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Modulation of Dendritic Cell Trafficking to and from the Airways

Claudia Jakubzick,* Frank Tacke,* Jaime Llodra,* Nico van Rooijen,† and Gwendalyn J. Randolph1*

We investigated the fate of latex (LX) particles that were introduced into mice intranasally. Macrophages acquired the vast majority of particles and outnumbered LX particle-bearing airway dendritic cells (DCs) by at least two orders of magnitude. Yet alveolar macrophages were refractory to migration to the draining lymph node (DLN), and all transport to the DLN could be ascribed to the few LX+ airway DCs. Upon macrophage depletion, markedly greater numbers of DCs were recruited into the alveolar space. Consequently, the number of DCs that carried particles to the DLN was boosted by 20-fold. Thus, a so far overlooked aspect of macrophage-mediated suppression of airway DC function stems from the modulation of DC recruitment into the airway. This increase in DC recruitment permitted the development of a robust assay to quantify the subsequent migration of DCs to the DLN. Therefore, we determined whether lung DCs use the same molecules that skin DCs use during migration to DLNs. Like skin DCs, lung DCs used CCR7 ligands and CCR8 for emigration to DLN, but the leukotriene C4 transporter multidrug resistance-related protein 1 did not mediate lung DC migration as it does in skin, indicating that pathways governing DC migration from different tissues partially differ in molecular regulation.

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Dendritic cells (DCs)2 are found throughout the airways (1) and comprise a very small fraction of the cells in bronchoalveolar lavage (BAL) (2), where the vast majority are alveolar macrophages. Pulmonary DCs initiate immune responses to Ags that enter the airways (1). Presentation of Ag to T cells can be observed in the draining lymph nodes (DLNs), and there is evidence that DCs can pick up soluble Ags or tracers and migrate from the airway to the lymph-draining mediastinal LNs (MLNs) to access naive T cells therein (3).

To date, very few molecules involved in the migration of airway DCs to LNs have been delineated (4–6), because in general, our knowledge of the molecules used by DCs to emigrate to LNs from the periphery is confined to studies in skin. Although assays that demonstrate DC migration from the lung to the DLNs have been developed, those that permit a strong quantitative evaluation are still needed, due to the small numbers of migratory DCs that can generally be found in the MLN. Molecules, including leukotriene C4, LTC4 and its transporter, multidrug resistance-related protein 1 (MRP1, also called ABCC1), and chemokine receptors such as CCR8 participate in DC migration from skin (7, 8). These molecules are pertinent to immune and allergic responses in lung (9, 10), but whether they regulate DC migration from the lung to the MLN is unknown.

Furthermore, the role of DCs in the transport of different types of Ag to LNs remains unclear. DCs transport soluble Ags to MLNs (3), but their role in the transport of particulate Ags has not been clarified. Particulates coming into the airways are very efficiently phagocytosed by alveolar macrophages, and these macrophages are reported to carry the particles to the LN (11). The suppressive nature of alveolar macrophages helps to ensure that harmless environmental particulates do not readily trigger damaging inflammatory reactions in the lung (12). Indeed, alveolar macrophages, in close proximity to resident DCs (13), suppress the function and maturation of pulmonary DCs (13–15).

In this study, we investigated the fate of particulate tracers that were introduced into mice intranasally (i.n.). We reveal that DCs, not alveolar macrophages, transport particulate Ags to the MLN for presentation to T cells. In the absence of macrophages, vastly greater numbers of DCs were recruited into the alveolar space, and T cell proliferative responses to Ag administered i.n. were augmented because subsequent mobilization of DCs to the MLN was, in turn, greatly increased. Thus, a so far unknown, significant aspect of alveolar macrophage-mediated suppression stems from the modulation of DC migration. The increased magnitude of DC mobilization achieved in the absence of macrophages further permitted us to develop a robust assay to quantify migration of DCs to the MLN and allowed us to ask whether lung DCs use the same subset of molecules that skin DCs use during migration to DLNs.

Materials and Methods

Mice

Wild-type (WT), plt/plt, CCR8−/−, MRP1−/−, and OT-II transgenic mice were all backcrossed at least 10 generations onto the C57BL/6 background. Mice were maintained under specific pathogen-free conditions. Procedures were approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine.

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Tracing latex (LX)-bearing cells

FITC-conjugated plain or carboxylate-modified LX microspheres (0.5-μm; Polysciences) were diluted to 0.1% (w/v) in PBS without calcium or magnesium. Fully anesthetized mice received 20 μl of FITC LX i.n. (iNLx). In a modified version of the assay, 30 μl of clodronate-loaded liposomes (CLs) was delivered i.n. 3 days before LX microspheres (iNcLlx). Clodronate was a gift from Roche and was incorporated into liposomes (www.clodronateliposomes.org). Mice were sacrificed 2 days after i.n. delivery of LX beads. MLN were excised from each mouse and digested with collagenase D (Roche) for 30 min. Single-cell suspensions were generated by pressing teased LN through a 70-μm cell strainer. Cells were stained and analyzed by flow cytometry. To compare results from different experiments, data were normalized by dividing the number of LX+ LN cells in WT controls from the same experiment. BAL was always analyzed in parallel with the corresponding MLN to ensure that LX beads were properly instilled i.n. In the infrequent instances when instillation failed, data from the relevant mouse were not considered.

Proliferation of OVA-specific transgenic CD4+ T cell

Unseparated spleen cells from OT-II mice in which the TCR of CD4+ cells is restricted to OVA were isolated and labeled with CFSE (Molecular Probes). Four million of these cells were transferred i.v. into recipient mice is restricted to OVA were isolated and labeled with CFSE (Molecular Probes). Four million of these cells were transferred i.v. into recipient mice. One day before i.n. delivery of LX that was covalently coupled with OVA or probes. mice were sacrificed 2 days after i.n. delivery of LX beads. Mice were sacrificed 3 days after delivery of LX. T cell proliferation (CFSE dye dilution) was measured by the loss of some Fluo-3 dye and corresponding fluorescence intensity (8). Gating on CD11b+ DCs was conducted during analysis.

Flow cytometry

Single-cell suspensions were obtained from BAL, whole lung, spleen, and MLN. BAL was obtained by flushing the airways four times with 1 ml of HBSS containing 0.5 mM EDTA in the BAL space. MLN, spleen, and lung were minced, digested with collagenase D for 30 min, and pressed through a 70-μm cell strainer. Lung and spleen were treated with 7.5 ml of ammonium chloride lysing reagent (BD Biosciences), followed rapidly by addition of the 7.5 ml of HBSS. Cells were washed twice in HBSS. Cells were resuspended in FACS blocking solution and stained for 30 min with PE-conjugated Abs to I-Ab, CD11b, CD11c, CD4; or CD3; and APC-conjugated mAbs to B220, Gr-1, or CD11b; and allophycocyanin-conjugated mAbs to CD11c or Gr-1. Appropriate isotype-matched control mAbs were also obtained from BD Biosciences.

Immunostaining

Nasal-associated lymphoid tissue (NALT) was excised by cutting open the jaws of the mouse and laying flat the upper portion of the jaw. Then, the tip of the nose from the mouse was cut off, and PBS/OCT (1:1) was injected into the nasal cavity. This process adheres the NALT along the epithelial layer of the upper palate via pressure from PBS/OCT. With a scalpel, the epithelial layer of the palate was cut along the edges of the teeth. Lastly, with a fine forceps, the epithelial layer was carefully lifted and placed in OCT for sectioning. Frozen sections of the NALT, cervical LN (CLN), MLN, and spleen were fixed in 3% paraformaldehyde, stained with rat anti-mouse B220 (Cedarlane Laboratories), rat anti-mouse DEC-205 (Serotec), or isotype-matched control mAbs and detected with Cy3-conjugated anti-rat mAb (Jackson ImmunoResearch Laboratories).

RT-PCR

Total RNA was prepared from BAL DCs that were isolated using I-Aα miniMACS beads (Miltenyi Biotec). RNA was isolated with TRIzol reagent according to the manufacturer’s instructions (Invitrogen Life Technologies). The purified RNA was subsequently reverse transcribed into cDNA using oligo(dT)12–18 primers (Invitrogen Life Technologies). The following oligo(dt) primers (5′-3′ sequences) were used for PCR analysis: GPDH sense, GTGGGGGCGCCGCGGAGCA; GPDH antisense, GCTCCTAAATGTCAGCAGGGTTT; Murine CCR8 primers were purchased from R&D Systems. These mixtures were incubated for 4 min at 94°C, amplified at 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s in 40 cycles. After amplification, the samples were separated on a 2% agarose gel containing ethidium bromide. Bands were visualized and photographed using a UV source.

Efluss assay to assess MRP1 activity

BAL was isolated from CL-treated mice so that the cell suspension was enriched in DCs. An aliquot of cells was separated at a negative control (e.g., no dye uptake). Remaining cells were loaded with Fluo-3 at 37°C for 30 min at a final concentration of 4 μg/ml (Molecular Probes), a fluorescent MRPI substrate. Cells were then placed on ice for 1 min and washed twice in cold RPMI 1640. An aliquot was kept at 4°C to record dye uptake. DCs were then incubated at 37°C for 60 min, washed twice in cold RPMI 1640, and immediately analyzed by flow cytometry for MRPI activity, as measured by the loss of some Fluo-3 dye and corresponding fluorescence intensity (8). Gating on CD11b+ DCs was conducted during analysis.

Results

Phenotype of LX+ and migratory pulmonary phagocytes

FITC+ LX particles (0.5-μm) were delivered i.n. into the alveoli. BAL, lung parenchyma, and various lymphoid tissues were examined for cells that engulfed LX. Lung macrophages are highly autofluorescent, CD11c+ and CD11b−, whereas DCs are scarcely autofluorescent, CD11c+, and CD11b+ (2, 3, 16) (Fig. 1A). The
CD11b⁺CD11c⁺ DCs express notably higher levels of MHC class II (MHC II) than their CD11b⁺ macrophage counterparts (Fig. 1A). Both cell types in lung express DEC-205 (17). Delivery of the LX beads i.n. did not induce inflammation in the BAL. Beads were engulfed by alveolar macrophages within 4 h. In lung sections, numerous LX⁺ DEC-205⁺ cells were seen around the alveolar epithelium (Fig. 1B). By day 2, >99% of the beads were contained within CD11c⁺CD11b⁻Gr-1⁻ MHC II⁺⁺ macrophages (Fig. 1C). A very small number of CD11b⁺ DCs also engulfed microspheres, because scanning LX⁺ cells in cytospins of the BAL and in situ of the whole lung revealed a few LX⁺ CD11b⁺ cells with DC morphology (Fig. 1D).

Next, we determined whether any LX-bearing cells emigrated to regional lymphoid tissue. It is thought that alveolar macrophages transport microspheres to the lung-DLNs (11). We examined NALT, CLNs, MLNs, and spleen for the presence of LX⁺ cells. Among these tissues, only MLN significantly accumulated LX⁺ cells (Fig. 2A). We detected 50 –100 bead⁺ cells in the pooled MLNs per mouse (Fig. 2B), >100 times fewer than the number of cells that engulfed beads in the airways, but not much less than the number of DCs that are normally found in BAL (around 200; see below). Infrequent LX⁺ cells were observed in the B cell-rich NALT and CLNs (Fig. 2A). Spleen was negative (data not shown).

In MLN, LX⁺ cells accumulated in the T cell zone (Fig. 2A), and their phenotype by flow cytometry was similar to the lung DCs (CD11c⁺, CD11b⁻) (Fig. 2B), rather than CD11b⁺ alveolar macrophages. Thus, either the LN LX⁺ cells derived from alveolar macrophages that acquired a DC phenotype in transit to the LN or the small fraction of DCs that engulfed particles in the airways selectively migrated to the MLN, leaving alveolar macrophages behind in the air spaces and lung interstitium.
Pulmonary DCs, but not alveolar macrophages, migrate to MLN and induce T cell proliferation proportional to the magnitude of migration

To determine whether alveolar macrophages or airway DCs carried the particles to the MLN, we assessed how depletion of alveolar macrophages affected the appearance of LX⁺ DCs in the MLN. If alveolar macrophages were precursors for the LX⁺ MLN DCs, then the number of LX⁺ cells in the MLN pool should be reduced after macrophage depletion.

To deplete alveolar macrophages, we administered CLs i.n. The i.n. delivery of these liposomes causes local and selective depletion of macrophages with little or no inflammation in the BAL or lungs of recipient animals (18–20). In agreement with these studies, very few if any neutrophils were found in BAL at any of the time points tested (data not shown). Peak depletion of alveolar macrophages occurred between days 3 and 5, when 80–90% were lost (Fig. 3A). During this time, there was a relative and absolute increase, by 10- to 30-fold, in the number of CD11c⁺CD11b⁺ MHC II⁺ DCs that entered the airway (Fig. 3, A and B), such that the number of DCs recovered in BAL jumped from ~200 to nearly 3000 (Fig. 3A). Under these conditions, up to 30% of the total live (propidium iodide-negative) alveolar cells were DCs, rather than the typical ≤1% observed in naive mice (Fig. 3C). The number of DCs surrounding airway epithelium was also greatly increased under these conditions (Fig. 3D). There was a striking inverse correlation between the relative number of alveolar macrophages in the BAL with the relative number of DCs recovered therein (Fig. 3C). For example, when half of the macrophages had rebounded following CL-mediated depletion (see day 11, Fig. 3C), the number of DCs in the BAL was correspondingly half the magnitude that it was at the nadir of macrophage depletion. When we administered liposomes i.n. that were loaded with PBS as a control, neither the number of macrophages nor the recruitment of DCs into BAL was altered (data not shown). These data suggest that the size of the DC population in the BAL is negatively regulated by alveolar macrophages.

When we compared the magnitude of DC migration to the MLN when alveolar macrophages were or were not depleted using CLs 3 days before LX administration i.n., we observed that LX⁺ DC migration to the MLN was increased by ~20-fold in mice with depleted macrophages (Fig. 4A). Recovery of LX⁺ cells in MLN was now >1000, reflecting the massive increase in DCs that entered the BAL. More than 80% of the bead⁺ cells in the MLN were CD11c⁺CD11b⁺MHCII⁺, indicating that, although the number of LX⁺ cells increased massively in the LN of CL-treated mice, the overall phenotype of migrating cells was similar to that in the untreated conditions (Fig. 4B, compare also to Fig. 2B). Under these conditions, migration remained largely confined to the MLN and did not increase LX⁺ cells in CLNs or NALT.

The increased migration of Ag-bearing DCs observed in mice depleted of alveolar macrophages led directly to a proportionally increased proliferative response by Ag-specific T cells. CFSE-labeled OT-II T cells were transferred into mice before i.n. immunization with LX. LX was covalently coupled with OVA or left uncoupled and was administered to unmanipulated mice or mice that received CLs. The i.n. delivery of OVA-coupled LX beads induced modest proliferation of the OT-II T cells 3 days after Ag delivery (Fig. 4C). Control noncoupled beads provoked no T cell proliferative response (Fig. 4C). By comparison, the mice treated with CLs 3 days before i.n. delivery of OVA-coupled LX beads had a markedly greater T cell proliferative response (Fig. 4C), as expected (21).

Pulmonary DC migration to the MLN

The use of CLs to amplify the detection of migrating DCs permits the development of a robust assay to quantify mobilization of DCs to the MLN. We, therefore, used this approach to determine whether lung DCs use the same molecules that skin DCs use during migration to the MLN. We quantified migration of lung DCs in plt/plt mice that lack expression of CCR7 ligands in LNs (22), CCR8 knockout (KO) mice, and MRP1 KO mice, because all of these mice and molecules have been reported to regulate DC migration from skin (7, 23, 24). CCR7 is expressed by mature lung DCs (5). CCR8 also was expressed by airway DCs (Fig. 5A), and these cells had low MRP1 activity (Fig. 5A). Thus, all of these molecules were candidates for regulating mobilization to the MLN.

Accordingly, migration was depressed by nearly 90% in plt/plt mice (Fig. 5B), and as in skin, DC migration was substantially but not fully impaired in CCR8 KO mice (Fig. 5B), revealing that both CCR7 and CCR8 are mediators of lung DC migration from the airway to the MLN. In contrast, MRP1 KO mice did not exhibit reduced DC migration from the lung (Fig. 5B). MRP1 is the transporter for LTC4 (25), and these KO mice fail to secrete LTC4 (26).

To be sure that the treatment of mice with CLs to amplify detection of migrating DCs did not alter the molecules involved in migration, we conducted experiments without these treatments. Similar trends were observed, in that migration was still impaired in plt/plt mice and CCR8 KO mice and was not affected in MRP1 KO mice (Fig. 5D). However, in strains like the CCR8 KO, where migration was only partially blocked, the effect was less apparent without the use of CLs to amplify baseline migration. Overall, differences between genotypes were much more evident and statistically significant often higher when the magnitude of migration was amplified in CL-treated mice (Fig. 5C).

Discussion

DCs are superior APCs, compared with macrophage (27). DCs possess many features that help to account for their potency as APCs. One of these features is the capacity to home to peripheral sites of Ag capture to the T cell zone of LNs (28), where the highest density of mature naive T cells is found. At least in skin, macrophages are poorly capable of homing to the T cell zone of LNs (29). It is unknown whether the paradigm in skin also is true in lung. It may not be reasonable to assume that the behavior of macrophages and DCs in other tissues will be relevant in lung. For example, lung DCs and lung macrophages differ significantly in cell surface markers like patterns of CD11c and CD11b expression from their counterparts in the other tissues (2, 16). Furthermore, alveolar macrophages have been described to be motile cells that ferry particulate Ag to the lung DLNs (11).

However, in this study, we show that alveolar macrophages are, at best, very inefficient at transporting particulates to the DLN. These macrophages engulf the vast majority of particles that enter the airway but cannot be demonstrated to transport the particles to the LN. At least when a critical threshold of particle load is reached (12), some particles also are acquired by airway DCs. In contrast to the behavior of alveolar macrophages, our data indicate that airway DCs very efficiently migrate to the MLN; a major fraction of the total airway DCs can be recovered as LX⁺ DCs in the MLN. Thus, in agreement with studies in skin (29) and in vitro (30), macrophages in lung are very unlikely to emigrate from the tissue where they have accumulated and proceed to the LN. This function is a distinguishing and apparently universal feature of DCs.
DC migration to the MLNs was markedly enhanced after procedures to eliminate macrophages were performed. Previous studies demonstrated a significant increase in the pulmonary immune response to an Ag challenge after alveolar macrophage depletion (13, 21, 31). They concluded that these observations were due to the elimination of the suppressive effects of alveolar macrophages, including TGF-β, IL-1, PGE₂, and particularly NO that may influence airway DC function and maturation (32). Some of the suppressive effects of alveolar macrophages are observed in vitro (33, 34), where there appears to be a direct effect on the maturation and/or Ag-presenting function of DCs. Our observations reveal a so far overlooked mechanism that could largely explain the increased response observed in vivo after alveolar macrophage elimination: the significantly increased DC migration into and then out of the airway under conditions when macrophages are strongly reduced in number. Studies are underway to determine whether the trafficking of DCs into the airway may be regulated by macrophage-derived factors like NO.

Like others, we used a long-standing method to eliminate alveolar macrophages in some experiments: CLs (13, 18–21, 31). This method selectively eliminates macrophages in various tissue compartments, depending upon how it is administered. A concern that may be raised in this approach is the possibility of bystander inflammation, triggered from the sudden death of numerous macrophages. However, like others (19), we observed only mild to no inflammation using this method, as measured by the low influx of CD11b⁻CD11c⁻ neutrophils into the alveolar space. In many respects, this elimination procedure is comparable to other methods.
of population-selective cell killing, such as diphtheria toxin-mediated killing of macrophages or DCs transgenically engineered to express diphtheria toxin receptor (35, 36). In these models, the elimination of DCs or macrophages by subsequent administration of diphtheria toxin also appears to provoke little if any inflammation, despite the large population of dying cells that ensues. Similarly, the elimination of lung DC subsets by Abs (37) has been done without reports of inflammatory reactions ensuing. An advantage of CLs over Ab-mediated or transgenic techniques of cell killing is that the effect of eliminating a macrophage population is inducible and locally restricted (38).

Although the use of CLs is not overtly inflammatory, elimination of macrophages leads to increased DC recruitment into the airway. The mechanism that permits more DCs to enter the airway is unclear, but the observation permitted us to develop a robust method to track DC migration out of the airway. In this study, we examined the roles of several different molecules in DC migration that were predicted from studies in skin to be relevant mediators of DC migration. We establish that pretreatment of mice with CLs to eliminate killing of macrophages or DCs transgenically engineered to express diphtheria toxin receptor (35, 36). In these models, the elimination of DCs or macrophages by subsequent administration of diphtheria toxin also appears to provoke little if any inflammation, despite the large population of dying cells that ensues. Similarly, the elimination of lung DC subsets by Abs (37) has been done without reports of inflammatory reactions ensuing. An advantage of CLs over Ab-mediated or transgenic techniques of cell killing is that the effect of eliminating a macrophage population is inducible and locally restricted (38).

Although the use of CLs is not overtly inflammatory, elimination of macrophages leads to increased DC recruitment into the airway. The mechanism that permits more DCs to enter the airway is unclear, but the observation permitted us to develop a robust method to track DC migration out of the airway. In this study, we examined the roles of several different molecules in DC migration that were predicted from studies in skin to be relevant mediators of DC migration. We establish that pretreatment of mice with CLs to improve the migratory response to the MLN does not obviously alter molecular events involved in DC migration. However, if products from macrophages regulate DC migration beyond suppressing their recruitment into the airway, these factors would not be present during this migration assay. Therefore, this method will best be applied to the identification of DC-intrinsic mediators that regulate migration from lung to the MLN.

Currently, only a few molecules have been shown to regulate DC migration from lung to MLNs. These include peroxisome proliferator-activated receptor γ and PGD2, both of which have been argued to impair DC migration from the lung to the MLNs (4, 6), as they do in skin (39, 40). However, the doses of peroxisome proliferator-activated receptor γ and PGD2 agonists needed to block lung DC migration were rather high (4, 6), and the effects were less evident than the actions of these compounds in modifying skin DC migration (39, 40). Another study has implicated CCR7 in the regulation of DC migration from the airway (5). All of the studies above used fluorescent soluble protein or dyes to follow DC migration. Taken together, a picture begins to form that mediators of DC migration from lung to MLNs are the same as those that mediate DC migration from skin. Our observations extend the connection between migration pathways in the two tissues to include a role for CCR8. We show here that CCR8-deficient KO mice manifest a diminished accumulation of particle-bearing DCs to MLNs, reminiscent of the reduced emigration of phagocytic DCs from skin (7). In skin, the role of CCR8 in DC migration is apparent when particle-bearing DCs are traced but not when soluble fluorescent contact sensitizers are used to track migration, because soluble stimuli and particulate Ags are acquired by distinct populations of DCs (29). It will be interesting to determine whether the method of tracking DC migration in lung influences, which DCs emigrate, or the molecular mechanism that regulates trafficking.

Although there are clearly many common mediators of DC migration between skin and lung, we identified MRP1, the transporter that mediates secretion of LTC4 (25), as a mediator that may control migration of DCs in a tissue-selective manner. LTC4 is a significant mediator in inflammatory and allergic reactions in the lung.
(41). We predicted that one of its roles in the lung may be to modulate DC migration. However, this connection is not evident. In skin and in vitro, LTC4 affects DC responsiveness to CCR7 ligands (8), as CCR7 requires additional extracellular signals to become functionally coupled (28). Because LTC4 is not needed for the migration of lung DCs, it may that other mediators, maybe even other lipids like PGE2 (42–44), take over this function or act redundantly with LTC4 in the lung. Future studies will be required to address this possibility, as additional mediators of DC migration from lung to MLNs are delineated.

Disclosures
The authors have no financial conflict of interest.

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31: 22–27.