{DTR}) but not controls (*Foxp3*^{gfp}) had a 50% reduction in epithelial proliferation when compared to the WT mice 7 days after PNX (Figure 5F). Similar to the role of 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, alveolar epithelial type 2 (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 approximately 10–20 intraepithelial

lymphocytes per 100 bronchial epithelial cells [32]. AT2 cells co-cultured for 24 hours with $CD4^+CD25^+$ ($Foxp3^+$ T_{reg}) cells had increased rates of proliferation when compared to those cultured with $CD4^+CD25^-$ lymphocytes (Figure 6). The augmentation of proliferation by $CD4^+CD25^+$ cells continued when the two cells 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 hours a greater number of AT2 cells cultured with $CD4^+CD25^+$ cells had continued expression of SPC-GFP and decreased levels of T1α expression by imaging (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 $Foxp3^+$ T_{reg} 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 $Foxp3^+$ T_{reg} cell effects on alveolar epithelial proliferation after ALI have not been previously described. Prior work has demonstrated an integral role for $Foxp3^+$ T_{reg} cells during LPS-induced lung inflammation resolution and that these cells exhibit suppressive function mainly on pro-inflammatory alveolar macrophages and neutrophils [8,14,33]. In this study we utilize both a depletive method (*Foxp3*^{DTR} mice) and an additive method (AT of lymphocyte subsets into *Rag-1*$^{-/-}$ mice) to determine the effect of $Foxp3^+$ T_{reg} cells on proliferation after injury. Furthermore, our PNX findings reinforce that $Foxp3^+$ T_{reg} cells exert an effect on the lung epithelium in a non-inflammatory context. Herein, we also show that the number of $Foxp3^+$ T_{reg} 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 $Foxp3^+$ T_{reg} cells expression of CD103 may play a role, and prior reports suggest proliferation and recruitment [8]. Likely, all three mechanisms play a role and the interplay with other immune cells such as macrophages and dendritic cells promoting development of $Foxp3^+$ T_{reg} cells are likely involved [34].

The LPS model was chosen as it has been widely characterized and elicits reproducible alveolar epithelial damage [23,35]. One limitation of the LPS model is that in the absence of lymphocytes (*Rag-1*$^{-/-}$ mice) or specifically $Foxp3^+$ T_{reg} cells (*Foxp3*^{DTR} mice depleted of $Foxp3^+$ cells) there is a sustained inflammatory response which may directly or indirectly dampen the epithelial reparative process. Persistent injury in the absence of $Foxp3^+$ T_{reg} cells led us to explore our findings into the PNX model to better determine $Foxp3^+$ T_{reg} cell effects on lung epithelial proliferation independent of sustained inflammation.

We identified specific lung epithelial subpopulations during resolution of lung injury by utilized multicolor flow cytometry. The cytometric scheme presented allows for identification of alveolar epithelial cells and builds on the previous literature [16,17,19]. In this report we use MHC class II molecules to identify AT2 cells within the $CD326^+$ population. MHC II has been previously described to be constitutively expressed on AT2 cells [22,36,37]. Lee et al. published the construction of a transgenic mouse that expresses GPF through a SP-

C promoter, and demonstrated that CD74 can be used to identify AT2 cells [19,36]. CD74 or invariant chain is part of the MHC II system [36]. 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. This decrease in cell number could be due to damage elicited by LPS as confirmed by elevated levels of soluble RAGE after injury [23]. 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 which also corresponded to the peaked increase in $Foxp3^+$ T_{reg} cells. Depletion of $Foxp3^+$ T_{reg} 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 to 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 [38]. 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 [39]. AT2 cells undergo hyperplasia and then differentiate into AT1 cells to reform an intact functional epithelial barrier [40]. This report supports that the presence of $Foxp3^+$ T_{reg} 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 [41]. 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 [41,42]. To our knowledge this is the first report demonstrating a role for $Foxp3^+$ T_{reg} cells exerting an effect on epithelial proliferation in general and specifically the lung. A previous study found that intraepithelial γδ T cells (DETCs) induced epithelial cell proliferation in a murine keratinocyte cell line (Balb/MK) through production of keratinocyte growth factor (KGF) [43]. $Foxp3^+$ T_{reg} 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 $Foxp3^+$ T_{reg} cells as the lung epithelium, and recent work demonstrate that $Foxp3^+CD4^+$ T_{reg} cells promotes muscle repair after injury [44]. Future work examining $Foxp3^+$ T_{reg} 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 $Foxp3^+$ T_{reg} cell mechanisms involved in epithelial repair and are the subject of ongoing investigation.

$Foxp3^+$ T_{reg} cells have been previously demonstrated to migrate to sites of infection, inflammation, and tumor microenvironments [45]. The α_E (CD103) chain is part of the integrin $\alpha_E\beta7$, 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 [29]. 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 [46,47]. Furthermore, CD103 expression is induced by TGF-β1 and may induce integrin $\alpha_E\beta7$ expression on T cells at epithelial surfaces along with inducing Foxp3$^+$ T_{reg} cell development [48]. The increased percentage of CD103$^+$ Foxp3$^+$ T_{reg} cells detected at days 3–4 days post IT LPS may be due in part to increased levels of TGF-β1 during injury [8].

We detected a decrease in Foxp3$^+$ T_{reg} 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$^+$ T_{reg} cells to the number of proliferating CD326$^+$ cells in these conditions we found a strong linear correlation. Moreover, in contrast to CD103$^+$ T_{reg} adoptively transferring CD103$^{-/-}$ T_{reg} cells into *Rag-1*$^{-/-}$ 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$^+$ T_{reg} cells still increased after CD103 blockade but not to the levels of isotype antibody control. The decrease Foxp3$^+$ T_{reg} 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$^+$ T_{reg} cells to dampen alveolar inflammation. Furthermore, mechanisms underlying Foxp3$^+$ T_{reg} modulation of epithelial repair (e.g. CD103) may be distinct from pathways used by Foxp3$^+$ T_{regs} to modulate inflammation or abrogate macrophage/neutrophil responses. Nevertheless, the adoptive transfer experiments with *Rag-1*$^{-/-}$ mice transferred CD103$^{-/-}$ Foxp3$^+$ T_{reg} cells, and our co-culture experiments with Foxp3$^+$ T_{reg} and AT2 cells in the presence of anti-CD103 antibodies support the a role for this integrin's contribution in Foxp3$^+$ T_{reg} 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$^+$ T_{reg} cell effects on AT2 epithelial cell proliferation *in vitro*. These findings strongly indicate an interaction between Foxp3$^+$ T_{reg} 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$^+$ T_{reg} cells to epithelial surfaces. The potential mechanism for decreased epithelial proliferation with antibody-mediated blockade *in vivo* is a decrease in the number of epithelial-localized Foxp3$^+$ T_{reg} cells which then exerts a CD103-independent effect on epithelial proliferation detached from whether Foxp3$^+$ T_{reg} cells specifically express CD103 or not. This potential mechanism is supported in part by the adoptive transfer experiments where *Rag-1*$^{-/-}$ *mice* were transferred CD103$^{-/-}$ Foxp3$^+$ T_{reg} cells (Figure 4). This subset of adoptive transfers had a lower percentage of epithelial proliferation and a lower number of Foxp3$^+$ cells in the lung compared to the WT Treg adoptive transfer subset. However, the *in vitro* co-culture experiment where anti-CD103 antibodies were added to the co-culture of Foxp3$^+$ T_{reg} cells and AT2 cells already in direct contact demonstrated a lower level of AT2 proliferation compared to IgG isotype controls. This strongly supports a role for Foxp3$^+$ T_{reg} cell expressed CD103 in AT2 proliferation (Figure 6). Another potential mechanism is blocking CD103 may down-

regulate Foxp3$^+$ T_{reg} cell expression of a pro-proliferative soluble mediator which enhances epithelial proliferation (such as a cytokine or growth factor). Foxp3$^+$ T_{reg} cells may demonstrate similar ability to induce epithelial cell proliferation through production of growth factors such as KGF similar to shown by intraepithelial γδ T cells [43,49], and may help explain a contact-independent mechanism for epithelial proliferation. This is supported by the contact-independent experiment where Foxp3$^+$ T_{reg} 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 which 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-mediate blockade *in vitro* experiments (Figure 6). The transwell *in vitro* experiments however suggest that this may not be the major mechanism.

In whole, the *in vivo* CD103 antibody-mediated blockade results suggest that retention of Foxp3$^+$ T_{reg} cells during time points of ALI resolution are directly correlated to increased levels of epithelial proliferation (see Figure 3F), i.e. more Foxp3$^+$ T_{reg} cells localized to the epithelium during resolution the greater the percentage of epithelial proliferation. *In vivo* and *in vitro* antibody-mediated blockade may affect this interaction through one or more several possible mechanisms listed above. The transwell contact-independent experiments performed suggest that that not all of the pro-proliferative effects of Foxp3$^+$ T_{reg} cells on AT2 cells *in vitro* are mediated through direct Foxp3$^+$ T_{reg} cells —alveolar epithelial communication and possibly other mechanisms besides CD103 may be important. Determining the extent of the contact-dependent and/or contact-independent factors are future efforts of the authors.

The co-culture data suggests that the interaction of AT2 cells and Foxp3$^+$ T_{reg} cells may also influence surfactant production and differentiation of AT2 cells after injury as detected by differences in SP-C GFP expression versus T1α expression by imaging. This interaction could extend to other subsets of epithelial cells in the lung such as Club or AT1 cells. Growth of purified AT2 cells *in vitro* have been complicated by their ability to proliferate along with the findings that AT2 cells trans-differentiate to a AT1-like phenotype in the absence of specific growth factors [50–52]. The role of AT1 cells in injury repair is unknown, and there have been reports suggesting that AT1 undergo proliferation *in vitro* [53]; however, the current thought is that AT1 cells are terminally differentiated and do not undergo proliferation [39]. If AT1 cells do proliferate after injury then further work investigating the interactions between AT1 and Foxp3$^+$ T_{reg} cells should be studied.

In reciprocal fashion, there have been reports that AT2 cells induce Foxp3$^+$ T_{reg} cells in an antigen dependent manner, and the presence of surfactant protein A (SP-A) has been demonstrated to enhance Foxp3$^+$ T_{reg} cell numbers after LPS-induced injury through a TGF-β dependent mechanism [22,54].

Lastly, the roles for Foxp3$^+$ T_{reg} cells in mediating the response to injury that indirectly impact AT2 proliferation cannot be excluded and is a future area of investigation. We cannot exclude that a contribution of Foxp3$^+$ T_{reg} cells contribute through modulation of a third cell

type such as dendritic cells or macrophages. For instance, Foxp3$^+$ T$_{reg}$ cells decrease TNF-α production by macrophages in a contact-dependent manner [8]. Furthermore, others have demonstrated in the PNX model that primary capillary endothelial cells increase MMP14 which in turn exposes epidermal growth factor receptor (EGFR) and elicits growth of epithelial cells after unilateral PNX [15]. The interactions of pulmonary capillary endothelial cells and Foxp3$^+$ T$_{reg}$ cells may also be important to the effect of proliferation by Foxp3$^+$ T$_{reg}$ cells in PNX [15]. However, the *in vitro* co-culture experiments lend support to a direct interaction and effect of Foxp3$^+$ T$_{reg}$ cells on the lung epithelium.

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$^+$ T$_{reg}$ cells may have additional functions in nonlymphoid tissues [55]. These studies suggest an important role for Foxp3$^+$ T$_{reg}$ cells in alveolar epithelial repair after LPS-induced ALI. Furthermore, Foxp3$^+$ T$_{reg}$ cells also promote CD326$^+$ epithelial cell proliferation in a non-inflammatory compensatory lung growth model. The integrin CD103 plays a role in retaining Foxp3$^+$ T$_{reg}$ cells in the lung after injury and blocking this interaction abrogates the Foxp3$^+$ T$_{reg}$ cell proliferative effects on the lung epithelium. This work highlights an undescribed role Foxp3$^+$ T$_{reg}$ cells in alveolar epithelial proliferation. A better understanding of the interactions of Foxp3$^+$ T$_{reg}$ 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.

MATERIALS AND METHODS

Mice

C57BL/6 wild type (WT), recombinase-activating gene-1–deficient (*Rag-1*$^{-/-}$), and α_E $^{-/-}$ (CD103$^{-/-}$) mice (6–8 weeks of age) were purchased from Jackson Laboratories and housed in a pathogen-free facility. *SP-C*^{GFP} mice were a gift from Dr. John K. Heath (University of Birmingham). *Foxp3*^{gfp} and *Foxp3*^{DTR} 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[8]. Additional detail is provided in an online data supplement.

Unilateral left lung pneumonectomy

Nine to twelve week old C57BL/6, *Rag-1*$^{-/-}$, *Foxp3*^{gfp}, and *Foxp3*^{DTR} mice underwent left pneumonectomy, and lung tissue obtained at 3, 7, or 14 day post procedure for endpoints as previously described [15].

Diphtheria toxin administration

Diphtheria toxin (List Biologicals Laboratories) was suspended in PBS. Stock solutions were thawed once and mice were injected intraperitoneally with 50 μg/kg or 10 μg/kg of diphtheria toxin similar as previously described [26].

In vivo blockade of CD103

WT animals were given 0.15 mg/dose/mouse of intraperitoneal injections of a blocking CD103 rat polyclonal antibody (M290; BioXcell) or isotype (rat IgG, Sigma-Aldrich) 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[8]. Additional detail is provided in an online data supplement.

Lung morphology

Lungs from animals were inflated to 25 cm H_2O with 1 % of low melting agarose (Invitrogen) for histological evaluation by hematoxylin and eosin staining[8].

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) and elastase 3 U/mL (Worthington Biochemical Corp) prior to 1% (wt/vol) low melting agarose (Invitrogen) infusion similar to previous descriptions [7,16]. Lungs were minced and filtered as described [8,16] and additional detail is provided in an online data supplement.

Isolation of $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^-$ T cells and adoptive transfer

Spleens were removed and prepared for single-cell suspensions. $CD4^+$ T cells were isolated from the resulting splenocytes using magnetic bead separation as previously described[8].

BAL RAGE Levels

BAL fluid was used in ELISA assays to determine levels of soluble RAGE (R&D Systems) as previously described[23].

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 FACSAria and FACSDiva software (Becton Dickinson) as previously described [7]. Additional detail is provided in an online data supplement.

Immunoblot Analysis

Whole lung samples were prepared and used for immunoblot analysis as previously described[56]. Additional detail is provided in an online data supplement.

Immunofluorescence

AT2 cell were grown on chamber slides (Lab-Tek) and at time points of interest the media was removed and the cells were washed with PBS. Then APC-conjugated anti-mouse podoplanin (Biolegend) was added to PBS with 3% albumin and incubated at 37 C for 30 minutes. The slides were then washed with PBS and then slides covered with Fluoromount (Sigma), sealed and visualized by immunofluorescence with a Leica SP2 Confocal Microscope.

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

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research Support: NIH NRSA F32HL110561 (JRM), NIH R00HL103973 (FRD), NIH R01HL089346 (LSK), and Johns Hopkins Bayview Scholars Program (LSK).

The authors would like to thank Andre Robinson and James Watkins for expert assistance with tissue processing for histological studies. We thank Dr. Mark Soloski, Joe Chrest, and Raffaello Cimbro for their assistance in the Johns Hopkins Bayview Flow Cytometry Core. We thank Dr. Enid Neptune for use of the *SP-C*^{GFP} mice, which were a gift from Dr. John Heath and Dr. Jo Rae Wright. We thank Dr. Alexander Rudensky for use of the *Foxp3*^{gfp} and *Foxp3*^{DTR} mice strains.

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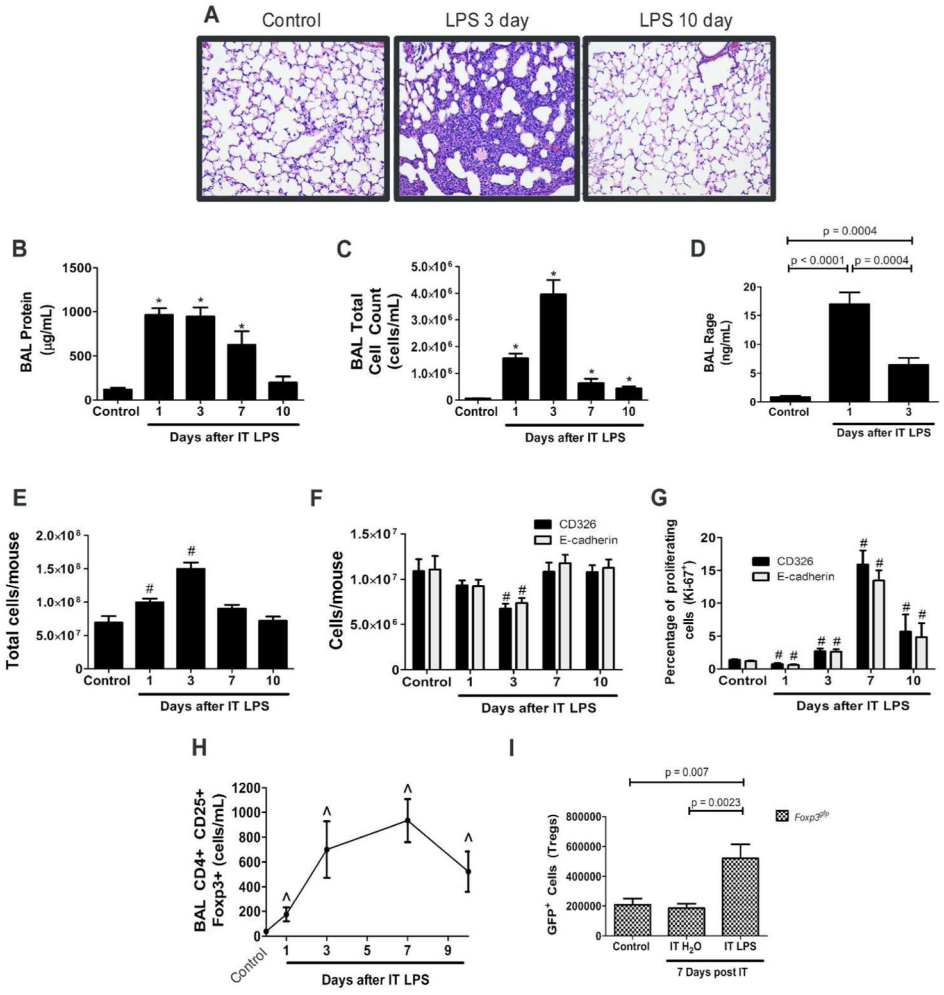

Figure 1. Lung epithelial cells are more proliferative after LPS-induced ALI

Epithelial proliferation levels peak during ALI resolution. WT mice (n = 8–14 per group per time point) were challenged with IT LPS. (A) Representative lung sections were stained with H&E to reveal morphologic changes at time points after IT LPS. Original magnification, x40. (B–D) BAL total protein (B), BAL total cell counts (C), and BAL soluble RAGE measured by ELISA (D) were determined in WT mice controls or after treatment with LPS. *$p < 0.001$ versus control by Mann Whitney. (E–G) Total lung cell count (E), Total $CD326^+$ or E-$cadherin^+$ cell counts (F), and percentage of proliferating ($ki\text{-}67^+$) $CD326^+$ or E-$cadherin^+$ cells (G) were determined by flow cytometry in WT mice controls or after treatment with IT LPS. #$p < 0.05$ versus control by Mann Whitney. (H) Alveolar Treg ($CD4^+CD25^+Foxp3^+$) numbers at baseline and after injury in WT mice. (I) Total $Foxp3^+$ T_{reg} numbers (GFP^+ cells) in single cell suspensions of *Foxp3*^{gfp} mice at baseline and 7 days after treatment with IT water or LPS. (n = 7 per group). ^$p < 0.01$ versus control by Mann Whitney. Figure p values determined by Mann Whitney.

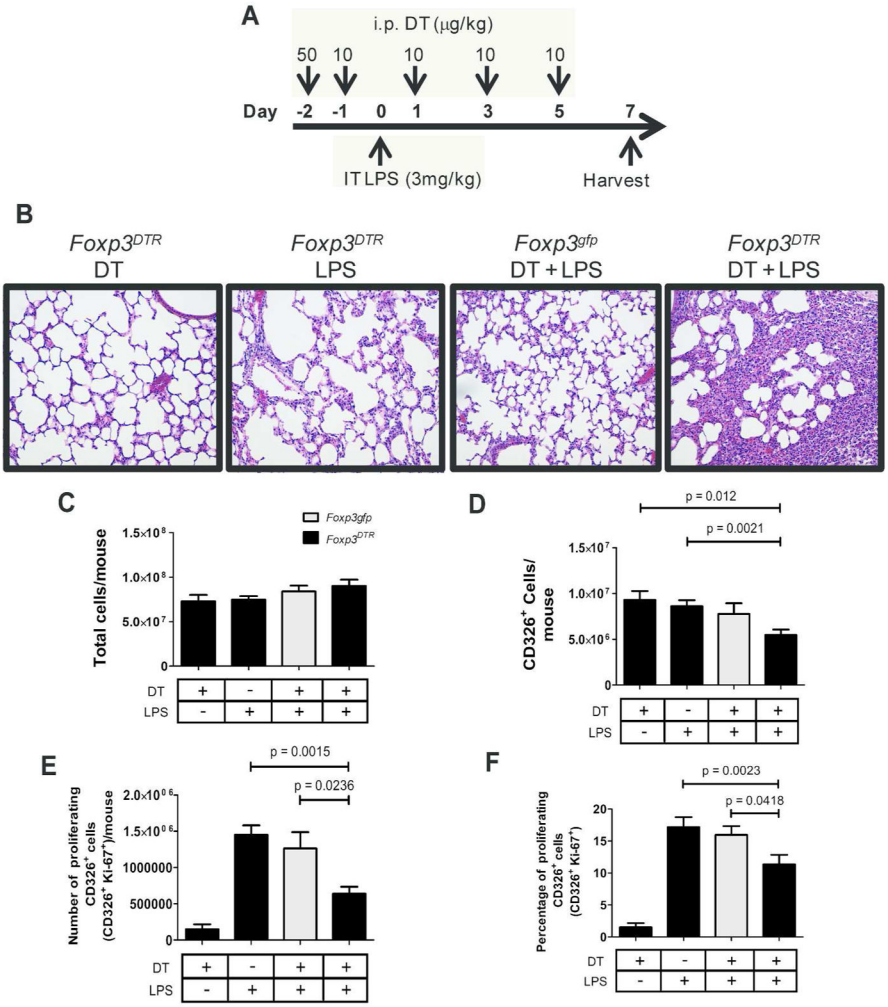

Figure 2. Epithelial proliferation during ALI resolution is impaired in T_{reg}-depleted mice

T_{reg}-depleted mice have decreased levels of epithelial proliferation. (A) *Foxp3^{gfp}* or *Foxp3^{DTR}* mice (n = 5–9 per group) were challenged with IT LPS (day 0) and/or diphtheria toxin (50 μg/kg or 10 μg/kg) administered at day -2, -1, 1, 3, and 5 and harvested at day 7 post LPS. (B) Representative lung sections were stained with H&E to reveal morphologic changes 7 days after IT LPS ± DT. Original magnification, x40. (C–F) Total lung cell count (C), Total CD326$^+$ cell numbers (D), proliferating epithelial cell (CD326$^+$ki-67$^+$) numbers (E) and percentage of proliferating CD326$^+$ cells compared to total CD326$^+$ cells (F) were determined by flow cytometry. p values determined by Mann Whitney.

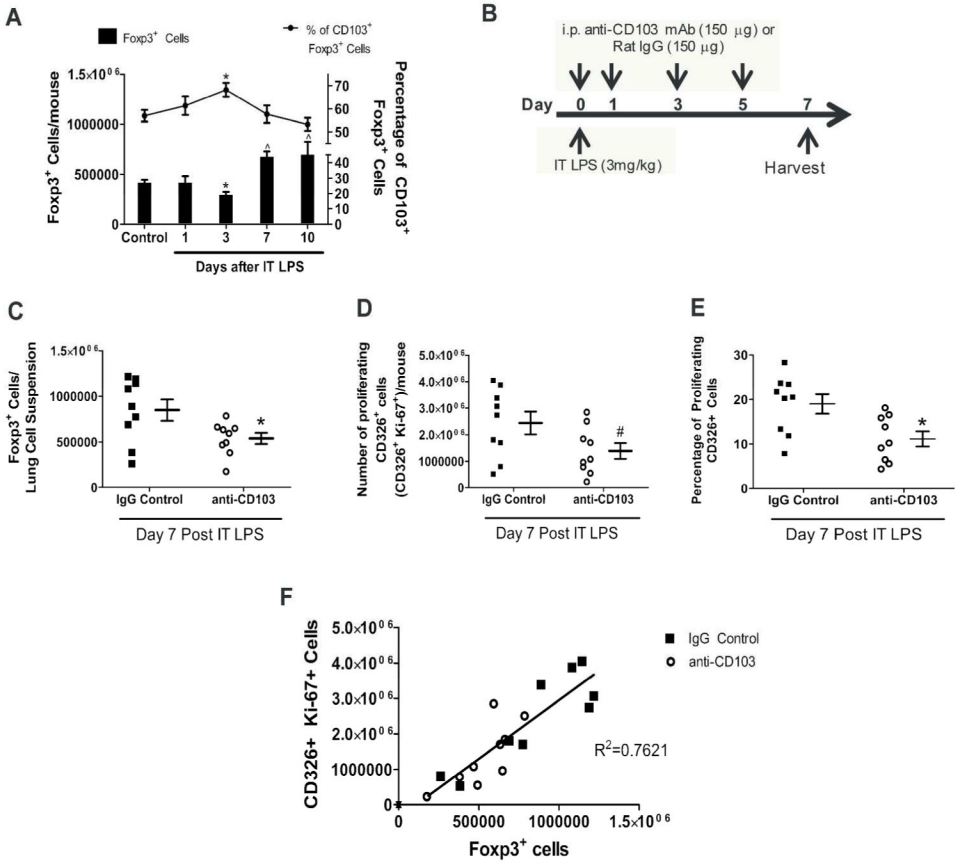

Figure 3. T_{reg} number and epithelial proliferation after ALI is abrogated with antibody blockade of CD103

CD103 blockade decreased lung Foxp3$^+$ T_{reg} number and subsequent epithelial proliferation after ALI. (A) WT mice (n = 10–12 per group per time point) were challenged with IT LPS and the number of Foxp3$^+$ T_{reg} cells in the lung cell suspensions were identified by flow cytometry along with the percentage of Foxp3$^+$ cells that co-expressed CD103 (n = 7–9 per group per time point). *p < 0.05; ^p < 0.005 versus control by Mann Whitney. (B–F) WT mice (n = 9 per group) were challenged with IT LPS along with administration of either an anti-CD103 antibody (150 μg) or isotype control (150 μg rat IgG) at days 0, 1, 3, and 5 post IT LPS (B). The number of Foxp3$^+$ cells in the lung cell suspensions were determined by flow cytometry (C) along with the total number (D) and percentage (E) of proliferating CD326$^+$ cells (CD326$^+$ki-67$^+$ cells) cells. (F) Total lung Foxp3$^+$ cell number positively correlated with CD326$^+$ki-67$^+$ epithelial cell number (Pearson r = 0.8730, R^2 = 0.7621, p < 0.0001 using linear regression). #p = 0.0939; *p < 0.05 by Mann Whitney.

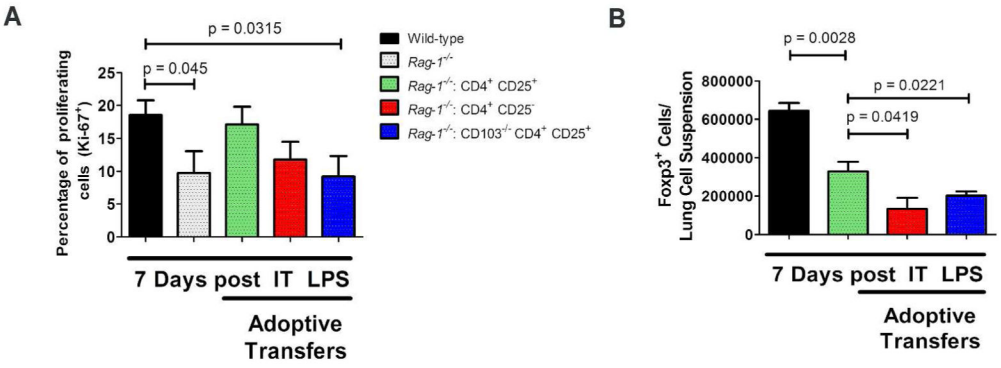

Figure 4. Adoptive transfer of T_{regs} augments epithelial proliferation after ALI in *Rag-1*$^{-/-}$ mice

AT of WT T_{regs} augments epithelial proliferation after ALI in *Rag-1*$^{-/-}$ mice. *Rag-1*$^{-/-}$ mice were challenged with IT LPS and 1 hour afterward received 1×10^6 WT $CD4^+CD25^+$, WT $CD4^+CD25^-$ or $CD103^{-/-}CD4^+CD25^+$ cells and lungs harvested at day 7 post LPS administration. (A) Percentage of proliferating $CD326^+$ki-67^+ 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 t-test.

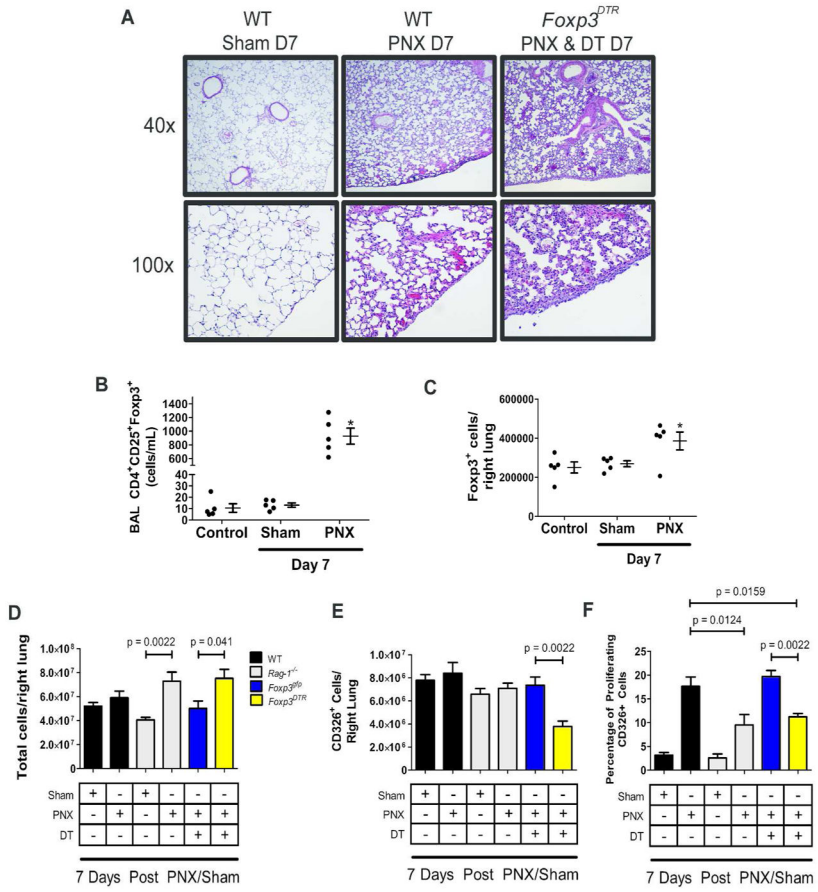

Figure 5. T_{regs} augment epithelial proliferation in mice after PNX

WT, *Rag-1*$^{-/-}$, *Foxp3*^{gfp}, and *Foxp3*^{DTR} mice underwent left lung pneumonectomy. *Foxp3*^{gfp} and *Foxp3*^{DTR} mice undergoing PNX were also administered DT at the time of PNX and at days 2, 4, and 6 post PNX. (A) Representative lung sections were stained with H&E to reveal morphologic changes 7 days post procedure in WT sham, WT PNX, and *Foxp3*^{DTR} mice (PNX and DT). Original magnification, x40 and 100x. (B–C) WT mice (n = 5 per group) alveolar (B) and lung (C) Foxp3^{+} T_{reg} number at baseline and at 7 days post sham or PNX. *$p < 0.05$ versus control/sham by 1 way ANOVA. (D–F) Total lung cell count (D), CD326^{+} cell numbers (E) and percentage of proliferating epithelial cells (CD326^{+}ki-67^{+}) number (F) were determined by flow cytometry 7 days after sham procedure or PNX (n = 6–13 per group). p values determined by Mann Whitney.

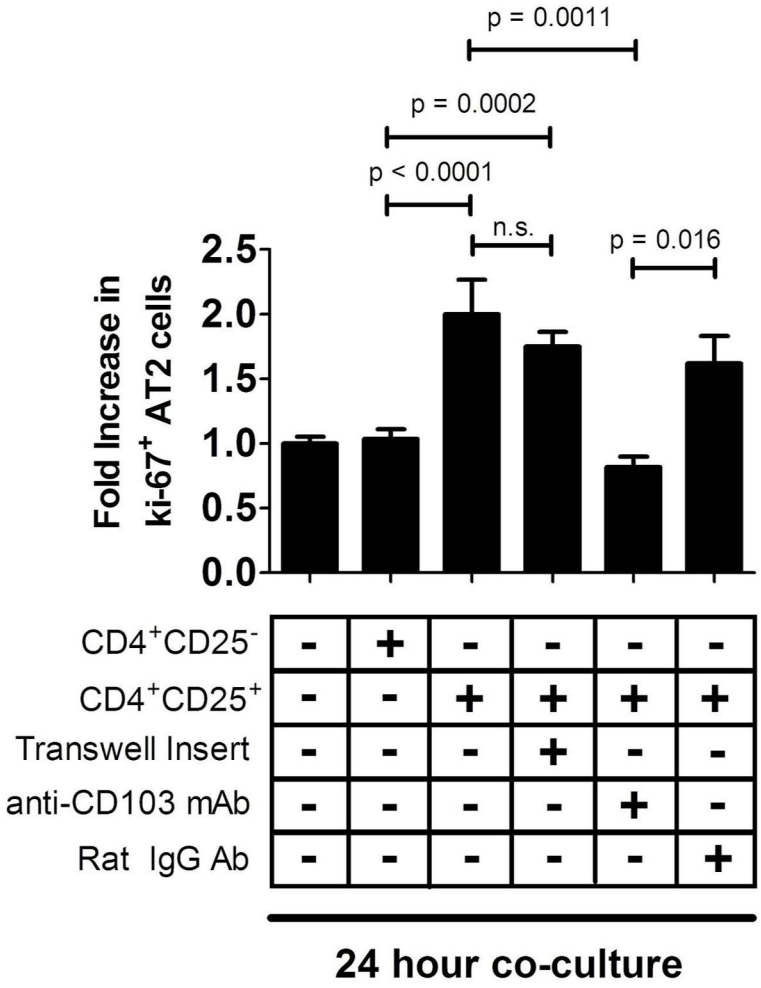

Figure 6. T_{regs} enhance proliferation of AT2 cells *in vitro*

Primary AT2 cells were isolated by sorting GFP^+ cells from *SP-C*^{GFP} mice and co-cultured with lymphocytes subsets (WT $CD4^+CD25^+$, WT $CD4^+CD25^-$) at a lymphocyte: AT2 ratio of 1:5, $n \geq 5$ for each condition. Fold increase (compared to media alone) in proliferation ($CD326^+$ki-67^+) was determined after 24 hours of co-culture of AT2 cells with either $CD4^+CD25^-$, $CD4^+CD25^+$, $CD4^+CD25^+$ plus addition of CD103 blocking antibody, or $CD4^+CD25^+$ separated from AT2 cells through a Transwell insert. p values determined by Mann Whitney.