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Alveolar Macrophages Transport Pathogens to Lung Draining Lymph Nodes

Alun C. Kirby, Mark C. Coles, and Paul M. Kaye

The first step in inducing pulmonary adaptive immunity to allergens and airborne pathogens is Ag acquisition and transport to the lung draining lymph nodes (dLN). Dendritic cells (DC) sample the airways, and active transfer of Ag to the lung dLN is considered an exclusive property of migratory DC. However, alveolar macrophages (AM) are the first phagocytes to contact inhaled particulate matter. Although having well-defined immunoregulatory capabilities, AM are generally considered as restricted to the alveoli. We show that murine AM constitutively migrate from lung to dLN and that following exposure to *Streptococcus pneumoniae*, AM rapidly transport bacteria to this site. Thus AM, and not DC, appear responsible for the earliest delivery of these bacteria to secondary lymphoid tissue. The identification of this novel transport pathway has important consequences for our understanding of lung immunity and suggests more widespread roles for macrophages in the transport of Ags to lymphoid organs than previously appreciated. *The Journal of Immunology*, 2009, 183: 1983–1989.

Alveolar macrophages (AM) are highly adapted to their role as the first cell of the immune system to encounter inhaled particulates and pathogens (2, 3). AM are not only excellent phagocytes capable of rapidly clearing large numbers of bacteria from the lung (1), but they have well-defined immunosuppressive capabilities. AM are able to suppress the responses of both T cells and dendritic cells (DC) in vitro (4–7), and several studies have demonstrated the constitutive immunosuppressive activity of AM in vivo (6, 8).

Adaptive immunity to inhaled material, including pathogens, is developed in the lung draining lymph nodes (dLN) following transport of Ag to this site. Despite their position as the primary Ag-exposed immune cell population and their range of immunoregulatory functions, AM are not commonly thought to contribute to adaptive immune responses. This is due mainly to the perceived inability of AM to migrate from the alveolar spaces to lung dLN. Instead, CCR7-dependent migration of pulmonary DC is thought to be the only mechanism by which particulate Ag is transported from the lungs to dLN (9, 10). While migration of AM to lung dLN has been proposed in three previous studies (11–13), two were before the ability to discriminate between AM and DC, and the methodologies employed were insufficient to unequivocally exclude DC-mediated transfer of labeled material. In one recent study where AM were investigated alongside DC, no migration of AM was reported (9), but this study did not specifically attempt to examine the migratory potential of these cells.

Several recent studies have demonstrated that LN macrophages have a hitherto unrecognized role in the acquisition and presentation of Ag to B lymphocytes in the dLN (14–16). These studies, which examined the transport of soluble and particulate Ag to the dLN by lymph and from the circulation, demonstrated Ag acquisition by resident subcapsular sinus macrophages. However, these experiments were not designed to address the potential contribution of migratory macrophages to Ag transport. In contrast to other tissue sites, however (14, 15), soluble Ags do not normally reach lung dLN through passive mechanisms (17, 18). Therefore, at early time points following intranasal challenge it is highly unlikely that particulate Ags such as noninvasive bacteria have free access to the pulmonary lymphatics. Thus, the early transport of particulates from the lung to the dLN is likely to be an active and host cell-mediated process.

Given the position of AM as the primary exposed immune cell, and the potential ramifications of an alternate mode of Ag transport on our understanding of pulmonary immunity, we have readressed the question of whether AM exit the lung and migrate to the dLN. We demonstrate that AM constitutively migrate to lung dLN. Following pathogen challenge, AM hold a temporal advantage over DC in Ag acquisition and AM containing *Streptococcus pneumoniae* appear in lung dLN before the onset of pathogen-induced DC migration.

Materials and Methods

*Mice, Abs, and bacteria*

C57BL/6j and B6J.CD45.1 mice were bred in house. B6.hCD2-DsRed mice, generated at the National Institute for Medical Research (London) and backcrossed for 10 generations before intercrossing to homozygosity, were a gift of D. Kioussis and A. Patel (National Institute for Medical Research (London)). Animal experiments were conducted with local ethical approval and under U.K. Home Office License. All Abs were from BD Pharmingen (Oxford, U.K.), except anti-CCR7 and anti-F4/80 (eBioScience), anti-CD68 (AbD Serotec), and anti-7/4 (Caltag Laboratories). Biotinylated anti-Ly49B and isotype control mAb were gifts of C. Brooks (University of Newcastle). The culture and intranasal delivery of encapsulated isolate of *S. pneumoniae* serotype 6B was as described previously (19). Where required for tracking assays, bacteria were labeled with PKH26 (Sigma-Aldrich) according to the manufacturer’s instructions.
Flow cytometry and cell sorting

Bronchoalveolar lavage (BAL) fluid, lung, and dLN were harvested, prepared as single-cell suspensions, and stained for the expression of surface and intracellular markers as previously described (19). Samples were acquired on a CyAn ADP cytometer and analyzed using Summit v4.3 software (both from Beckman Coulter). Staining for the expression of CCR7 (clone 4B12 or isotype control) was conducted at 37°C. All other flow cytometric staining procedures were conducted on ice. Purified AM and lung DC were obtained by initial enrichment using anti-CD11c magnetic beads (Miltenyi Biotec) before surface labeling and sorting on a MoFlo cell sorter (Beckman Coulter) utilizing a cooled, automated SmartSampler, fluorescence-activated cell sorting procedures were conducted on ice. Purified AM and lung DC were obtained by initial enrichment using anti-CD11c magnetic beads (Miltenyi Biotec) before surface labeling and sorting on a MoFlo cell sorter (Beckman Coulter) utilizing a cooled, automated SmartSampler. Staining for the expression of CCR7 (clone 4B12 or isotype control) was conducted at 37°C. All other flow cytometric staining procedures were conducted on ice. Purified AM and lung DC were obtained by initial enrichment using anti-CD11c magnetic beads (Miltenyi Biotec) before surface labeling and sorting on a MoFlo cell sorter (Beckman Coulter) utilizing a cooled, automated SmartSampler.

Immunofluorescence and confocal microscopy

Paraformaldehyde-fixed cytospins or acetone-fixed 8-μm frozen tissue sections were stained for CD11c and MHC-II using directly conjugated mAbs. Siglec-F was detected using purified anti-Siglec-F (or isotype control), followed by biotinylated anti-mouse IgG2a and visualized with streptavidin-Alexa 647. Samples were mounted in ProLong Gold (Invitrogen) and imaged using a Zeiss Axioplan LSM 510 NLO confocal microscope as single optical slices (0.8–1.0 μm). Images were analyzed using Zeiss LSM Image Browser software and Adobe Photoshop CS.

Results

AM migrate to lung dLN in the steady-state

Since AM share some phenotypic characteristics with other cell types, most notably with DC (20), it was critical to be absolutely precise in our phenotypic definition of AM. Therefore, before examining dLN, we first reconfirmed the phenotype of AM in naive animals based on previously defined characteristics of AM (19, 20). In naive mice, AM are autofluorescent, CD11c⁺, and regarded as MHC-II⁺⁺ (20). We report that while the majority of autofluorescent, CD11c⁺ AM are indeed MHC-II⁺⁺ (Fig. 1A, B; R2-gated), they were reconfirmed as MHC-II⁺⁺ (Fig. 1B; R3-gated), a fraction (5.75 ± 0.96%; n = 12) of AM in BAL fluid from naive animals express significant levels of MHC-II at the cell surface (Fig. 1A, B; R2-gated). In comparison, the few DC found in the alveolar spaces were nonautofluorescent and MHC-II⁺⁺ (Fig. 1B; R3-gated). Using additional phenotypic parameters, including forward light scatter (FSC) and side light scatter (SSC), and by examining the morphology of each population following flow cytometric sorting, the identity of MHC-II⁺⁺ and MHC-II⁺ cells as AM was confirmed (Fig. 1, C and D, and data not shown). Furthermore, not only did MHC-II⁺⁺ and MHC-II⁺ AM share an identical phenotype and morphology, but these differed significantly from that associated with alveolar DC (Fig. 1E).

We hypothesized that constant exposure to inhaled Ags would be sufficient to drive constitutive migration of AM from unchallenged lungs, should this pathway exist. Using the basic parameters defined above, we examined naive lung dLN for cells with a phenotype corresponding to AM found in BAL fluid. Pooled, enriched CD11c⁺ cells from lung dLN were divisible into small, nonautofluorescent, B220⁺ cells (G4) and large, autofluorescent B220⁺ cells (G5). D–G, Populations phenotypically defined by regions G1, G2, G4, and G5 were purified and Giemsa stained. Bar, 10 μm. Original magnification ×630. H and I, CD11c⁺-enriched cells from inguinal, mesenteric, and mediastinal (lungs-draining) LN were examined in parallel for the presence of cells with an AM phenotype. H, Within each LN the CD11c⁺ population comprised three distinct subpopulations based on MHC-II expression. I, Only within the CD11c⁺ MHC-II⁺⁺ cells of lung dLN (R3-gated) was an autofluorescent FSC⁺⁺ population corresponding to AM detectable. Three to 8 × 10⁶ CD11c⁺ cells were analyzed in each case. Representative of three separate experiments in which LN from 4 non-lung draining sites were examined.

FIGURE 1. Identification of AM in BAL fluid. A, CD11c⁺⁺ cells comprise >80% of naïve BAL cells. B, Among CD11c⁺⁺-gated cells, AM were identified as predominantly MHC-II⁻⁻ autofluorescent (AF) cells (R1), with a minority being MHC-II⁺⁺ (R2). DC in BAL samples were AF⁻ and MHC-II⁺⁺ (R3). AM and DC were further differentiated based on forward and side scatter characteristics (not shown). C–E, Purified, Giemsa-stained MHC-II⁺⁺ (R1-gated) and MHC-II⁺⁺ (R2-gated) AM exhibited distinct AM morphology compared with MHC-II⁺⁺ DC (R3-gated). Original magnification ×630.

FIGURE 2. Identification of mAM in dLN. A, CD11c⁺ MACS-enriched cells from pooled naive lung dLN comprised three distinct populations separable based on expression of CD11c and MHC-II. We identified homogeneous mature (MHC-II⁺⁺; G1) and immature (MHC-II⁺⁺; G2) conventional DC populations, and a mixed MHC-II⁺⁺ population (G3). B and C, G3-gated cells were divisible into small, nonautofluorescent, B220⁺ cells (G4) and large, autofluorescent B220⁺ cells (G5). D–G, Populations phenotypically defined by regions G1, G2, G4, and G5 were purified and Giemsa stained. Bar, 10 μm. Original magnification ×630. H and I, CD11c⁺-enriched cells from inguinal, mesenteric, and mediastinal (lungs-draining) LN were examined in parallel for the presence of cells with an AM phenotype. H, Within each LN the CD11c⁺ population comprised three distinct subpopulations based on MHC-II expression. I, Only within the CD11c⁺ MHC-II⁺⁺ cells of lung dLN (R3-gated) was an autofluorescent FSC⁺⁺ population corresponding to AM detectable. Three to 8 × 10⁶ CD11c⁺ cells were analyzed in each case. Representative of three separate experiments in which LN from 4 non-lung draining sites were examined.

(1A, B; R2-gated). In comparison, the few DC found in the alveolar spaces were nonautofluorescent and MHC-II⁺⁺ (Fig. 1B; R3-gated). Using additional phenotypic parameters, including forward light scatter (FSC) and side light scatter (SSC), and by examining the morphology of each population following flow cytometric sorting, the identity of MHC-II⁺⁺ and MHC-II⁺ cells as AM was confirmed (Fig. 1, C and D, and data not shown). Furthermore, not only did MHC-II⁺⁺ and MHC-II⁺ AM share an identical phenotype and morphology, but these differed significantly from that associated with alveolar DC (Fig. 1E).

We hypothesized that constant exposure to inhaled Ags would be sufficient to drive constitutive migration of AM from unchallenged lungs, should this pathway exist. Using the basic parameters defined above, we examined naive lung dLN for cells with a phenotype corresponding to AM found in BAL fluid. Pooled, enriched CD11c⁺ cells from lung dLN were divisible into three populations based on MHC-II expression (Fig. 2A). Additional phenotypic analysis indicated that the MHC-II⁺⁺ and MHC-II⁺ populations (Fig. 2A; G1 and G2, respectively) were homogeneous, whereas MHC-II⁺⁺ cells (Fig. 2A; G3) were further
divisible. G3-gated cells comprised a nonautofluorescent, FSClow, B220/H11001 population likely to be plasmacytoid DC (Fig. 2, B and C; G4-gated) and an autofluorescent, FSC high, B220/H11002 population (Fig. 2, B and C; G5-gated). Cells defined by G5 confirmed that a population of dLN cells corresponding precisely to our initial six-parameter definition of naive BAL-derived AM (CD11chighMHC-IIlowCD11blowFSChighSSChigh and autofluorescent) was present at low frequency in naive lung dLN (Fig. 2 A–C). We refer to these cells hereafter as “migratory AM” (mAM).

Following flow sorting, morphological examination confirmed the identity of G1-, G2-, and G4-gated cells as mature conventional DC, immature DC, or plasmacytoid DC (Fig. 2D–F). Moreover, the morphological appearance of mAM was clearly distinct from these DC populations and further demonstrated their comparability with BAL-derived AM. Together, conventional DC, immature DC, plasmacytoid DC, and mAM populations appeared to comprise the entire CD11c+ population (of 1.51 ± 0.91 × 10^4 cells/dLN; n = 12) in dLN, with each naïve dLN containing 286 ± 124 (n = 9 pooled samples) mAM.

Constitutive migration of AM would be expected to selectively populate lung dLN with mAM. We therefore examined lymph nodes (LN) draining other tissue sites for the presence of cells with a phenotype corresponding to that of mAM seen in lung dLN. In none of the examined peripheral dLN, including superficial inguinal, deep inguinal, and cervical LN (Fig. 2, H and I, and data not shown), nor in the mesenteric LN, selected as a site of mucosal drainage, was such a population observed (Fig. 2, H and I). Taken together, these data demonstrate the presence of a population of cells exhibiting the precise phenotype of AM in the lung dLN, and confirm that this population is not present in LN other than those draining the lungs.

**AM in lung and dLN share a detailed and exclusive phenotype**

Given the phenotypic similarities of AM and DC (20), and the known ability of lung DC to migrate to dLN (9, 10), it was critical that we identified further properties of AM to facilitate their explicit identification and to unequivocally distinguish them from other populations.

Having found that a proportion of AM in BAL fluid express significant levels of MHC-II at the cell surface, we conducted a more detailed examination of MHC-II expression by lung CD11c+ cells. Surprisingly, flow cytometric analysis revealed constitutive expression of intracellular MHC-II in AM, as well as in DC, from naïve BAL fluid (Fig. 3A). This was confirmed using confocal microscopy, demonstrating intracellular MHC-II in AM from naïve mice, but not in peritoneal macrophages from the same animals (Fig. 2, B and C). The expression of intracellular MHC-II is therefore a further phenotypic characteristic of AM, as well as of lung DC. Confocal microscopy examination of cells sorted from each of the four defined CD11c+ populations in lung dLN (Fig. 2) revealed that mAM shared the characteristic expression of intracellular MHC-II with BAL AM.

To identify additional markers that were potentially AM-restricted, rather than shared between AM and DC, we conducted
whole genome Affymetrix expression profiling of AM from naive BAL fluid (data not shown). Among the panel of surface molecules thus obtained were Siglec-F (21) and Ly49B (22), whose expression by AM has recently been independently confirmed. In flow cytometric analyses, both Siglec-F and Ly49B were expressed by naive BAL AM and by mAM in lung dLN. Twelve wild-type and 6 B6.CD2-DsRed lung dLN were assessed and representative images are shown. Original magnifications ×630 (left panels) and ×400 (right panels). D–F, Representative images from frozen sections of lung dLN from naive B6.CD2-DsRed mice stained with anti-Siglec-F, anti-Ly49B, or appropriate isotype control mAb. C, Left panels, AM purified from naive BAL fluid and prepared as cytospins and (right panels) frozen sections of lung dLN from naive wild-type animals were stained for expression of CD11c and Siglec-F by confocal microscopy. mAM were localized by their dual expression of CD11c and Siglec-F. In the right panels the gate indicates the location of a CD11c⁺Siglec-F⁺ (mAM) cell. Adjacent DC are apparent as CD11c⁺Siglec-F⁻ cells. Twelve wild-type and 6 B6.CD2-DsRed lung dLN were assessed and representative images are shown. Original magnifications ×630 (left panels) and ×400 (right panels). D–F, Representative images from frozen sections of lung dLN from naive wild-type animals were stained for expression of CD11c and Siglec-F are shown to demonstrate that (D) CD11c⁺Siglec-F⁻ cells were not observed within T cell-rich (densely CD2⁺) areas of dLN, (E) CD11c⁺Siglec-F⁻ cells were not observed at the subcapsular sinus region, or within the immediate subcapsular areas (dotted line indicates edge of dLN), and (F) occasional SiglecF⁺CD11c⁻ cells, most likely eosinophils, were observed in dLN (indicated by box). All images are representative and original magnifications are ×400 (D and E) and ×630 (F).
The proportion of CD11chighMHC-IIhighFSClowSSClow, nonautofluorescent DC was identified within BAL samples from S. pneumoniae challenge. MHC-IIhigh BAL DC was shown to be a distinct, nonoverlapping population (R3-gated in Fig. 1A). Representative images are shown; original magnification ×630. A. The proportion of MHC-II+ AM (R2-gated in A, above) among total AM (B) and the proportion of CD11cMHC-IIhighFSClowSSC low, nonautofluorescent DC (R3-gated in Fig. 1B) among CD11c+ BAL cells (C) were determined in BAL samples from naïve mice and from mice 90 min and 12 h following S. pneumoniae challenge. Bars represent mean ± SEM; n = 6–12 at each time point. Numbers over bars indicate p values vs naive (Student’s t test). B. Left plot shows CD11c+ BAL cells from naïve mice stained for expression of CD11b and MHC-II. CD11b+ and CD11b− MHC-IIhigh BAL DC (R1) and total AM were further analyzed following staining with isotype control mAb (center panels) or anti-CCR7 (right panels). Percentages of cells within each quadrant are shown. Plots are representative of at least two experiments in which pooled BAL fluid from six individuals was examined.

MHC-II+ population was confirmed as AM by additional phenotypic parameters, and by morphology following cell sorting (Fig. 5A and data not shown). MHC-II up-regulation by AM appeared to be independent of phagocytosis, as many cells without visible bacteria were MHC-II+, and vice versa. Furthermore, we were able to identify MHC-IIhigh BAL DC as a distinct, nonoverlapping population within BAL samples from S. pneumoniae-challenged mice.

A significantly increased proportion of MHC-II+ AM was maintained for at least 12 h postchallenge, while no significant increase in the proportion of cells phenotypically identified as BAL DC was observed over the same time period (Fig. 5, B and C). At up to 4 h postchallenge, those AM up-regulating MHC-II appeared to be previously resident AM, in that they lacked expression of CD11b associated with newly influxing AM (19).

As DC migration to lung dLN is CCR7 dependent (9), and this is the only defined pathway of cell migration from lung to dLN, we also examined the expression of CCR7 by AM. CCR7 expression was not observed on naïve BAL AM, although it was clearly detectable on a subset of DC present in the BAL fluid (Fig. 5D). S. pneumoniae infection did not induce CCR7 expression on AM in BAL at up to 24 h following challenge (data not shown).

Having confirmed that S. pneumoniae challenge did not impair our ability to distinguish AM, the cellular association of fluorescently labeled (PKH26+) bacteria was examined at early time points following intranasal challenge. MHC-II was excluded as a discriminatory marker in these assays due to the potential effects of S. pneumoniae challenge on MHC-II expression by AM. At 2–4 h postchallenge, >40% of all CD11c+/74°F4/80−FSC highSSC high, autofluorescent AM contained bacteria, and ~95% of all PKH26+ cells in whole-lung digestes were AM (Fig. 6A). The remaining PKH+ cells were Ly6CG+CD11b− neutrophils and CD11c+/74− cells (data not shown). Significantly, no association of PKH26+ bacteria with lung DC was observed at this time point (Fig. 6A). Taken together, our data clearly demonstrate that AM hold a significant temporal advantage over pulmonary DC populations with regard to the acquisition of intranasally delivered bacteria.

The dLN of the same animals taken 2–4 h postchallenge were examined flow cytometrically by the same criteria applied to the lung, above. At this time point the only cell population that contained PKH26+ bacteria exhibited the phenotype of mAM, precisely matching that of PKH+ AM in the lung (Fig. 6B). Based on flow cytometry data, an estimated maximum of 40–50 pathogen-bearing mAM were present in each dLN at this time point. Therefore, not only are AM capable of transporting bacteria to lung dLN, but these pathogen-bearing AM reach the lung dLN before significant migration of pathogen-bearing lung DC.

FIGURE 5. Effect of acute S. pneumoniae challenge on AM and DC in BAL fluid. A. Plots show gated CD11c+ cells within (left) naïve BAL and (right) BAL fluid taken 90 min following intranasal S. pneumoniae challenge. CD11c+ autofluorescent cells (AM) are divisible into MHC-II low (R1) and MHC-II high (R2) populations in each case. Other phenotypic characteristics were used to confirm the identity of AM and to exclude DC. R1- and R2-gated cells were purified in each case and the morphology was examined following Giemsa staining (lower panels). Representative images are shown; original magnification ×630. B and C. The proportion of MHC-II AM (R2-gated in A, above) among total AM (B) and the proportion of CD11cMHC-IIhighFSClowSSC low, nonautofluorescent DC (R3-gated in Fig. 1B) among CD11c+ BAL cells (C) were determined in BAL samples from naïve mice and from mice 90 min and 12 h following S. pneumoniae challenge. Bars represent mean ± SEM; n = 6–12 at each time point. Numbers over bars indicate p values vs naive (Student’s t test). D. Left plot shows CD11c+ BAL cells from naïve mice stained for expression of CD11b and MHC-II. CD11b+ and CD11b− MHC-IIhigh BAL DC (R1) and total AM were further analyzed following staining with isotype control mAb (center panels) or anti-CCR7 (right panels). Percentages of cells within each quadrant are shown. Plots are representative of at least two experiments in which pooled BAL fluid from six individuals was examined.

Discussion
This study has readdressed the question of whether alveolar macrophages, phagocytes assumed to be alveolus-restricted, can migrate from the alveolar spaces and contribute to Ag transport to the lung dLN. We have shown that cells phenotypically and morphologically identical to AM in the BAL fluid are found in the dLN of naïve animals, indicating a constitutive migration pathway for these cells. Moreover, we show that AM acquire inhaled pathogens before lung DC, and that a fraction of pathogen-bearing AM migrate to the dLN.

Migration of AM to lung dLN has been proposed in three previous studies (11–13). These studies instilled labeled particles or cells into lungs and demonstrated the appearance of label predominantly within paracortical T cell areas of dLN, to which DC migrate (18). However, the earliest two studies suffered from the inability to distinguish AM from DC, while the later study failed to address the possibility of DC-mediated transport. More recently, Jakubzick et al. clearly demonstrated the CCR7-dependent migration of lung DC to dLN (9) and no migration of AM was reported in their model. However, this study did not set out to address AM migration directly and, as the current study has demonstrated, the numbers of mAM in dLN are relatively small and may have been easily overlooked.
In the lung dLN of unmanipulated, naive mice we found four populations within CD11c-enriched cells (Fig. 2). Three of these corresponded to known DC populations, while the fourth was identical in phenotype and general morphology to AM found in the BAL fluid, and has been named mAM. Importantly, we found no similar population in LN taken from other peripheral sites, suggesting that this population was not a previously unrecognized population of LN cells. Additionally, this population was absent from mesenteric LN, suggesting that the phenotype was not broadly associated with mucosal draining sites. Taken together, these data demonstrate the specificity of mAM to lung dLN.

Having made our initial observation based on existing, defined phenotypic and morphological parameters associated with AM and lung DC, we subsequently extended the phenotypic characterization to include the constitutive expression of intracellular MHC-II by AM and mAM. Flow cytometric observation of intracellular MHC-II was confirmed by confocal microscopy of highly purified AM and mAM (Fig. 3). The potential explanations for constitutive expression of intracellular MHC-II by AM and mAM are currently under investigation, as most macrophage populations do not constitutively express significant amounts of MHC-II intracellularly. With regard to phenotype, the expression of intracellular MHC-II was shared by AM and DC. However, we also identified Ly49B and Siglec-F as surface markers expressed at very similar levels by both AM and mAM (Fig. 4A). Ly49B is the only member of the Ly49 family not expressed by NK cells, but it has a relatively broad distribution among myeloid cells (22). Siglec-F is predominantly a marker of eosinophils in the naive mouse (24), although its expression by AM has recently been confirmed in passing by other groups (23, 26). Most importantly for this study, neither Ly49B nor Siglec-F was expressed by any other CD11c+ population in either lung or dLN (Fig. 4B). In total, we have defined a 12-parameter phenotype and a distinctive morphology shared exclusively by AM and mAM, which unambiguously distinguishes AM and mAM from any other identifiable DC or tissue macrophage population.

In a model in which labeled AM were adoptively transferred intratracheally, AM have been proposed to migrate to paracortical T cell areas of dLN (13). However, this study did not determine the nature of the cells containing label that were found in dLN subsequent to transfer. Utilizing highly purified donor AM and rigorous phenotyping of dLN populations, we were unable to detect significant AM migration in adoptive transfer models similar to those of Thepen et al. (13), despite viable transferred AM being detectable in BAL fluid up to 48 h posttransfer (our unpublished observations). The inability to detect migration of transferred AM is most likely due to the extremely low frequency of AM migration. In contrast, using unmanipulated naive mice, mAM were detectable by both flow cytometry and confocal microscopy. Furthermore, the microscopy data suggest that constitutively migrating AM are predominantly found in B cell regions in dLN, rather than to the paracortical T cell areas (Fig. 4C–E). This indicates that, in contrast to that recently demonstrated for resident subcapsular sinus macrophages (14–16). However, the specific function of mAM in dLN during homeostasis remains under investigation.

We have demonstrated that AM in the lung have a significant temporal advantage over lung DC in the acquisition of intranasally delivered bacteria (Figs. 5 and 6). Moreover, S. pneumoniae challenge did not induce the influx of DC into the alveolar spaces, with no significant increase in the proportion of DC within the alveolar spaces observed up to 12 h following S. pneumoniae challenge (Fig. 5C). This contrasts with the reported increase in alveolar DC 48 h following instillation of latex beads (9) or following repeated allergen challenge in an asthma model (28). It is most likely that differences in the models used are responsible for these observations. Importantly, AM did not express CCR7 in naive animals, nor was CCR7 expression on AM induced in the 12 h following S. pneumoniae challenge (Fig. 5D). This indicates that, in contrast to lung DC, AM migration to dLN utilizes an as-yet unidentified CCR7-independent pathway.

Affymetrix whole genome analysis of naive AM did not reveal any obvious candidate receptors that may direct AM migration to dLN (our unpublished data). However, given the low level migration of AM to dLN during homeostasis, it is possible that migratory receptor expression is restricted to a very small proportion of AM in BAL fluid and consequently difficult to identify among unfractionated populations using this approach. Although a recent study (29) demonstrated that inflammatory stimuli enhanced macrophage migration to LN following i.v. transfer, factors directing macrophage migration remain relatively undefined. Further studies of enriched subsets of BAL AM are clearly required and are currently underway.

Finally, we show that AM and not DC are the first pathogen-bearing population to arrive in the lung dLN (Fig. 6B). The proportion of AM containing S. pneumoniae that reach the dLN in the
References

The authors have no financial conflicts of interest.

Disclosures


