Control of Regulatory T Cells and Airway Tolerance by Lung Macrophages and Dendritic Cells

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Abstract

Airway tolerance, a state of immunological surveillance, suppresses the development of lung inflammatory disorders that are driven by various pathological effector cells of the immune system. Tolerance in the lung to inhaled antigens is primarily mediated by regulatory T cells (Treg cells) that can inhibit effector T cells via a myriad of mechanisms. Accumulating evidence suggests that regulatory antigen-presenting cells are critical for generating Treg cells and/or maintaining the suppressive environment in the lung. This review focuses on the control of airway tolerance by Treg cells and the role of regulatory lung tissue and alveolar macrophages, and lung and lymph node dendritic cells, in contributing to airway tolerance that is associated with suppression of allergic asthmatic disease.

Keywords: airway tolerance; regulatory T cells; lung; macrophages; dendritic cells

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Airway exposure to environmental antigens can lead to a state of immunological tolerance. This can occur in the lungs of normal individuals on inhalation of antigen, or in the gut or other organs, depending on how antigen is encountered and the form of the antigen. Tolerance is defined as a state of unresponsiveness to the antigen whereby an effective or strong immune response is not promoted. In the case of the lung, this prevents the unwanted development of airway inflammation, and can even restrict the development of inflammation when the same antigens are subsequently reencountered in a form that is normally immunogenic. Much of the tolerogenic mechanism occurs at the level of T cells that are reactive against the environmental antigens. Although evidence shows that deletion and anergy (unresponsiveness) of antigen-specific T cells likely contribute to airway tolerance and the inability to mount an inflammatory immune response, mouse and human studies have highlighted the key role of regulatory $CD4^+$ T cells (Treg cells) that inhibit the activity of pathogenic cells in keeping the lung in a quiescent state and limiting inflammation associated with allergic asthmatic disease (1–6). In humans, the appearance of Treg cells expressing forkhead box P3 (Foxp3) or IL-10, or both molecules, have been correlated with the suppression of lung inflammation, and these Treg cells have been seen to accumulate in patients with asthma or allergic rhinitis undergoing specific allergen immunotherapies (1–6). For example, Treg cell numbers were observed to increase in patients undergoing immunotherapy to escalating doses of house dust mite (7), grass pollen (8), and venom (9). Enhanced numbers of $Foxp3^+$ Treg cells have also been found in glucocorticoid-treated patients with asthma (10). In mouse models, two types of Treg cell can suppress asthmatic lung inflammation, so-called natural or thymic-derived $Foxp3^+$ Treg (nTreg) cells, and inducible Treg (iTreg) cells that develop in the periphery in response to antigen (11–14). iTreg cells are likely the most important subset as they are specific for the inhaled antigen and quantitatively better at limiting an asthmatic-type lung inflammatory response on a per-cell basis (11–14). In mice and humans, it is presently difficult to definitively separate nTreg from iTreg cells. Expression of Foxp3 and IL-10 as determined by intracellular staining can be used to identify Treg cells, but both nTreg and iTreg cells have the ability to bear these molecules. A number of molecules such as helios, neuropilin-1, programmed cell death (PD)-1, and CD73 have been suggested as possible distinguishing markers, with nTreg cells thought to differentially express these molecules or to express higher levels compared with iTreg cells (15–17). However, the data at present suggest there is not yet a set of molecules that can easily allow analysis of iTreg cells in the absence of knowing the antigen with which they react.

In the mouse, iTreg cells are induced and predominate when pure protein antigen, such as ovalbumin free of any contaminants, is inhaled into the lungs of unsensitized animals. In contrast, when extracts of allergens are inhaled, such as from house dust mite or *Aspergillus fumigatus*, or when a pure protein is inhaled along with an innate pattern recognition receptor ligand (such as a Toll-like receptor-4 ligand), effector/pathogenic $CD4^+$ T cells are primarily induced with few if any iTreg cells (13, 18–20). As such it is then important to fully understand how these lung iTreg cells are generated, as this has potential implications for the treatment of asthmatic lung inflammatory disease.

Extensive *in vitro* studies have demonstrated that the generation of iTreg cells from naive $CD4^+$ T cells involves engagement of antigen by the T-cell receptor together with the synergistic activity of signals from IL-2, transforming growth factor (TGF)-β, retinoic acid (RA), and/or IL-10 (21–24). It is likely that this combination of factors will be provided naturally when a responding T cell interacts with an antigen-presenting cell (APC), but whether there is only one type of APC that is responsible for promoting the development of iTreg cells in the lung or elsewhere is not clear. Indeed, *ex vivo* analysis has found that several populations of APC have the potential to promote the differentiation of iTreg cells (reviewed below). As such these cells can be termed regulatory APCs or tolerogenic APCs to distinguish them from stimulatory APCs that drive the differentiation of effector/pathogenic T cells. However, the nature of these specific APCs may differ depending on the tissue that is central to the particular immune response, and there may be several different subsets of regulatory APC that participate in the overall tolerogenic response. This review focuses on the role of lung macrophages in promoting airway tolerance through induction of Treg cells and also discusses how lung and lung-draining lymph node dendritic cells (DCs) may contribute to this process.

Mechanisms Regulating Airway Tolerance

Studies suggest that Treg cells serve as the most critical cellular mechanism for promoting tolerance to antigens inhaled into the airways. In particular, iTreg cells, generated in the lung or periphery, and expressing the transcription factor Foxp3, have been shown to control helper T-cell type 2–driven lung inflammation in several models of tolerance in mice (12, 13, 25). $Foxp3^+CD4^+$ Treg cells expressing TGF-β on their surface have also been reported to inhibit the development of allergic inflammation in the lung (11), and these may be a subset of the Treg cells described in the former publications. In addition, a new subset of $Foxp3^-CD4^+$ iTreg cells, bearing membrane latency-associated peptide (LAP), a marker of the capacity to make TGF-β, was described to be generated in response to inhaled pure soluble antigen and similarly suppressed asthmatic responses in the lung (19). Last, IL-10–producing $CD4^+$ iTreg cells that also lack Foxp3, but can be induced during allergen immunotherapy, likewise suppress helper T-cell type 2 responses, lung eosinophilia, and airway hyperresponsiveness (2, 4–6, 26).

Whether these various types of Treg cell have truly distinct requirements for their generation is not clear. Induction of Foxp3 expression in peripheral naive $CD4^+$ T cells is facilitated by high amounts of TGF-β (21). TGF-β antagonizes the recruitment of DNA methyltransferase I to the Foxp3 locus, thus allowing Foxp3 to be active (27). TGF-β through Smad3 also facilitates activity of conserved noncoding sequence-1 (CNS1), an intronic Foxp3 enhancer (22, 25). RA furthermore synergizes with TGF-β to enhance or stabilize the induction of Foxp3 through nuclear RA receptor–binding sites in CNS1 (28, 29). IL-10 can also aid the formation of $Foxp3^+$ iTreg cells in ways not completely understood, but perhaps by limiting inflammatory activity in APCs. On the other hand, IL-10–producing $CD4^+$ Treg cells that lack Foxp3 can be induced by a combination of vitamin D and corticosteroids, and by IL-10 or IL-27, and IL-10 may be most relevant under physiological conditions (2, 30, 31). Thus, arguably, TGF-β, RA, and IL-10 may be the primary factors for allowing iTreg cells to develop in any tissue. *In vivo* studies with lung tolerance models have produced data in line with this conclusion, in that TGF-β has been found to be required for the induction of $Foxp3^+$ iTreg cells (13, 32) as well as LAP^+Foxp3^- iTreg cells (19); RA was shown to contribute to the development of $Foxp3^+$ iTreg cells (20); and IL-10 was additionally found to be required for the accumulation of both of these iTreg cell subsets (19). In the latter studies, IL-10–secreting iTreg cells were not found even though IL-10 was active *in vivo* (19), but other reports of suppression of allergic lung inflammation have found that IL-10 production can contribute to the generation of $IL\text{-}10^+Foxp3^-$ iTreg cells (33, 34). As costimulatory ligands in the tumor necrosis factor (TNF) family, such as OX40 ligand (OX40L), and inflammatory cytokines, such as IL-6, block the induction of iTreg cells in the lung environment (13, 18, 19), a generalization is then that an APC that is immature or undifferentiated, lacking expression of certain costimulatory ligands and inflammatory cytokines, and producing TGF-β, RA, and/or IL-10, may be an APC that can promote or favor the generation of iTreg cells and would result in keeping the response to environmental antigens in the lungs, or in fact any tissue, in check and at a level that does not result in pathology.

Lung Regulatory Macrophages Promote iTreg Cells and Suppress Lung Inflammation

Macrophages and DCs are classical APCs for $CD4^+$ T cells as they constitutively express class II MHC; however, their state of activation and differentiation can vary dramatically. Several populations of macrophages and DCs have been described in the lung and are likely to play an important role in lung protection, or lung pathology, or both, depending on their activation and differentiation state (35, 36). Moreover, populations of these cells also exist in lung-draining lymph nodes and will also contribute to any pulmonary response given that antigen can drain to the lymph nodes and that many of these APCs can migrate to and from the lung. Therefore, what is a primary APC and what is a secondary APC in the lung environment are the subject of speculation.

Tissue macrophages (sometimes termed interstitial macrophages) and alveolar macrophages represent the two main macrophage subsets in the lung. We reported that mouse lung tissue macrophages intrinsically have the ability to

promote the generation of Foxp3$^+$ iTreg cells and contribute to airway tolerance as they can produce both TGF-β and RA (20). These tissue macrophages express CD11c, Siglec F (a sialic acid–recognizing lectin), F4/80, and CD68, and exhibit high autofluorescence (AF), but have low levels of class II MHC, and are negative for CD11b, CD205, and CD24, thus distinguishing them from the majority of lung tissue DCs that are CD11c$^+$MHCII^{hi}CD11b$^+$CD205$^+$CD24$^+$, AF low, and negative for Siglec F, F4/80, and CD68. In the steady state lung of unmanipulated mice, the tissue-resident macrophages constitutively express TGF-β that drives Foxp3 expression, and retinaldehyde dehydrogenase types 1 and 2 (RALDH1 and RALDH2), the enzymes that regulate RA production that synergizes with TGF-β to stabilize Foxp3. We found that these macrophages could present a pure version of a protein antigen to naive CD4$^+$ T cells and efficiently promote the generation of Foxp3$^+$ iTreg cells in a TGF-β and RA–dependent manner in the absence of any exogenous factor, when isolated directly from the lungs of unimmunized mice. Moreover, this activity was retained when they took up soluble pure antigen after it was inhaled by an unsensitized animal. This implies that the macrophages function as an integral component of the tolerance mechanism that maintains normal homeostasis in the lung under steady state conditions. Indeed, transferring the antigen-bearing tissue macrophages into the airways of naive mice induced a state of tolerance whereby the development of asthmatic lung inflammation was prevented on subsequent challenge with antigen in a normally immunogenic form. Of significance, when mice inhaled several allergen extracts, which contained antigens that intrinsically have protease activity as well as containing pattern recognition receptor ligands, these blocked the induction of airway tolerance to a pure protein antigen and led to asthmatic lung inflammation. Explaining the defect in tolerance in this scenario, the tissue macrophages lost their ability to induce Foxp3$^+$ iTreg cells and instead took on an inflammatory phenotype, producing cytokines such as IL-1, IL-6, and TNF. Interestingly, these activated macrophages continued producing high levels of TGF-β and RALDH, showing that the natural tolerogenic activity of these factors was overridden by uptake of allergen in the context of the stimulatory effects of the protease activity and pattern recognition receptor ligands (20). Together, these data suggest that the lung tissue macrophages likely function as a central component of the suppressive mechanisms active within the lung environment, at least in the steady state.

Alveolar macrophages, generally isolated from bronchoalveolar lavage by adherence, high AF, and/or sorted as CD11c$^+$CD11b$^-$, have been more extensively studied than lung tissue macrophages and also exhibit intrinsic suppressive activity when purified from naive unimmunized animals, suggesting that they additionally contribute to maintaining tolerance in the lung in the steady state. Because many studies were done before iTreg cells were recognized as a major cell type that could promote tolerance, previous experiments with alveolar macrophages from mice or rats focused on their inability to efficiently activate T cells; their capacity to induce a state of T-cell hyporesponsiveness, termed anergy; or their propensity to suppress the activity of lung DCs (37–40). Similar observations were reported for human alveolar macrophages obtained by bronchoalveolar lavage (39, 41–44). In some studies, neutralization of TGF-β, IL-10, or CTLA-4 (cytotoxic T-lymphocyte antigen-4) did not alter their activity on T cells, but exogenous provision of CD28 signals, or addition of IL-2, or of granulocyte-macrophage colony-stimulating factor and TNF, did allow a productive T-cell response, suggesting that the suppressive activity may be due to poor expression of costimulatory molecules or poor antigen presentation rather than through inhibitory molecules or induction of Treg cells (42, 45). However, other studies found that production of nitric oxide, TGF-β, or prostaglandins by alveolar macrophages contributed to their suppressive effect in some experimental protocols (37–41). Moreover, alveolar macrophages from mice and humans were also reported to be able to induce the generation of Foxp3$^+$ iTreg cells from naive CD4$^+$ T cells *in vitro* in a TGF-β and RA–dependent manner (46), although it is not clear whether they exert the same level of activity in this regard as lung tissue macrophages. Similar to tissue macrophages, exposure to allergens or isolated pattern recognition receptor ligands can antagonize the suppressive function of alveolar macrophages and only unstimulated *ex vivo* cells protect against development of asthmatic lung inflammation when transferred into other mice (47–50).

Intratracheally administered clodronate (dichloromethylene diphosphonate)–containing liposomes have been used as a means of depleting lung macrophages in mouse models, with the result that significantly greater lung inflammatory and systemic T-cell–dependent responses were observed on antigen challenge (37, 40, 50–52). Although the focus of these reports was the alveolar macrophage, clodronate-containing liposomes may also deplete a proportion of lung tissue macrophages. Collectively, these results demonstrate a strong role for both lung tissue and alveolar macrophages in dampening the pathogenic response to inhaled pure protein antigens. However, if the antigens contain protease activity, as many allergens appear to do, or if the antigens are inhaled together with an undefined level of pattern recognition receptor ligands, such as LPS, which activates Toll-like receptor-4, the lung macrophages may lose their tolerogenic activity.

Regulatory DCs Participate in Promoting Lung Tolerance

In contrast to lung resident macrophages that might be intrinsically suppressive in the steady state, it is not clear whether lung DCs also possess this capacity, or whether they need to be mobilized or induced to participate in promoting the generation of iTreg cells. Several types of lung resident DCs have been described. Conventional DCs (cDCs) in the resting mouse lung are CD11c$^+$, but have low AF, high levels of class II MHC, and are Siglec F negative as described previously and have been divided into CD103$^-$CD11b^{hi} and CD103$^+$CD11b^{lo} subsets. At least in the steady state mouse lung, both subsets appear to be more stimulatory than regulatory, primarily inducing the development of effector T cells (53–55). Similarly, human lung DCs were reported to be strong stimulators of allogeneic T cells in relation to human lung macrophages (43, 44). Indeed, in our studies, isolating these cells from the lungs

of unsensitized mice, based on being $CD11c^+AF^{lo}$Siglec F^-MHC II^{hi} (without subsetting based on CD103 or CD11b), revealed that they were weak at promoting $Foxp3^+$ iTreg cells from naive $CD4^+$ T cells when compared with lung tissue macrophages (20). Nevertheless, in the lungs of unsensitized mice we did find that as a population the lung tissue, $CD11c^+$ cDCs expressed RALDH2 (but not RALDH1 and with little TGF-β). This implies that these lung cDCs might be able to promote the generation of $Foxp3^+$ iTreg cells by making some RA as long as there is a sufficient amount of TGF-β produced locally from other cellular sources. *In vitro*, these cDCs when cultured in the presence of exogenous TGF-β did in fact efficiently induce $Foxp3^+$ iTreg cells, albeit with approximately fourfold less activity than lung tissue macrophages cultured under the same conditions (20). Their lower regulatory activity even when TGF-β was supplied was probably reflective of weaker production of RA, and higher expression of class II MHC and other costimulatory/inflammatory factors. This suggests that lung macrophages are likely to play a more dominant role than lung cDCs in promoting iTreg cells under steady state conditions where no sensitization has previously occurred.

However, this may change after inhalation of an antigenic or nonantigenic insult. One study reported that in mice that had inhaled a relatively pure ovalbumin antigen preparation multiple times, but one that may have contained a low level of LPS, lung $CD103^+$ cDCs became strongly tolerogenic and produced both TGF-β and RA, allowing the induction of $Foxp3^+$ iTreg cells (56). Therefore, lung cDCs might gain the capacity to be regulatory or stimulatory depending on the context in which antigen is encountered. Another DC that may also contribute to promote iTreg cells is the plasmacytoid DC (pDC). In one study, an antibody was used to delete pDCs immediately before pure soluble antigen was inhaled, which normally results in a tolerogenic response. In this case, tolerance was abrogated and lung inflammation was induced (57). Other reports injected Flt3 ligand (Flt3L) into mice to mobilize or promote the accumulation of pDCs, and found that this suppressed the development of both primary and secondary lung inflammatory responses when antigen was injected in a normally immunogenic form (58). Whether these results reflected the activity of lung-resident pDCs or lymph node–derived pDCs was not clear. The nature of suppression was not investigated at the level of induction of iTreg cells, but it was found to be dependent on expression of the inhibitory ligand PD-L1, and thus may have been different from that afforded by macrophages (58). Another related study, however, isolated pDCs from peripheral lymph nodes of mice injected with Flt3L and demonstrated that cells expressing CD8α were poor at promoting the generation of effector T cells and instead favored the generation of $Foxp3^+$ iTreg cells. These pDCs could suppress lung inflammation when immunogenic antigen was used to sensitize mice, whereas $CD8\alpha^-$ pDCs lacked this inhibitory activity (59). $CD8\alpha^+$ pDCs expressed RALDH1 and RALDH2, although they did not appear to make significant amounts of TGF-β as they efficiently induced $Foxp3^+$ iTreg cells only when cultured with exogenous TGF-β. Thus, pDCs in the lung or draining lymph nodes may also be intrinsically suppressive in the steady state or they may be promoted to be suppressive by exposure to relatively pure antigens that lack protease or pattern recognition receptor ligand activity, when factors such as Flt3L are available.

Both macrophages and DCs also have the capacity to produce the cytokine IL-10, which may be either directly suppressive or function to promote IL-10–producing iTreg cells from naive T cells. However, whether IL-10 is normally an initial product of these cells in the resting lung environment is still not clear. In the steady state mouse lung, we found no evidence of constitutive IL-10 expression by tissue macrophages or tissue cDCs (20). We did, however, find that IL-10 was active at some stage during the initial development of $Foxp3^+$ and $Foxp3^-LAP^+$ iTreg cells in response to inhaled soluble antigen, in that blocking IL-10 receptor suppressed the generation of these iTreg cells similar to blocking TGF-β, and this consequently prevented airway tolerance (19). The source of IL-10 was unknown. In contrast, in the presence of weak antigenic or inflammatory signals (e.g., low levels of pattern recognition receptor ligand activity), macrophages and DCs may gain the capacity to make IL-10. Exposure to low-dose LPS *in vitro*, or inhalation of pure protein antigen mixed with a low dose of LPS, was found to promote IL-10 production in lung tissue macrophages, leading to their ability to suppress cDC activity and lung inflammation in an IL-10–dependent manner (60). Alveolar macrophages can also make IL-10 in response to LPS (61, 62). Similarly, lung-draining lymph node cDCs isolated 3–4 days after inhalation of soluble protein antigen that may have contained a low level of LPS were found to express IL-10 and were shown to suppress a normally immunogenic response that results in lung inflammation through inducing the generation of IL-10–expressing Treg cells (26, 33). Therefore, IL-10–producing macrophages or cDCs may be induced after a certain type of antigenic insult via the airways and play a role at a later time in either amplifying an existing tolerogenic condition or limiting ongoing lung inflammation. Exactly how to promote IL-10 in these cells, and what type of antigenic insult is required, is not clear.

Lung and Lung-Draining Lymph Node Macrophages and DCs May Sequentially Present Antigen to Promote iTreg Cells and Airway Tolerance

Given the potential of lung and lung-draining lymph node DCs as well as lung macrophages to promote iTreg cells in the resting lung when environmental antigens are inhaled, the development of airway tolerance might then logically encompass sequential interactions between antigen-responding T cells and these alternative APCs. What the order of events might be is not clear.

Current hypotheses largely state that DCs in the lung-draining lymph nodes initiate the activation of T cells that then migrate to, and accumulate in, the lungs after inhalation of antigen, under either tolerogenic or inflammatory conditions. In the absence of migration to the lymph nodes in $CCR7^{-/-}$ mice, tolerance is not induced to inhaled pure protein antigen, implying that some level of movement between lung and lymph nodes is necessary (63, 64). Certainly, antigen is transported into the draining lymph nodes by cDCs after it is inhaled; however, a majority of lung tissue macrophages and alveolar macrophages

also uptake inhaled antigen (20, 65). In the steady state, naive T cells can traffic into lung tissues but with low frequency (66). Memory T cells also can traffic to, and exist in, the lungs, particularly the effector memory and tissue-resident memory subsets. Therefore, it is certainly feasible that lung tissue macrophages and alveolar macrophages, as they display intrinsic regulatory activity in the steady state, will be the initial APCs and first program the differentiation of $Foxp3^+$ and LAP^+Foxp3^- iTreg cells, as well as contribute to tolerance by promoting T-cell deletion or anergy within the lung. This hypothesis is furthered given the location of macrophages in the airways and alveolar spaces, or in the connective tissue of the alveoli, and their ability to capture inhaled antigen immediately. Our studies of lung tissue macrophages transferred into $CCR7^{-/-}$ mice showed a similar percentage of conversion of T cells into $Foxp3^+$ iTreg cells, but their accumulation in high numbers was impaired (20), correlating with the studies concluding that both migration to the lymph nodes and antigen presentation on other APCs is required for the overall tolerogenic response. In this scenario, the tolerogenic program would then be initiated by lung macrophages but maintained by subsequent interactions in the lymph nodes with either pDCs or cDCs that are capable of providing more RA and/or TGF-β to further $Foxp3^+$ and LAP^+ iTreg cell development (Figure 1).

Alternatively, regulatory lymph node pDCs or cDCs after taking up antigen in the nodes, or lung cDCs after taking up antigen in the lungs and migrating to the nodes, may start the series of events that results in the development of large numbers of iTreg cells. This is more likely to be the scenario for initiation of a naive T-cell response, as perhaps the majority of these antigen-reactive T cells will traffic through lymph nodes rather than the lung. We found that lung tissue macrophages were capable of

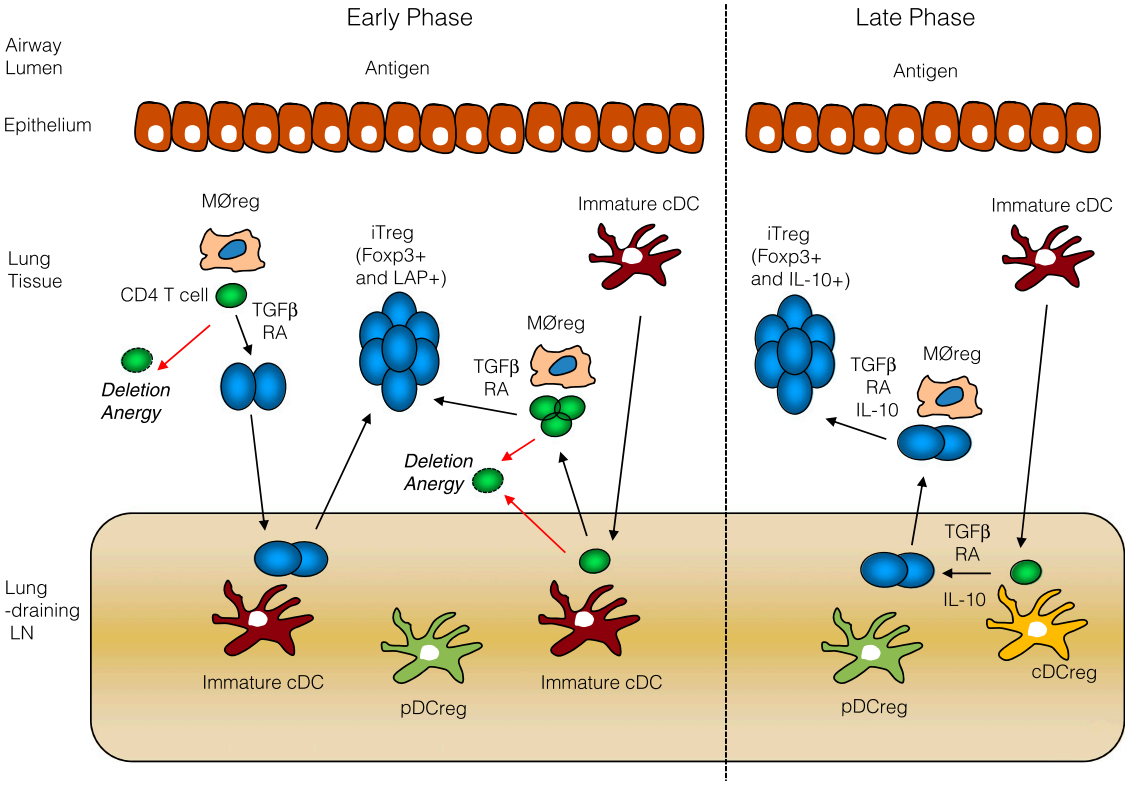

Figure 1. Schematic depicting possible interactions between T cells, macrophages, and dendritic cells that result in the formation of inducible Treg cells (iTreg cells) in the lung and lung-draining lymph nodes (LNs). For simplicity, alveolar and tissue macrophages have been grouped together, but it is still to be determined whether they exhibit divergent activities. Tolerance may be afforded by both early and late interactions. Early interactions would involve intrinsic activities of antigen-presenting cells (APCs) in the steady state when soluble pure protein antigen is taken up in the lung. Two primary scenarios are possible. *Left*: Regulatory macrophages (MØreg) in the lung that constitutively make transforming growth factor (TGF)-β and retinoic acid (RA) directly present antigen to naive $CD4^+$ T cells (*green*), resulting in the formation of forkhead box P3–positive ($Foxp3^+$) or latency-associated peptide–positive (LAP^+) iTreg cells (*blue*). These iTreg cells migrate to the lymph nodes and encounter antigen presented on immature conventional DCs (cDCs), which results in their expansion and movement back to the lung. *Right*: Immature cDCs in the lung take up antigen and migrate to the lymph nodes, where they activate naive $CD4^+$ T cells. These undifferentiated cells expand in numbers, move back to the lung, and then are stimulated by antigen presented on lung MØreg. This results in differentiation into $Foxp3^+$ or LAP^+ iTreg cells driven by TGF-β and RA. Regulatory plasmacytoid DCs (pDCreg cells) in the lymph nodes may also aid these processes. Some T cells are additionally induced to become anergic by interaction with MØreg and immature cDCs, or undergo deletion. At a later time, likely under the influence of low-level signals from antigen and innate stimuli such as LPS, immature cDCs differentiate into regulatory cells (cDCreg) in the lymph nodes and gain the capacity to make TGF-β, RA, and/or IL-10. Similarly, lung MØreg also gain the capacity to make IL-10. Together, this may either maintain the existing population of iTreg cells, or promote the generation of more iTreg cells that could be $Foxp3^+$ or $IL\text{-}10^+$.

promoting Foxp3 expression in T cells activated initially on cDCs (20), suggesting a different order of events where the lung macrophages then provide the secondary signals that solidify the differentiation program of Foxp3$^+$ iTreg cells, and/or promote the generation of more Foxp3$^+$ iTreg cells, when recently activated but undifferentiated T cells populate the lung tissue (Figure 1).

Other nonmutually exclusive scenarios are that tolerance initially afforded by Foxp3$^+$ iTreg cells is further reinforced at a later time by the induction of additional tolerogenic activity in macrophages and cDCs either in the lungs or lymph nodes. Here, uptake of antigen over time in the presence of weak inflammatory signals, such as low doses of LPS, may promote either RA or TGF-β activity in cDCs, or IL-10 activity in macrophages and cDCs, leading to the generation of more Foxp3$^+$ iTreg cells or a new population of IL-10$^+$ iTreg cells. These lung tissue and alveolar macrophages and DCs might then be considered inducible regulatory APCs rather than intrinsic regulatory APCs as their suppressive function is promoted later in a response on recognition of intermediate danger-type signals that are seen as tolerogenic rather than immunogenic (Figure 1). Regardless of the exact sequence of events, it is likely that airway tolerance is afforded by the concerted action of both of the primary populations of lung macrophages, as well as cDCs and pDCs that either reside in the lymph nodes or lungs or migrate between these organs.

Outlook

The knowledge that macrophages and DCs in the steady state lung or lung-draining lymph nodes can exert intrinsic or inducible regulatory APC activity by making TGF-β, RA, and/or IL-10 forms a framework for understanding how tolerance is generated and might be sustained in the airways. The primary question going forward is how to maintain tolerogenic mechanisms in these APCs and promote new development of iTreg cells in the face of insults from inhaled immunogenic antigens, particularly those that are classified as allergens. Increasing evidence suggests that allergens either contain protease activity that can elicit inflammatory molecules, or contain homologs or mimics of pattern recognition receptor ligands that also promote inflammatory molecules. Strong danger-type signals from allergen extracts that contain pattern recognition receptor ligand and/or protease activities (e.g., house dust mite, *Aspergillus*), or from isolated Toll-like receptor or Nod-like receptor ligands (e.g., LPS, muramyl dipeptide), have been found to block the induction of lung iTreg cells and prevent airway tolerance (13, 18–20). It is then likely that inhalation of extrinsic stimuli with normally nonimmunogenic protein antigens, or inhalation of allergens, converts regulatory macrophages and DCs into inflammatory APCs. Interestingly, in the case of lung tissue macrophages, this was not accompanied by down-regulation of the expression of RA or TGF-β, but rather resulted in up-regulated inflammatory activity, including cytokines such as IL-1, IL-6, and TNF, while retaining the ability to make the aforementioned molecules (13, 20). The inflammatory molecules then in effect antagonized or negated the suppressive molecules and prevented iTreg cells from being generated. Similar effects may be likely in regulatory pDCs and cDCs, although this needs to be studied. The key to promoting and/or restoring tolerance when confronted with these immunogenic insults may then lie in determining how to suppress the inflammatory activity of these APCs while still retaining the suppressive molecules TGF-β, RA, and IL-10. Simply blocking the inflammatory cytokines made by the APCs (e.g., IL-1, IL-6), or neutralizing membrane-expressed stimulatory ligands (e.g., TNF family members such as OX40L or TL1A) that together promote the formation of pathogenic effector T cells, may produce the desired effect. However, it is not clear how many inflammatory molecules might need to be targeted to allow the activity of the iTreg-promoting molecules to predominate. Moreover, whether this would be possible during or after airway disease has been initiated is not known. It has been well documented that naive CD4$^+$ T cells are readily convertible into iTreg cells, whereas preexisting pathogenic effector or memory CD4$^+$ T cells are more resistant to becoming iTreg cells. Thus, simply blocking the inflammatory molecules might not be sufficient to reveal the tolerogenic activity of the lung and lung-draining lymph node APCs in previously sensitized, allergic, individuals who already have lung inflammatory disease. In this case, the APCs might need to be targeted directly to promote greater expression of the suppressive molecules that favor the induction of Treg cells. A better understanding of the generation and maintenance of these regulatory APCs might then provide significant insight into strategies that could be efficacious at inducing tolerance in patients with lung disease. ■

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