Relationships between Distinct Blood Monocyte Subsets and Migrating Intestinal Lymph Dendritic Cells In Vivo under Steady-State Conditions

Ulf Yrlid, Christopher D. Jenkins and G. Gordon MacPherson

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The origins of dendritic cells (DCs) are poorly understood. In inflammation, DCs can arise from blood monocytes (M\(\text{Os}\)), but their steady-state origin may differ, as shown for Langerhans cells. Two main subsets of M\(\text{Os}\), defined by expression of different chemokine receptors, CCR2 and CX3CR1, have been described in mice and humans. Recent studies have identified the inflammatory function of CCR2\(^{\text{high}}\)/CX3CR1\(^{\text{low}}\) M\(\text{Os}\) but have not defined unambiguously the origin and fate of CCR2\(^{\text{low}}\)/CX3CR1\(^{\text{high}}\) cells. In this study, we show that rat M\(\text{Os}\) can also be divided into CCR2\(^{\text{high}}\)/CX3CR1\(^{\text{low}}\) and CCR2\(^{\text{low}}\)/CX3CR1\(^{\text{high}}\) subsets with distinct migratory properties in vivo. Using whole body perfusion to obtain M\(\text{Os}\), including the marginating pool, we show by adoptive transfer that CD43\(^{\text{low}}\) M\(\text{Os}\) can differentiate into CD43\(^{\text{high}}\) M\(\text{Os}\) in blood without cell division. By adoptive transfer of blood M\(\text{Os}\) followed by collection of pseudoafferent lymph, we show for the first time that a small proportion of intestinal lymph DCs are derived from CCR2\(^{\text{low}}\)/CX3CR1\(^{\text{high}}\) blood M\(\text{Os}\) and indicate that they may contribute to migratory intestinal DCs in vivo in the absence of inflammatory stimuli.


Abbreviations used in this paper: M\(\text{O}\), monocyte; DC, dendritic cell; LN, lymph node; DLN, draining LN; SS, steady state; MLNX, mesenteric lymphadenectomy; iL-DC, intestinal lymph DC; BM, bone marrow; PMN, polymorphonuclear cell; TDL, thoracic duct leukocyte; PLN, parathymic LN.

The Sir William Dunn School of Pathology, South Parks Road, Oxford, United Kingdom

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1 Address correspondence and reprint requests to Dr. G. Gordon MacPherson, The Sir William Dunn School of Pathology, South Parks Road, Oxford, OX1 3RE, U.K. E-mail address: gordon.macpherson@path.ox.ac.uk

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Materials and Methods

Rats and surgical procedures

PVG RT1<sup>e</sup> and congenic RT1<sup>e</sup>Rt<sup>b</sup> (Rt<sup>b</sup>) male rats were maintained under specific pathogen-free conditions and used at 12–24 wk of age in accordance with Home Office guidelines. Mesenteric lymphadenectomy, thoracic duct cannulation, and whole body perfusions were performed as previously described (12, 14).

Reagents

Twenty-five micrograms of R-848 (InvivoGen) or 50 μg of Salmonella typhimurium LPS (Sigma Aldrich) were injected i.v. in 0.5 ml of PBS. Peritonitis was induced by i.p. injection of 5 ml of sterile 4% thiglycolate broth and recruited cells were collected by peritoneal lavage. 7-aminocoumarinycin D (7AAD) was purchased from Sigma Aldrich.

Antibodies

mAbs to rat Ags: CD4 (OX35), CD5 (OX19), CD6 (OX52), CD8a (OX8), CD11b/c (OX42), CD43 (W3/13), CD45RA (OX33), CD90 (OX7), CD103 (OX62), CD172a (OX41), CD200R (OX102), and RT<sup>+</sup> (His41) purified from cell culture supernatants were used for depletions or conjugated to biotin, FITC, or Alexa-647. Anti-CD32 (D34-485), later biotinylated, anti-PMN (RP-1-PE), anti-MHC-II (OX6-PerCP), and streptavidin-PE and -allophycocyanin were all purchased from BD Pharmingen.

Labeling for FACS was performed in staining buffer for 15 min on ice after reaction was stopped by adding an equal volume of cold medium containing 20% FCS. The cells were washed twice in staining buffer before analyzed their migration into inflamed tissues. To this end, CD43<sup>high</sup> and low blood and BM M<sub>C</sub> were sorted by FACS. Given the low CD11b levels on some CD43<sup>low</sup> BM M<sub>C</sub>, these were subsorted into CD43<sup>low</sup>CD11b<sup>+</sup> and CD43<sup>low</sup>CD11b<sup>−</sup> subsets. Sorted M<sub>C</sub> from RT7<sup>b</sup> rats were given i.v. to RT1<sup>e</sup> rats with peritonitis and peritoneal cells examined 21 h later. Strikingly, no CD43<sup>high</sup> M<sub>C</sub> were detected in the peritoneum but significant and similar numbers of CD43<sup>low</sup> blood M<sub>C</sub> and CD43<sup>low</sup>CD11b<sup>−</sup> were present (Fig. 1B). Even after giving five times as many CD43<sup>high</sup> blood M<sub>C</sub>, very few were recovered from the cavity (Fig. 1C). In addition, only CD43<sup>low</sup> BM M<sub>C</sub> gave rise to CD11b<sup−</sup> MHC-II<sup+</sup> cells in the draining PLNs (Fig. 1D).

These experiments define two phenotypically and functionally distinct subsets of rat M<sub>C</sub>; CCR2<sup>high</sup>CD43<sup>−</sup> and CD43<sup>low</sup>CD11b<sup>−</sup> subsets using a MoFlo (Cytomation). The purity was then >98%.

Thoracic duct leukocytes (TDLs) were collected on ice and depletes of lymphocytes as above. Parathymic LNs (PLNs) were mashed through a cell strainer, washed once with PBS containing 2% FCS and 10 mM EDTA (staining buffer) before FACS analysis.

FACS and labeling of cells

Labeling for FACS was performed in staining buffer for 15 min on ice after blocking for 10 min in 10% rat serum. The cells were fixed in 2% paraformaldehyde and analyzed with a FACSCalibur (BD Biosciences). M<sub>C</sub> were labeled with 5 μM CFSE (Molecular Probes) by incubating them without serum for 10 min at 37°C at 5 X 10<sup>7</sup> cells/ml. The labeling reaction was stopped by adding an equal volume of cold medium containing 20% FCS. The cells were washed twice in staining buffer before injection.

Results

Rat M<sub>C</sub> comprise two subsets with distinct migratory properties

Rat blood M<sub>C</sub> are identified phenotypically as mononuclear cells expressing CD172a and CD11b/c (12, 15). There are currently no reagents available for detection of rat CD115. CD172a and CD11b/c are also expressed by PMNs and DCs but M<sub>C</sub> do not stain with the granulocyte-specific mAb RP-1 nor do they express the high levels of CD103 and MHC-II which define rat DCs. As for murine M<sub>C</sub> (7), rat M<sub>C</sub>s comprise two subsets differing in CD43 expression (1, 15).

To obtain all M<sub>C</sub> expressing CD43, including the marginalizing pool, rats were perfused with 1 L of buffer. M<sub>C</sub> were subsequently purified from the collected blood perfusate on density gradients or directly from BM, followed by depletion of B, T, and NK cells. FACS analysis of the recovered cells confirmed the phenotype of blood M<sub>C</sub> and additionally identified M<sub>O</sub> populations in BM (Fig. 1A). The two main M<sub>O</sub> subsets defined by CD43 expression were then further characterized (Fig. 1A). In comparison with CD43<sup>high</sup> M<sub>C</sub>, CD43<sup>low</sup> M<sub>C</sub> are larger, express more CD32, CD200R, and CD62L but less CD4 and CD11c. Moreover, CD43<sup>high</sup> M<sub>C</sub> express CCRxCR1 but are CCR2<sup>−</sup> while CD43<sup>low</sup> M<sub>C</sub> are CCRxCR1<sup>−</sup> but CCR2<sup>−</sup>. The ratio of CD43<sup>low</sup> M<sub>C</sub> in blood is 1:8 but in BM is ~8:1. The only consistent phenotypic difference between M<sub>C</sub> isolated from blood and BM is that some CD43<sup>low</sup> BM M<sub>C</sub> express less CD11b.

To assess the functional properties of the two subsets, we examined their migration into inflamed tissues. To this end, CD43<sup>high</sup> and low blood and BM M<sub>C</sub> were sorted by FACS. Given the low CD11b levels on some CD43<sup>low</sup> BM M<sub>C</sub>, these were subsorted into CD43<sup>low</sup>CD11b<sup>+</sup> and CD43<sup>low</sup>CD11b<sup>−</sup> subsets. Sorted M<sub>C</sub> from RT7<sup>b</sup> rats were given i.v. to RT1<sup>e</sup> rats with peritonitis and peritoneal cells examined 21 h later. Strikingly, no CD43<sup>high</sup> M<sub>C</sub> were detected in the peritoneum but significant and similar numbers of CD43<sup>low</sup> blood M<sub>C</sub> and CD43<sup>low</sup>CD11b<sup>−</sup> were present (Fig. 1B). Even after giving five times as many CD43<sup>high</sup> blood M<sub>C</sub>, very few were recovered from the cavity (Fig. 1C). In addition, only CD43<sup>low</sup> BM M<sub>C</sub> gave rise to CD11b<sup−</sup> MHC-II<sup+</sup> cells in the draining PLNs (Fig. 1D).

These experiments define two phenotypically and functionally distinct subsets of rat M<sub>C</sub>; CCR2<sup>high</sup>CD43<sup>−</sup> and CD43<sup>low</sup>CD11b<sup>−</sup> BM M<sub>C</sub> are recruited to inflamed tissues while CCR2<sup>low</sup>CD43<sup>high</sup> BM M<sub>C</sub> are not.

CD43<sup>low</sup> M<sub>C</sub> differentiate into CD43<sup>high</sup> M<sub>C</sub> in vivo

The developmental relationships between subsets of M<sub>C</sub> are uncertain, particularly in the SS. To examine M<sub>C</sub> differentiation in vivo, CFSE-labeled M<sub>C</sub> subsets were sorted (Fig. 2A) and transferred i.v. into normal rats. After 18 h, some transferred CD43<sup>low</sup> blood M<sub>C</sub> had up-regulated CD43 to intermediate levels whereas the majority of transferred CD43<sup>low</sup> BM M<sub>C</sub> were still CD43<sup>low</sup> (Fig. 2B). Sixty hours after transfer, the same rats that had been bled at 18 h were perfused and recovered M<sub>C</sub> were analyzed for CD43 and CFSE. All donor CD43<sup>high</sup> and low blood M<sub>C</sub> and CD43<sup>low</sup>CD11b<sup>−</sup> BM M<sub>C</sub> were now CD43<sup>high</sup> (Fig. 2C), Imporantly, their CFSE content showed that none of these M<sub>C</sub> had divided. In contrast, some donor CD43<sup>low</sup>CD11b<sup−</sup> BM M<sub>C</sub> had divided and had also up-regulated CD43.

To determine whether up-regulation of CD43 was accompanied by changes in other markers, blood M<sub>C</sub> were sorted by CD43 expression and BM M<sub>C</sub> were sorted into CD11b<sup−</sup>CD43<sup>high</sup> to avoid the dividing cell population. Sorted congenic M<sub>C</sub> subsets were transferred i.v. into naive recipients. After 40 h, recipients were perfused and donor M<sub>C</sub> numbers and phenotypes were assessed. CD43<sup>high</sup> M<sub>C</sub> retained their original phenotype (Fig. 3A), whereas all donor CD43<sup>low</sup> M<sub>C</sub>, independent of origin (blood or BM), had become CD43<sup>high</sup> expressed uniform high levels of CD4, and had up-regulated CCRxCR1 (Fig. 3, B and C). In addition, the adoptively transferred CD43<sup>low</sup> M<sub>C</sub> had down-regulated CD62L, CCR7, and to a lesser extent CCR2.
These experiments show that after adoptive transfer under SS conditions, CD43low Møs gradually acquire the phenotype of CD43high Møs without cell division.

Some CD43high blood Møs differentiate into SS iL-DCs without cell division

The fate of murine CCR2 high CX3CR1 low Møs in tissues has been examined recently (3, 9, 11) but the fate of CCR2 low CX3CR1 high Møs in tissues is less understood. Immature CX3CR1+/H11001 DCs present in the lamina propria of the small intestine constitutively sample intestinal contents (16). Their origins and migratory fate(s) are however unclear. To determine whether both the subset Møs can become lymph-borne migratory intestinal DC, we transferred CFSE-labeled CD43 high blood Møs or CD43 low BM Møs, into thoracic duct-cannulated MLNX rats (Fig. 4A). Eighteen hours after transfer of 4.5 × 10⁶ Møs, donor Møs represented 3–4% of total blood monocytes, and at the time of cannulation they constituted 1–2% (data not shown). Analysis of iL-DCs showed that a small but clearly identifiable proportion derived from the transferred CD43low blood Møs (Fig. 4B, top row of panels). Furthermore, this differentiation had occurred without cell division. A small population of donor-derived iL-DCs, where the majority had undergone cell division, could also be observed in rats that had received CD43low BM Møs (Fig. 4B, top row of panels).

Donor-derived cells in lymph with a Mø phenotype (SSCD43lowCD172a+CD103+ MHC-IIlow) were also detected and as noted with the monocyte-derived iL-DCs, only the lymph Møs originating from the CD43low BM Møs had divided (Fig. 4B, middle row of plots). No transferred CD43high blood Møs and very few, if any, CD43low BM Møs developed into PMNs in lymph (Fig. 4B, bottom row of plots).

These experiments show that Møs can give rise to DCs that migrate via intestinal lymph and that the Mø-derived iL-DCs originating from CD43high blood Møs do this without cell division.

Blood Møs can differentiate into both CD172a⁺ and CD172a⁻ iL-DCs

We have previously shown that rat iL-DC comprise two functionally distinct subsets distinguished by CD172a expression (14, 17) and wanted to determine whether both subsets could arise from blood Møs. As numbers of recovered iL-DCs were limiting for analysis, to maximize cell recovery and to minimize changes induced by in vitro handling, Møs were enriched by a single depletion from blood (Fig. 5A). CFSE-labeled CD43high blood Møs or CD43low BM Møs, into thoracic duct-cannulated MLNX rats (Fig. 4A). Eighteen hours after transfer of 4.5 × 10⁶ Møs, donor Møs represented 3–4% of total blood monocytes, and at the time of cannulation they constituted 1–2% (data not shown). Analysis of iL-DCs showed that a small but clearly identifiable proportion derived from the transferred CD43high blood Møs (Fig. 4B, top row of panels). Furthermore, this differentiation had occurred without cell division. A small population of donor-derived iL-DCs, where the majority had undergone cell division, could also be observed in rats that had received CD43low BM Møs (Fig. 4B, top row of panels).

Donor-derived cells in lymph with a Mø phenotype (SSCD43lowCD172a⁺CD103+ MHC-IIlow) were also detected and as noted with the monocyte-derived iL-DCs, only the lymph Møs originating from the CD43low BM Møs had divided (Fig. 4B, middle row of plots). No transferred CD43high blood Møs and very few, if any, CD43low BM Møs developed into PMNs in lymph (Fig. 4B, bottom row of plots).

These experiments show that Møs can give rise to DCs that migrate via intestinal lymph and that the Mø-derived iL-DCs originating from CD43high blood Møs do this without cell division.

**FIGURE 1.** Two phenotypically and functionally distinct subsets of Møs exist in blood and BM. A, Leukocytes were collected from blood by perfusion or from BM, lymphocyte-depleted, and analyzed by FACS. PMN were removed on a gradient (blood) or gated out on FACS by high SSC (BM). Numbers in the dot plots show percentages of CD43high or CD43low Møs. CD172a⁺CD43high (filled histograms) and CD43low Møs (open histograms) were analyzed for surface markers. B and C, RT1⁺ rats were given thioglycollate i.p. and 2 h later adoptively transferred i.v. with 4 × 10⁶ sorted congenic blood or BM Møs. Twenty-one hours later percentages and total numbers of donor cells in peritoneal washouts were determined. D, Rats were treated as in B and 21 h after i.v. transfer of 9.5 × 10⁶ CD43high blood or CD43low BM Møs. PLN cells (gated on CD11b⁺ cells) were analyzed by FACS for expression of the congenic marker and MHC-II expression. The results are representative of three independent experiments.
of observations made after administering TLR ligands to cannulated rats (13, 14). In these studies, we have shown that after giving i.v. LPS (13, 14) or R-848 (27), TLR4 and TLR7/8 ligands, respectively, there is virtually a complete loss of intestinal lamina propria DCs. This is accompanied by a TNF-α-dependent 15- to 30-fold increase in numbers of iL-DCs. Thus, following transfer of labeled MOs, cannulated rats were given i.v. LPS or R-848 and lymph collected for 18 h. LPS and R-848 both stimulated a large increase in the frequency (see included histograms of CD103 expression in density plots, Fig. 5C) and total numbers of iL-DC.

Importantly, of the LPS and R-848-released iL-DCs, 0.15% and 0.21%, respectively, were donor-derived compared with 0.12% in the SS (Fig. 5C). As suggested by SS experiments (PBS), the frequencies of the CD172a high and CD172a low subsets were almost identical between donor- and host-derived iL-DCs (Fig. 5C).

MOs express TLR4 and 7/8 and we could detect up-regulation of MHC-II expression on both donor derived and host MOs 18 h after i.v. injection of LPS (Fig. 5B). In contrast, no change in MHC-II expression was observed on MOs after R-848 administration (Fig. 5B). However, we could detect a dramatic increase in the frequency of CD43 low blood MOs in R-848-treated rats. This was not due to down-regulation of CD43 as transferred CD43 high blood MOs remained CD43 high and most likely reflects increased release of CD43 low MOs from the BM (Fig. 5D).

As 10–15% of blood MOs are CD43 low, we had thus transferred around $15 \times 10^6$ CD43 low CD11b+ MOs when total blood MOs were transferred (Fig. 5C). We repeated the experiment transferring $18 \times 10^6$ CD43 low CD11b+ BM MOs and $70 \times 10^6$ CD43 high blood MOs into separate rats and then administered R-848 i.v. CD43 high CFSE+ RT7b+ MOs developed into iL-DCs that both contain the dye and express the congenic marker (Fig. 5E, left panel). This confirms that the CFSE+ iL-DCs we recover (Figs. 4B and 5C) have not acquired the label through phagocytosis of transferred MOs. Moreover, no donor-derived CFSE+ cells were detected among iL-DCs in rats that had received CD43 low CD11b+ BM MOs (Fig. 5E, middle panel).

These experiments show that a small number of blood MOs, most likely the CCR2 low CX3CR1 high (CD43 high) subset, can enter the intestine and, within 3–4 days, differentiate into the two main subsets of iL-DCs.

**Discussion**

MOs constitute a heterogeneous population of mononuclear leukocytes that play a critical role in host innate immune responses as they are rapidly recruited to inflamed tissues. MOs are also crucial for the replenishment of tissue macrophages but their role in DC production is controversial. Phenotypically distinct MO subsets have been described in humans, mice, rats, and swine but most studies have focused on humans and mice and as different markers have been used between the species, there is a lack of clarity. We have therefore carefully examined the phenotype of the two main rat blood MO subsets, previously defined as CD43 high and CD43 low...
Because of the amenability of rats to experimental manipulation not possible in the mouse, we also followed their fate in tissues under SS and inflammatory conditions. Our phenotypic analysis shows that CD43\textsuperscript{high} MOs express CX\textsubscript{3}CR1 and CD11c but low levels of CD62L and CCR2 whereas in contrast the CD43\textsuperscript{low} subset expresses low levels of CX\textsubscript{3}CR1 and CD11c but high levels of CD62L and CCR2. The subset-specific expression of these chemokine receptors has also been described in mice and humans and are associated with distinct mechanisms of recruitment into tissues in naive and immunologically challenged animals, resulting in the classification of “resident” CCR2\textsuperscript{low}CX\textsubscript{3}CR1\textsuperscript{high} and “inflammatory” CCR2\textsuperscript{high}CX\textsubscript{3}CR1\textsuperscript{low} subsets (3). Consistent with our data in rats, CD43 has recently also been shown to be expressed at higher levels by murine CCR2\textsuperscript{low}CX\textsubscript{3}CR1\textsuperscript{high}(Ly6C\textsuperscript{low}) MOs compared with the Ly6C\textsuperscript{high} subset (7). Moreover, we show that CD32 is expressed at higher levels by the CCR2\textsuperscript{high}CX\textsubscript{3} CR1\textsuperscript{low} (CD43\textsuperscript{low}) which correlates with human peripheral blood MOs where CCR2\textsuperscript{high}CX\textsubscript{3}CR1\textsuperscript{low}(CD16\textsuperscript{+}) MOs have been shown to express high levels of this FcR (4). In rats, the proportion of CCR2\textsuperscript{low}CX\textsubscript{3}CR1\textsuperscript{high} MOs in blood is significantly higher than in mice and humans. Although this could be because we perfuse the animals and therefore obtain the marginating pool of MOs, this is most likely not the explanation as even after bleeding rats, the CCR2\textsuperscript{low}CX\textsubscript{3}CR1\textsuperscript{high}(CD43\textsuperscript{high}) MOs constitute the major proportion of blood MOs.

It is clear from our study as well as from studies in mice (7) that the major subset of MOs in BM is the CCR2\textsuperscript{high}CX\textsubscript{3}CR1\textsuperscript{low}(CD43\textsuperscript{low}) subset. This observation, together with finding that the CCR2\textsuperscript{high}CX\textsubscript{3}CR1\textsuperscript{low}(Ly6C\textsuperscript{high}) subset appears before CCR2\textsuperscript{low}CX\textsubscript{3}CR1\textsuperscript{high}(Ly6C\textsuperscript{low}) cells during repopulation of blood MOs after depletion by clodronate, has led to the suggestion that CCR2\textsuperscript{high}CX\textsubscript{3}CR1\textsuperscript{low} murine MOs may be precursors of CCR2\textsuperscript{low}CX\textsubscript{3}CR1\textsuperscript{high} MOs (7). Additionally, human CCR2\textsuperscript{high}CX\textsubscript{3}CR1\textsuperscript{low}(CD16\textsuperscript{+}) MOs come to resemble CD16\textsuperscript{−} blood MOs after in vitro culture with TGF\textbeta\textsubscript{1} (18). To directly address this point, we transferred sorted subsets of MOs and followed their differentiation in blood. Our results show for the first time that in vivo under SS conditions CD43\textsuperscript{low} MOs from blood or BM mature into CD43\textsuperscript{high} MOs without cell division. This differentiation is not just restricted to CD43 as expression of CD4, CD62L, CX\textsubscript{3}CR1, CCR7, as well as CCR2, albeit to a lesser degree, are also modulated on the transferred CD43\textsuperscript{low} MOs. Importantly, the donor CD43\textsuperscript{high} MOs recovered in rats receiving CD43\textsuperscript{low} MOs cannot be preferentially surviving CD43\textsuperscript{high} contaminants for two reasons. First, the numbers of transferred and recovered MO subsets are incompatible with this. Forty hours after transfer of sorted donor CD43\textsuperscript{high} or low MOs, 1–2% or 0.5–1%, respectively, of the transferred cells were recovered by perfusion in three independent experiments. In these experiments, the contamination of sorted donor

**FIGURE 3.** CD43\textsuperscript{low} MOs from blood and BM acquire the phenotype of CD43\textsuperscript{high} MOs in blood in the SS. Blood or BM MOs from RT7\textsuperscript{b} rats were FACS-sorted into (A) CD43\textsuperscript{high} blood MOs, (B) CD43\textsuperscript{low} blood MOs, (C) CD43\textsuperscript{low}CD11b\textsuperscript{+} BM MOs. A total of 6.5 × 10\textsuperscript{5} MOs of each subset were then injected i.v. into naive RT1\textsuperscript{c} rats. Upper rows, Surface phenotype of the sorted subsets at the time of transfer. Lower rows, The surface phenotype of all blood MOs recovered by perfusion 40 h after transfer. Numbers show the percentage of congenic MOs expressing the surface receptors. The results are representative of four independent experiments.
CD43low MØs with CD43high MØs was 0.5–2%. Given a similar recovery of contaminating CD43high MØs, these cells cannot account for >8% of the recovered CD43high MØs. Second, the increase in CD43 expression is gradual—18 h after transfer of CD43low MØs, the recovered MØs are CD43int but at 60 h they are CD43high in the same rats.

MØs enter tissues and differentiate into macrophages and possibly DCs but the replenishment of these cells is greatly influenced by inflammation or injury. The differentiation of human MØs into migratory DCs under different levels of inflammatory conditions has been extensively studied in vitro in a system of transendothelial migration (18, 19) but only recently have attempts been made to address this question in vivo in mice (9–11). In this study, we have shown that CCR2highCX3CR1low (CD43low) MØs are selectively recruited to the peritoneum during induced sterile peritonitis. Very few, if any, transferred CCR2lowCX3CR1high (CD43high) MØs were recruited to the site of inflammation. Some CD43low MØs also gave rise to CD11b/CD11cMHC-IIint cells in the draining PLNs. A similar subset-specific appearance of CD11c+ cells derived from transferred CCR2highCX3CR1low MØs has been reported previously (3). This suggests that these MØs express CCR7. However, these cells could also have entered the LN from blood via high endothelial venules as in both species the MØs express CD62L. In addition, murine CCR2highCX3CR1low MØs are recruited from the blood to inflamed LNs via high endothelial venules in a CCL2-dependent manner (5).

MØs have been shown to give rise to cells with DC characteristics in the spleen (3, 20). In one of these studies, it was shown that within 2 days after transfer into naïve mice, both subsets of blood MØs could enter the spleen, where some CCR2lowCX3CR1high(Ly6Clow) MØs up-regulated CD11c and MHC-II (3). In the skin, some murine CCR2highCX3CR1low(Ly6Chigh) MØs, recruited by intracutaneous bead injection, become bead-containing CD11c+MHC-IIhigh cells, strongly suggesting their differentiation into migratory lymph DCs in vivo (10, 11). These DCs were suggested to preferentially arise from Ly6C+, CCR7/8+ MØs (9). These studies could not however determine the fate of CCR2lowCX3CR1high MØs in vivo after entering nonlymphoid tissues as such cells were not recruited. To determine directly whether MØs, and in particular the CCR2lowCX3CR1high MØs, can enter tissues and differentiate into migratory DCs under SS conditions, we transferred labeled congenic subsets of MØs into cannulated MLNX rats. Using this model, we show for the first time that CCR2lowCX3CR1high(CD43high) MØs can differentiate into a small number of DCs that migrate from the gut toward the MLN in the absence of any inflammatory stimuli and that this occurs without cell division. We cannot however exclude the possibility that an unidentified subset of CD43high MØs gives rise to lymph DCs. iL-DCs that derive from CCR2highCX3CR1low(CD43low) MØs could also be identified, some of which had gone through several rounds of cell division. We do not know whether these transferred CD43low MØs had entered the intestine and then differentiated into intestinal DCs or if they had first differentiated into CD43high cells, which we could detect in blood, before entering the tissue. DCs that originate from CD43high BM MØs that had divided probably represent the CD11b− subset as in blood these cells divide and up-regulate CD11b. This suggests that

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**FIGURE 4.** MØs differentiate into iL-DCs in the SS. A, RT7bCD43high blood or CD43low BM MØs were enriched and analyzed for the expression of surface markers. B, The MØ subsets were then CFSE-labeled and 45 × 10⁶ of each subset was injected i.v. into two RT1+ MLNX rats/group. Sixty-five hours later, the MLNX rats were cannulated and lymphocyte-depleted TDLs were analyzed by FACS individually. iL-DC, -MØs, and -PMNs were gated out as indicated by the gates and arrows and analyzed for CFSE content. The frequency of donor MØ-derived cells in each gated population is indicated. The control rat received no MØs. The results are representative of three independent experiments.
CD11b−CD43high cells are at an earlier state of maturation than the CD11b+CD43low Mφs.

After transfer of approximately the number of CD43high Mφs obtained by perfusing one rat into a MLNX rat, the frequency of Mφ-derived DCs was 0.02–0.04%. However, 18 h after transfer, the frequency of donor cells among total recipient blood Mφs was 3–4% and at the time of cannulation they represented 2%. Several explanations for the low frequency of donor-derived DCs need to be considered. The low frequency may relate to the effects of in vitro handling on Mφs as when this was minimized higher frequencies of both donor-derived Mφs present in blood at the time of cannulation and Mφ-derived iL-DCs were detected. Additionally, it may reflect the normal proportion of CD43high Mφs that gives rise to DCs rather than macrophages. Moreover, we are sampling the number of Mφ-derived DC exiting only the gut and for a limited amount of time. Most likely, Mφs also enter a number of other tissues. As we in this study wanted to address DC differentiation under SS conditions, the rats were not irradiated. The transferred DC precursors will therefore have to compete with host precursors, which will presumably occupy most of the available niches and that have not been handled in vitro. Finally, it is possible that a large proportion SS iL-DCs do not derive from Mφs but from an as yet unidentified blood-borne precursor. What this study does however demonstrate is that iL-DCs can derive from Mφs under SS.

After transfer of both subsets of Mφs, we could detect cells with a M1, phenotype in lymph. These cells were not iL-DCs or PMNs as they were CD103+MHC-II− and SSClow. This is an interesting population of Mφs as their existence in pseudopotent lymph implies that at least some Mφs migrate via afferent lymph under SS conditions. Importantly, we did not detect any donor-derived PMNs in lymph and the Mφs we identified were not contaminating cells from blood as they were found in lymph free of RBC. In addition, these lymph-Mφs were in contrast to blood Mφs all CD43high (U. Yrlid and G. G. MacPherson, unpublished observation). We are currently further investigating the migration pattern of lymph Mφs.

Heterogeneity among afferent lymph DCs has been described in a number of species (17, 21–23) but whether they have common origins is not clear. We have previously shown that rat iL-DCs can be divided into individual rats then received 50 μg of LPS or 25 μg of R-848 i.v. and lymph was collected for an additional 18 h, at which time point blood samples were taken from the individual rats. B, FACS analysis of the frequency of CFSE−Mφs among total CD172a+ mononuclear cells in blood samples taken at the time of cannulation (Pre) and at the end of the cannulation 18 h after LPS or R-848 injection. The histogram in the top left corner shows the expression of CD103 by collected 7AAD−TDLs (after partial depletion of B and T cells). Indicated is also the gate used to allow for analysis of host (CFSE−) and donor Mφ-derived (CFSE+) iL-DCs shown in the large density plot. The right two histograms shows the expression of CD172a by the CFSE− and −iL-DCs gated out as indicated by the gates and arrows (note that the CFSE labeling of Mφs already in blood in this experiment was lower but homogeneous and does not reflect any cell divisions). D, A total of 10×10^6 CD43low sorted congenic blood Mφs were injected i.v. into RT1+ rats. Forty-two hours later, recipients were bled (Pre R-848), then given 25 μg of R-848 i.v. and 1 h later perfused (Post R-848). CD172a+ mononuclear cells were gated out and CD43 expression by either (all Mφs) or (transferred Mφs) was assessed by FACS. E, RT7bCD43high blood Mφs (70×10^6) and RT7bCD11b+CD43low BM Mφs (18×10^6) were enriched, CFSE-labeled and injected i.v. into individual RT1+MLN rats. The MLN rat were then given R-848 i.v., cannulated as in C, and CD103+MHC-II−TDLs were analyzed by FACS. Plots represent individual rats when not stated otherwise and control rats received no Mφs. Numbers show the percentage of cells in each gate or quadrant. The results are representative of three independent experiments.
into two phenotypically and functionally distinct subsets distinguished by the expression levels of CD172a (17, 24). CD172a<sup>low</sup> iL-DCs selectively carry intestinal apoptotic intestinal material to the T cell areas of the MLN while CD172a<sup>high</sup> DCs are largely excluded from this part of the MLN (24). This subset-specific function and localization in lymphoid tissue is shared with murine DC subsets from lymphoid tissues where CD8α<sup>+</sup>, but not CD8α<sup>-</sup> DCs engulf apoptotic material and are primarily detected in T cell areas of spleen and LN (25). In this study, we show that both the major subsets of iL-DCs can be derived from M<sub>S</sub> in the absence of cell division and that the frequencies of the two subsets are very similar to those of total iL-DCs. This suggests that there is no preference for M<sub>S</sub> to differentiate into either of the subsets under SS conditions. To be able to analyze iL-DC subset frequencies more accurately, we administered TLR4 or TLR7/8 ligands which stimulate virtually total emptying of DC from the lamina propria of the gut. This procedure had only a minor effect on the overall frequency of M<sub>S</sub>-derived iL-DCs, we recovered but the significant increase in iL-DCs recovered enabled us to confidently confirm that subset frequencies among donor- and host-derived iL-DCs were essentially identical. Even though the M<sub>S</sub> transferred were not enriched for either subset, it is very unlikely that CD43<sup>low</sup> M<sub>S</sub> would contribute significantly to either subset of iL-DCs, as when equivalent numbers of purified CD11b<sup>-</sup>CD43<sup>low</sup> BM M<sub>S</sub> were transferred no donor-derived iL-DCs could be detected. This also suggests that those iL-DCs detected after transfer of CD11b<sup>-</sup>/CD43<sup>-</sup> BM M<sub>S</sub> most likely derive from the dividing, CD11b<sup>-</sup> subset of CD43<sup>low</sup> M<sub>S</sub> which could contain cells equivalent to the novel CD11b<sup>-</sup> progenitor of mononuclear phagocytes described in mice (26). CCR2<sup>high</sup>CX<sub>C</sub>-CR1<sup>low</sup>(Ly6C<sup>high</sup>) M<sub>S</sub> enriched from BM have also been shown to give rise to both the major subsets of murine splenic DCs (20). This study however used irradiated recipients and CD8α<sup>-</sup> and CD8α<sup>+</sup> donor-derived CD11c<sup>-</sup>MHC-II<sup>-</sup> splenic DCs were recovered 2 wk after transfer, whereas in our study blood M<sub>S</sub> were transferred to naive rats and M<sub>S</sub>-derived DCs were analyzed after 3–4 days.

In conclusion, we show that CD43 expression divides rat M<sub>S</sub> into two distinct subsets. CD43<sup>high</sup> M<sub>S</sub>, the major blood population, do not express CCR2, CCR7, or CD62L but express CX<sub>C</sub>-CR1 and CD11c. In contrast to CD43<sup>low</sup> M<sub>S</sub>, CD43<sup>high</sup> M<sub>S</sub> do not migrate to inflamed peritoneum. Furthermore, we demonstrate unambiguously that in blood, in the absence of any phagocytic or inflammatory stimuli, CD43<sup>high</sup> M<sub>S</sub> mature into CD43<sup>high</sup> M<sub>S</sub> without division. Finally, we show that a small number of blood M<sub>S</sub> can enter the intestine and differentiate into the two main subsets of iL-DCs. This study therefore provides evidence for one of the possible origins of CCR2<sup>high</sup>CX<sub>C</sub>-CR1<sup>high</sup> blood M<sub>S</sub> and demonstrates their ability to differentiate into migratory intestinal DCs in vivo in the absence of inflammatory stimuli.

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Disclosures

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