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Epigenetic Control of Ccr7 Expression in Distinct Lineages of Lung Dendritic Cells

Timothy P. Moran,*1 Hideki Nakano,† Hrisavgi D. Kondilis-Mangum,‡ Paul A. Wade,‡ and Donald N. Cook†

Adaptive immune responses to inhaled allergens are induced following CCR7-dependent migration of precursor of dendritic cell (pre-DC)–derived conventional DCs (cDCs) from the lung to regional lymph nodes. However, monocyte-derived (moDCs) in the lung express very low levels of Ccr7 and consequently do not migrate efficiently to LN. To investigate the molecular mechanisms that underlie this dichotomy, we studied epigenetic modifications at the Ccr7 locus of murine cDCs and moDCs. When expanded from bone marrow precursors, moDCs were enriched at the Ccr7 locus for trimethylation of histone 3 lysine 27 (H3K27me3), a modification associated with transcriptional repression. Similarly, moDCs prepared from the lung also displayed increased levels of H3K27me3 at the Ccr7 promoter compared with migratory cDCs from that organ. Analysis of DC progenitors revealed that epigenetic modification of Ccr7 does not occur early during DC lineage commitment because monocytes and pre-DCs both had low levels of Ccr7-associated H3K27me3. Rather, Ccr7 is gradually silenced during the differentiation of monocytes to moDCs. Thus, epigenetic modifications of the Ccr7 locus control the migration and therefore the function of DCs in vivo. These findings suggest that manipulating epigenetic mechanisms might be a novel approach to control DC migration and thereby improve DC-based vaccines and treat inflammatory diseases of the lung. The Journal of Immunology, 2014, 193: 4904–4913.

The migration of lung dendritic cells (DCs) to lymph nodes (LN) is critical for orchestrating adaptive immune responses against inhaled Ags or microbes (1–4). Mobilization of lung DCs to LN is dependent on the chemokine receptor CCR7 and its ligands, CCL19 and CCL21, which are produced inafferent lymphatic vessels and the T cell zones of LN (5). Under homeostatic conditions, CCR7-dependent migration of lung DCs to draining LN is important for the induction of regulatory T cells, which help maintain peripheral tolerance to innocuous environmental Ags (6). During infection or other inflammatory insults, migratory DCs become licensed to induce cytotoxic T cells or effector Th cell differentiation (7). These effector T cells not only assist with pathogen clearance but can also mediate maladaptive immune responses leading to inflammatory lung diseases such as asthma (3, 4, 8). Because migratory DCs play a central role in shaping immune responses in the lungs, manipulating Ccr7 expression is a potential strategy for either augmenting or suppressing adaptive immunity. However, such a strategy requires an improved understanding of the molecular mechanisms responsible for regulating Ccr7 expression.

As in other nonlymphoid tissues, DCs in the lungs are composed of two major lineages: conventional DCs (cDCs) and monocyte-derived DCs (moDCs) (9, 10). Lung cDCs arise from circulating progenitor cells referred to as precursor of DCs (pre-DC), which descend from the common DC progenitor in the bone marrow (BM) via a pathway dependent on Flt3 ligand (FL) (11). In contrast, lung-resident moDCs develop from emigrating peripheral blood monocytes independently of FL (10). There is growing evidence that DC functional specialization is dependent on their developmental lineage (12). In agreement with this, we recently reported that lung cDCs and moDCs differ significantly in their ability to express Ccr7 and migrate to regional LN (10). During steady-state conditions, Ccr7 is expressed at low levels in lung cDCs and is markedly upregulated by microbial products, such as LPS and polyinosinic:polycytidylic acid [poly (I:C)]. By contrast, moDCs in the lung fail to express Ccr7, even after treatment with these microbial products. Consequently, cDCs, but not moDCs can efficiently ferry Ag from the lung to regional LN. These findings are in agreement with studies showing that moDCs from other peripheral tissues, such as the skin and gut, also express low levels of Ccr7 and migrate poorly to LN (13, 14). Taken together, these studies suggest that the capacity for peripheral DCs to express Ccr7 and migrate to LN is regulated in a lineage-specific manner.

The unique transcriptional profiles of DC subsets are likely important for dictating their functional and migratory properties. Although the transcriptomes of DC subsets are clearly dependent on lineage-specific transcription factors (15), epigenetic mechanisms are also essential for regulating gene expression during DC development (16). Epigenetic mechanisms, which include DNA methylation and posttranslational modifications of histone tails, regulate gene expression by governing chromatin accessibility to the transcriptional machinery (17). Although epigenetic
modifications of multiple genes have been well studied in the context of Th cell differentiation (18–20), remarkably little is known of epigenetic mechanisms that might be expected to influence DC development or function (16). In the current study, we investigated whether epigenetic modifications at the Ccr7 locus influence the migratory properties of different DC subsets. We found that moDCs have increased repressive histone modifications at the Ccr7 locus, which was associated with decreased Ccr7 expression and a nonmigratory phenotype. Surprisingly, these repressive epigenetic marks are not established during lineage commitment within the BM but rather during monocyte differentiation into moDCs in peripheral tissues. Our findings suggest that epigenetic modifications at the Ccr7 locus closely correlate with the migratory properties, and hence the functional roles, of nonlymphoid DC subsets. The transcriptional silencing of Ccr7 during moDC differentiation might also have implications for the development of effective DC-based vaccines for immunotherapy.

Materials and Methods

Mice

C57BL/6j and OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ccr7−/− reporter mice were generated in our laboratory and have been described previously (10). Mice were housed in specific pathogen-free conditions at the National Institute of Environmental Health Sciences (NIEHS) and used between 6 and 12 wk of age in accordance with guidelines provided by the Institutional Animal Care and Use Committees.

Flow cytometric analysis

Cells were incubated with a nonspecific-binding blocking reagent mixture of anti-mouse CD16/CD32 (2.4G2) and normal mouse and rat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 5 min. Staining was added during the last 24 h of culture with fluorochromes, phycocyanin, allophycocyanin-Cy7, Alexa Fluor 488, Alexa Fluor 647, eFluor 450, eFluor 605 NC, FITC, PerCP-Cy5.5, or PE) or biotin-conjugated Abs against mouse CD3ε (145-2C11), CD11b (M1/70), CD11c (N418), CD14 (Sa2-8), CD19 (6D5), CD40 (1C10), CD49b (DX5), CD86 (GL1), CD88 (20/70), CD103 (M290), CD115 (AFS98), CD115 (AF210), F4/80 (BM8), Ly-6C (AL-21), Ly-6G (1A8), MHC class II (Abich25), Sca-1 (D7), and TER-119 (BD Biosciences [San Jose, CA), BioLegend [San Diego, CA], and eBioscience [San Diego, CA]). Staining with biotinylated Abs was followed by fluorochrome-conjugated streptavidin. Stained cells were analyzed on a five-laser LSRII (BD Biosciences) or sorted on a five-laser ARIA-II flow cytometer (BD Biosciences). FlowJo (Treestar, Ashland, OR) software, or BD (Becton, Dickinson and Company, Franklin Lakes, NJ) software was used for data analysis. Doublet cells were excluded based on their forward and side scatter or uptake of 7-aminoactinomycin D. Only single cells were analyzed.

Generation and analysis of BM-elicited DCs

Marrow was collected from pulsedervized bones, and RBCs were lysed with 0.15 M ammonium chloride and 1 mM potassium bicarbonate. BM cells were then cultured in complete RPMI 1640 medium (RPMI 1640, 10% FBS [Gemini, West Sacramento, CA], penicillin/streptomycin, and 50 mg ml−1 2-mercaptoethanol (2-ME), supplemented with either 100 ng ml−1 recombinant human FL to generate FL-DCs or 5 ng ml−1 recombinant human FLT to generate FLT-DCs. Recombinant human FL was produced by the NIEHS Protein Expression Core Facility using previously described methods (21). Media was replaced every 3 d, at which time fresh cytokines were also added. In some experiments 100 ng ml−1 LPS (Sigma-Aldrich, St. Louis, MO) or 10 μg ml−1 poly(I:C) (InvivoGen, San Diego, CA) was added the last 24 h of culture to induce DC maturation. Cells were harvested on day 8 and stained with biotinylated anti-CD11c Ab (eBioscience), followed by incubation with streptavidin Microbeads (Miltenyi, Auburn, CA), and finally isolated with an automated magnet-activated cell sorter to a purity of >90% CD11c+ cells. To assay T cell stimulatory activity, BM-derived DCs were purified using MACS and then cultured with naive CD4+ OT-II cells (DC:T cell ratio = 1:2) in the presence of 10 μg ml−1 OVA257–264 peptide (New England Peptide, Gardner, MA) as described previously (8). Proliferation was inferred from recovery of viable (trypan blue negative) T cells after 5 d of coculture. In some experiments, DCs were centrifuged onto glass slides and photographed using an Olympus BX51 microscope, DP70 digital camera, and DP software (Olympus, Center Valley, PA).

Using flow cytometry and confocal microscopy, we investigated whether DCs of distinct developmental stages express Ccr7 and migrated to chemoattractants. BM cells were harvested on day 8 and stained with biotinylated anti-CD11c Ab (eBioscience), followed by incubation with streptavidin Microbeads (Miltenyi, Auburn, CA), and finally isolated with an automated magnet-activated cell sorter to a purity of >90% CD11c+ cells. To assay T cell stimulatory activity, BM-derived DCs were purified using MACS and then cultured with naive CD4+ OT-II cells (DC:T cell ratio = 1:2) in the presence of 10 μg ml−1 OVA257–264 peptide (New England Peptide, Gardner, MA) as described previously (8). Proliferation was inferred from recovery of viable (trypan blue negative) T cells after 5 d of coculture. In some experiments, DCs were centrifuged onto glass slides and photographed using an Olympus BX51 microscope, DP70 digital camera, and DP software (Olympus, Center Valley, PA).

Chromatin immunoprecipitation

Native chromatin was prepared from cells by small-scale micrococcal nuclease digestion as previously described with modification (23). Briefly, CD11c+ BM-elicited DCs (4 × 106) or sorted lung DCs (0.2–1 × 106) were washed and incubated in lysis buffer containing 0.4% Nonidet P-40 for 10 min on ice. Nuclei were then washed and resuspended in digestion buffer containing 25 U/ml micrococcal nuclease (Worthington Biochemical, Lakewood, NJ) and incubated at 37°C for 5 min to generate mono- and dicentric chromosomes. The reaction was stopped by the addition of 200 mM NaCl (pH 8), 5 mM EDTA, and 10 mM sodium butyrate, and samples were incubated overnight at 4°C. After centrifugation, the supernatant was harvested and Triton X-100 was added to a concentration of 1% (v/v). For BM-elicited DC samples, chromatin was precleared with protein A–Sepharose/salmon sperm DNA (EMD Millipore, Billerica, MA) and then incubated overnight at 4°C with 2.5 μg of either anti-trimethylated H3 lysine 27 (anti-H3K27ac; Active Motif, Carlsbad, CA), anti-acetylated H3 lysine 27 (anti-H3K27ac; Active Motif), or nonspecific anti-rabbit IgG Ab (Santa Cruz Biotechnology, Dallas, TX). After incubation with protein A–Sepharose/salmon sperm DNA for 1 h, immunoprecipitates were washed vigorously, and DNA was purified. For lung DC samples, chromatin was immunoprecipitated with the above Abs using the MAGnify ChIP System (Life Technologies) per the manufacturer’s instructions. Bound and input fractions were quantified by QPCR using an Mx3000p QPCR system (Agilent Technologies) with Power SYBR Green PCR Master Mix (Life Technologies) and PCR primers listed in Supplemental Table I. PCR conditions were as follows: 10 min at 95°C, 15 s at 95°C and 60 s at 62°C for 40 cycles; and dissociation curve analysis to verify specific PCR product labeling. Analysis was performed using the efficiency-corrected ΔCt method, with amplification efficiency determined from a standard curve that was generated from genomic DNA. Data are presented as the signal from bound fractions normalized to the input fraction.

Statistics

Data are expressed as mean ± SD or SEM as indicated. Statistical differences between groups were calculated using a two-tailed Student t test, unless indicated otherwise. A p value < 0.05 was considered significant.
Results
BM-elicited conventional DCs, but not moDCs, express functional CCR7

Lung DCs are rare compared with many other cell types in that organ. Accordingly, we first used different culture conditions to generate cDCs and moDCs from BM progenitors ex vivo (Fig. 1A) (24). Because FL is required for cDC development, we cultured BM progenitors in the presence of this cytokine to generate cDCs (FL-DCs). To generate moDCs, we cultured progenitors with GM-CSF and IL-4 (GM-DCs). Light microscopic and flow cytometric analyses revealed that both cultures contained CD11c<sup>hi</sup>I-Ab<sup>hi</sup> DCs having similar morphologies, including the presence of dendrites (Fig. 1B, 1C). FL-DCs and GM-DCs were similarly responsive to maturation stimuli, as both upregulated expression of MHC class II and costimulatory molecules following LPS treatment (Fig. 1D). Furthermore, LPS treatment induced similar expression of Il6 and Il12b mRNA in FL-DCs and GM-DCs (data not shown). Importantly, LPS-matured FL-DCs and GM-DCs were equivalent in their ability to stimulate naive T cells (Fig. 1E). Thus, by these standard assays, FL-DCs and GM-DCs each displayed the characteristics of bona fide DCs. However, FL-DCs and GM-DCs differed considerably with regard to lineage-associated gene expression. Compared with GM-DCs, FL-DCs expressed significantly higher levels of mRNA for the cDC-specific transcription factor Zbtb46 (Fig. 1F) (25, 26). In contrast, activated GM-DCs highly expressed Nos2, consistent with a moDC phenotype (Fig. 1F) (27). On the basis of these observations, we concluded that FL-DCs and GM-DCs corresponded to cDCs and moDCs, respectively.

CCR7<sup>gfp</sup> reporter mice are a convenient resource for assaying Ccr7 expression in DCs (10). Using these mice as a source of BM, we compared Ccr7<sup>gfp</sup> expression in FL-DCs and GM-DCs. Following LPS stimulation, FL-DCs rapidly upregulated Ccr7<sup>gfp</sup> (Fig. 2A, 2B). In contrast, GM-DCs expressed very low levels of Ccr7<sup>gfp</sup> following LPS treatment (Fig. 2A, 2B). Similar results were observed when FL-DCs and GM-DCs were stimulated with the TLR3 ligand poly(I:C) (Fig. 2B). To confirm that the GFP fluorescence correlated with functional CCR7 protein, we studied the abilities of FL-DCs and GM-DCs to undergo chemotaxis in response to CCL19. FL-DCs migrated very efficiently to various concentrations of CCL19, confirming that they expressed functional CCR7 (Fig. 2C). Conversely, GM-DCs migrated poorly to CCL19 (Fig. 2C), which was consistent with their low expression of Ccr7<sup>gfp</sup>. Thus, although FL-DCs and GM-DCs were similar in many measures of DC function, only FL-DCs expressed functional CCR7 at high levels.

FIGURE 1. Culturing BM precursors with FL or GM-CSF/IL-4 yields DCs that resemble conventional or moDCs, respectively. (A) Schematic protocol for the generation and maturation of BM-elicited DCs using FL (FL-DCs) or GM-CSF/IL-4 (GM-DCs). (B) Representative light micrographs of cytospin preparations of FL- or GM-DCs. (C) Representative cytograms depicting surface I-A<sup>b</sup> and CD11c expression on FL- and GM-DCs after maturation with LPS. (D) Histograms depicting I-A<sup>b</sup>, CD40, and CD86 expression on unstimulated (shaded histograms) or LPS-matured (bold lines) DCs. Data are from a single experiment, representative of two. (E) Stimulation of naive CD4<sup>+</sup> OT-II cells by LPS-matured FL- or GM-DCs in the presence or absence of OVA<sub>323-339</sub> peptide. Bar graphs represent the mean ± SD of viable T cells (duplicate samples). Data are from a single experiment, representative of two. (F) Zbtb46 and Nos2 mRNA expression by MACS-purified CD11c<sup>+</sup> FL- and GM-DCs. Bar graphs represent the mean ± SD of mRNA amounts in arbitrary units (AU) after normalization to Gapdh mRNA. Data are from a single experiment, representative of two.
The Ccr7 locus is enriched for repressive histone modifications in BM-elicited moDCs

The inability of GM-DCs to express Ccr7 despite having otherwise normal responsiveness to LPS (Fig. 1D) suggested that the Ccr7 locus in these cells might be inaccessible to transcriptional activators. It is well established that histone modifications help govern chromatin accessibility to the transcriptional machinery, thereby regulating gene expression (17). In particular, trimethylation of lysine 27 on histone 3 (H3K27me3), which is primarily mediated by the polycomb repressive complex 2 (PRC2), is associated with transcriptional repression in various cells including DCs and macrophages (28, 29). Analysis of available chromatin immunoprecipitation (ChIP)-sequencing data obtained from the Encyclopedia of DNA Elements (ENCODE) project (30) revealed that the Ccr7 promoter is enriched for H3K27me3 in tissues that generally lack CCR7 expression (Fig. 3A), suggesting that H3K27me3 might also be associated with Ccr7 repression in moDCs. Therefore, using ChIP-QPCR assays, we investigated H3K27me3 modifications at the Ccr7 promoter in LPS-stimulated FL-DCs and GM-DCs. We found that the Ccr7 promoter is significantly enriched for H3K27me3 in GM-DCs but not FL-DCs (Fig. 3B). GM-DCs also had similar enrichment of H3K27me3 at the first intron of Ccr7. To exclude the possibility that our findings at the Ccr7 locus were due to a widespread nonspecific increase in H3K27me3 in GM-DCs, we studied a distant locus containing the Magea2 gene. No significant differences were seen in H3K27me3 at that locus, indicating that repressive histone marks were preferentially increased at the Ccr7 locus (Fig. 3B). The level of H3K27me3 at the Ccr7 promoter was inversely related to Ccr7 mRNA expression, consistent with transcriptional repression of Ccr7 (Fig. 3C). Similar differences in Ccr7-associated H3K27me3 were observed in unstimulated DCs, indicating that the repressive histone marks were established prior to DC activation (Supplemental Fig. 1A). Because histone acetylation has been associated with transcriptional activation in human DCs (28), we also analyzed H3K27 acetylation at the Ccr7 locus. We did not find significant differences in Ccr7-associated H3K27 acetylation between FL- and GM-DCs (Supplemental Fig. 1B), suggesting that Ccr7 expression was predominantly regulated by repressive histone marks. Taken together, these findings suggest that lineage-specific repressive histone modifications help govern Ccr7 expression in BM-elicited DCs.

Nonmigratory lung-resident moDCs have increased levels of Ccr7-associated H3K27me3

Having established that the Ccr7 locus is associated with repressive histone marks in moDCs expanded from BM progenitors, we next investigated whether similar repressive histone marks are associated with Ccr7 in lung-resident moDCs. Lung-resident DCs can be phenotypically divided into CD103+ or CD11bhi DCs. CD103+ DCs arise from pre-DC precursors and thus represent a pure population of cDCs (10). By contrast, CD11bhi DCs contain a mixture of FL-dependent cDCs and FL-independent moDCs, with the latter cells having a higher display level of CD14 (10). However, because some CD11bhi DCs express intermediate levels of CD14, this molecule cannot reliably distinguish cDCs from moDCs. CD64 expression is reported to discriminate between cDCs and moDCs (3, 31), but we recently found that display levels of a different marker, CD88, is a more reliable marker to distinguish CD11bhi moDCs from CD11bhi cDCs (H. Nakano, T.P. Moran, K. Nakano, C.D. Bortner, and D.N. Cook, submitted for publication). To confirm that CD88 can also distinguish nonmigratory moDCs from migratory cDCs, we isolated lung-resident DCs from Ccr7 reporter mice by flow cytometric cell sorting (Fig. 4A). As expected, all lung-resident DC subsets expressed low levels of Ccr7 mRNA immediately after isolation from naive mice (Fig. 4B). Stimulation with LPS resulted in significant induction of Ccr7 mRNA in CD103+ and CD11bhiCD88hi DCs (Fig. 4B). In contrast, CD11bhiCD88lo DCs expressed very low levels of Ccr7 after activation, which is consistent with their monocytic lineage (Fig. 4B). All lung DC subsets expressed high levels of CD86 after LPS stimulation (data not shown), indicating normal responsiveness to TLR4 ligands. Analysis of Ccr7 mRNA in wild-type mice confirmed that this gene is more highly expressed in activated CD103+ cDCs and CD11bhiCD88hi cDCs compared with CD11bhiCD88lo moDCs (Fig. 4C). Thus, CD88 display can identify nonmigratory CD11bhi moDCs in the lung.

Using CD88 as a marker for moDCs, we purified migratory CD103+ and CD11bhiCD88lo cDCs, as well as nonmigratory CD11bhiCD88hi moDCs, from the lungs of LPS-treated mice. Chromatin was prepared from each DC subset and analyzed for
Ccr7-associated H3K27me3. Compared with CD103+ DCs and CD11b+CD88lo cDCs, CD11bhiCD88hi moDCs had significantly elevated levels of H3K27me3 at the Ccr7 promoter (Fig. 4D). The amount of Ccr7-associated H3K27me3 was inversely related to the expression of this gene, consistent with transcriptional silencing of Ccr7 in CD11bhiCD88hi moDCs (Fig. 4C). Levels of H3K27me3 at a distant locus (Hoxc10) were similar among all DC subsets, confirming that the epigenetic differences seen at the Ccr7 promoter are not due to a nonspecific genomewide increase of H3K27me3 in lung moDCs (Fig. 4D). Overall, these findings indicate that the nonmigratory status of lung-resident moDCs is associated with repressive histone modifications at the Ccr7 promoter.

BM monocytes have low levels of Ccr7-associated H3K27me3

Our observation that cDCs and moDCs have disparate levels of Ccr7-associated H3K27me3 is consistent with lineage-specific epigenetic repression of Ccr7 expression. This prompted us to investigate whether the divergence in H3K27me3 at the Ccr7 locus in cDCs and moDCs occurs during lineage commitment. Monocyte-DC progenitors can give rise to both monocytes and pre-DCs (32). We therefore compared histone modifications in the latter two types of progenitor cells following their purification from BM. Unexpectedly, monocytes and pre-DCs had similar amounts of H3K27me3 at the Ccr7 promoter (Fig. 5A), and these amounts were much lower than that seen for either BM-elicited or lung moDCs (Figs. 3B, 4D). Thus, differentiation of monocyte-DC progenitors to monocytes in the BM is not immediately accompanied by epigenetic repression of Ccr7.

Epigenetic repression of the Ccr7 promoter is gradually established during monocyte differentiation into DCs

The similar amounts of H3K27me3 at the Ccr7 locus of monocytes and pre-DCs suggested that this epigenetic modification is acquired after monocytes enter peripheral tissues and differentiate into moDCs. We first investigated whether monocyte emigration into pulmonary tissues triggers epigenetic repression of Ccr7. Airway exposure to LPS results in the recruitment of Ly-6Chi monocytes to the lungs, which rapidly differentiate into Ly-6Chi CD11bhi inflammatory DCs (33). Inflammatory DCs also express CD88 but can be distinguished from lung-resident moDCs by their high-level expression of Ly-6C (Fig. 5B). ChIP-QPCR analysis of inflammatory DCs revealed that they had significantly lower levels of Ccr7-associated H3K27me3 compared with lung-resident moDCs (Fig. 5C). Indeed, inflammatory DCs and BM monocytes had similar low levels of H3K27me3 at the Ccr7 locus (Fig. 5A, 5C). Thus, extravasation of monocytes into the lung parenchyma does not immediately result in epigenetic repression of Ccr7.

We next investigated whether epigenetic repression of Ccr7 is established during the later stages of monocyte differentiation into...
moDCs. We have previously shown that only a very small percentage of adoptively transferred monocytes enter the lungs and differentiate into resident moDCs (10). Therefore, it was not possible to accurately track the fate of large numbers of monocytes in the lung for an extended period. As an alternative approach, we purified Ly-6Chi monocytes from BM and followed epigenetic changes at the Ccr7 promoter during ex vivo differentiation of these cells into moDCs (34). As expected, during 6 d of culture in the presence of GM-CSF and IL-4, monocytes differentiated into CD11chiI-Abhi moDCs (Fig. 6A). As we had seen previously with BM progenitors cultured under these same conditions, monocyte-derived cells failed to express Ccr7, even after their stimulation with LPS (Fig. 6B). During ex vivo moDC differentiation, we observed a significant increase in Ccr7-associated H3K27me3 (Fig. 6C). In contrast, the level of H3K27me3 at the Hoxc10 locus remained stable during the culture period (Fig. 6C), indicating the observed changes at the Ccr7 locus were not due to a genomewide increase in H3K27me3. Consistent with this, we did not detect increased expression of the PRC2 core subunits EZH2, EED, SUZ12, and RbAp48 during moDC differentiation (Supplemental Fig. 2). Overall, these findings suggest that epigenetic silencing of Ccr7 occurs in a specific and gradual manner during the differentiation of monocytes into lung-resident moDCs.

Discussion

The migration of DCs to lung-draining LN is critical for shaping nascent immune responses to inhaled Ags (1, 3, 10). Given that the DC network is composed of populations with distinct functional roles, it is not surprising that only certain subsets possess the ability to express Ccr7 and migrate to regional LN. We have previously demonstrated that the developmental lineage of DCs is important for determining their migratory potential (10). In the current study, we investigated whether epigenetic mechanisms might contribute to lineage-specific Ccr7 expression. We found that in BM-elicited or lung moDCs, the Ccr7 promoter is enriched for the repressive histone mark H3K27me3. Consequently, Ccr7 induction is transcriptionally repressed in moDCs but not cDCs. The repressive histone modifications are not established during lineage commitment in the BM but are formed during monocyte differentiation into moDCs in the periphery. Our results suggest

![FIGURE 4.](http://www.jimmunol.org/) Nonmigratory lung-resident moDCs have increased levels of Ccr7-associated H3K27me3. (A) Gating strategy for flow cytometry-based sorting of lung-resident DC subsets from wild-type or Ccr7gfp reporter mice. (B) Analysis of Ccr7 mRNA expression on sorted lung-resident DCs before (shaded histograms) or after (bold lines) ex vivo stimulation with 100 ng ml⁻¹ LPS for 24 h. Data are from a single experiment, representative of two. (C) Ccr7 mRNA expression in lung-resident DCs isolated from mice 16 h post-LPS inhalation. Bar graphs represent the mean ± SEM (after normalization to Gapdh mRNA) of combined data from two independent experiments. (D) ChIP-QPCR analysis of the Ccr7 promoter or Hoxc10 locus (reference gene) in lung-resident DCs after immunoprecipitation with either anti-H3K27me3 (■) or nonspecific IgG (□) Abs. Bar graphs represent the mean ± SEM (after normalization to input DNA) of combined data from three independent experiments. ***p < 0.001. Auto, autofluorescence; AU, arbitrary unit.
that a combination of lineage-specific epigenetic mechanisms and cues within the tissue microenvironment help determine the migratory capacity, and consequently the functional role, of lung DC subsets. The nonmigratory status of moDCs suggests that they do not function during Ag sensitization, which is thought to require DC migration, but rather during the effector phase of immune responses within the lung. Indeed, a recent report found that moDCs were largely responsible for Ag presentation and proinflammatory cytokine secretion in the lungs during allergen challenge (3). Whether lung moDCs are more efficient than cDCs at stimulating effector or memory T cells is an important question for future studies.

Although there has been recent progress in identifying the transcriptional profiles of different DC subtypes (9, 12, 35), little is known regarding the epigenetic mechanisms regulating gene expression in DCs. This is in contrast to Th cell differentiation, where epigenetic changes at lineage-specific genes have been well documented (36). Epigenetic mechanisms are likely responsible for regulating several aspects of DC immunobiology. For example, DCs treated with histone deacetylase inhibitors have altered expression of proinflammatory cytokines and costimulatory molecules, arguing that dynamic changes in histone modifications can influence DC function during adaptive immune responses. Splenic DCs from offspring of allergen-sensitized mothers had altered genomic DNA methylation patterns, which was associated with a Th2-skewing phenotype when compared with DCs from pups of naive mothers (38). The current study provides further insight into how epigenetic changes can influence DC function, specifically through regulation of their migration. By epigenetically silencing the Ccr7 gene, moDCs assume a nonmigratory phenotype within the lung.

Unexpectedly, we found that epigenetic repression of Ccr7 is not established early during DC lineage commitment, but rather during monocyte differentiation into moDCs. Using an ex vivo model for generating moDCs from BM monocytes, we observed a gradual but specific increase in H3K27me3 at the Ccr7 promoter. It is possible that ex vivo generation of moDCs from monocytes does not accurately reflect in vivo differentiation of moDCs in the lung. However, ex vivo–generated and lung-resident moDCs had comparable surface marker phenotypes and histone modifications at the Ccr7 locus, suggesting that they undergo similar differentiation pathways. Improvements in cellular fate-mapping and ChIP analyses using small cell numbers will help facilitate future epigenetic studies of lung-resident moDCs and other rare cell types.

Interestingly, we observed low levels of H3K27me3 at the Ccr7 locus in monocytes, implying that Ccr7 is not expressed in peripheral blood monocytes in humans. Because LN homing by DCs is considered necessary for optimal stimulation of Ag-specific T cells (45), understanding how GM-CSF and FL influence the migratory potential of DCs will be essential for designing effective DC vaccines.
at the indicated time points. (A) 6 d. Cells were analyzed by flow cytometry (porter mice (B) analysis of the Ccr7 representative of two. (C) Cytograms showing CD11c and I-A\(^{b}\) during culture. Cells were treated with 100 ng ml\(^{-1}\) LPS for 24 h prior to flow cytometric analysis. Data are from a single experiment, representative of two. (B) Cytograms depict Ccr7\(^{gfp}\) expression by monocytes at the indicated time points during culture. Cells were treated with 100 ng ml\(^{-1}\) LPS for 24 h prior to flow cytometric analysis. Data are from a single experiment, representative of two. (C) Quantification of H3K27me3 by ChIP-QPCR analysis of the Ccr7 promoter or Hoxc10 locus (reference gene) during monocyte differentiation into moDCs. Bar graphs represent the mean ± SEM (after normalization to input DNA) of combined data from two independent experiments. The line graph depicts the mean ratio (± SEM) of Ccr7-associated H3K27me3 to Hoxc10-associated H3K27me3. **p < 0.01.

Although our studies demonstrate that Ccr7-associated H3K27me3 increases during monocyte differentiation into moDCs, the mechanisms by which occurs are currently unclear. Because trimethylation of H3K27 is mediated by the chromatin modification complex PRC2, we studied expression of genes encoding its core subunits (EZH2, EED, SUZ12, and RbAp48) during monocyte differentiation to moDCs (46). We found that expression of these genes generally decreased during moDC development (Supplemental Fig. 2), which is consistent with the previously reported decrease in Ezh2 expression during cellular differentiation (47). Therefore, it is likely that the increased Ccr7-associated H3K27me3 results from a selective recruitment of PRC2 to the Ccr7 locus by specific DNA-binding proteins or transcription factors (48). Identification of such factors should further our understanding of how the Ccr7 locus is regulated in moDCs.

Although our observations suggest that H3K27me3 leads to silencing of Ccr7 in moDCs, demonstrating direct causality is difficult. It is possible to inhibit H3K27me3 by targeting the histone methyltransferase EZH2, but this can affect multiple aspects of cell differentiation and survival (49). Not surprisingly, we found that the EZH2 inhibitor 3-deazaneplanocin A interfered with DC development from BM progenitors and monocytes, thus precluding any specific studies on Ccr7 expression (T.P. Moran, unpublished observations). Strategies that specifically inhibit EZH2 activity at the Ccr7 locus will be important for addressing this issue. It is also likely that H3K27me3 is but one of many mechanisms involved with regulating Ccr7 expression. Monocytes and monocyte-derived Ly-6C\(^{hi}\) inflammatory DCs have low levels of Ccr7-associated H3K27me3, yet they do not express Ccr7 after stimulation with LPS (Fig. 6A) (10). This implies that in addition to a receptive chromatin configuration at the Ccr7 promoter, lineage-specific transcription factors are also necessary for driving gene expression. Several transcriptional factors have been reported to regulate Ccr7 expression, including NF-\(\kappa\)B (50), peroxisome proliferator-activated receptor \(\gamma\) (51), Runx3 (52), liver X receptor (53), and IFN regulatory factor 4 (54). The migratory properties of cDCs and moDCs may be dependent on differential expression of these transcription factors. Additional epigenetic mechanisms, such as DNA methylation and noncoding RNAs, might also regulate Ccr7 expression. The Ccr7 promoter does not contain any CpG islands, but it is possible that DNA methylation in upstream enhancers or intronic regions may influence Ccr7 expression. Noncoding RNAs that inhibit CCR7 production have been described in human T lymphocytes and breast cancer cells (55, 56) but not in APCs. Future studies will help clarify the relative contribution of lineage-specific transcription factors and other epigenetic marks to Ccr7 regulation.

Overall, our findings suggest that by controlling Ccr7 expression, lineage-specific epigenetic mechanisms can influence the function of specific DC subsets. Identifying the endogenous and cell-intrinsic factors that direct Ccr7-specific epigenetic changes will likely uncover pathways that can be targeted to modulate DC migration. In turn, the ability to manipulate the migratory properties of DCs represents a novel strategy for improving DC-based immunotherapies and for treating immune-mediated diseases.

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Disclosures

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