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*J Immunol* 2006; 176:4155-4162; doi: 10.4049/jimmunol.176.7.4155
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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

The origins of dendritic cells (DCs) are poorly understood. In inflammation, DCs can arise from blood monocytes (M<sub>0</sub>s), but their steady-state origin may differ, as shown for Langerhans cells. Two main subsets of M<sub>0</sub>s, 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<sup>high</sup>CX3CR1<sup>low</sup> M<sub>0</sub>s but have not defined unambiguously the origin and fate of CCR2<sup>low</sup>CX3CR1<sup>high</sup> cells. In this study, we show that rat M<sub>0</sub>s can also be divided into CCR2<sup>high</sup>CX3CR1<sup>low</sup> (<i>CD43</i>) and CCR2<sup>low</sup>CX3CR1<sup>high</sup> (<i>CD43</i>) subsets with distinct migratory properties in vivo. Using whole body perfusion to obtain M<sub>0</sub>s, including the marginating pool, we show by adoptive transfer that CD43<sup>low</sup> M<sub>0</sub>s can differentiate into CD43<sup>high</sup> M<sub>0</sub>s in blood without cell division. By adoptive transfer of blood M<sub>0</sub>s followed by collection of pseudoafferent lymph, we show for the first time that a small proportion of intestinal lymph DCs are derived from CCR2<sup>low</sup>CX3CR1<sup>high</sup> blood M<sub>0</sub>s and indicate that they may contribute to migratory intestinal DCs in vivo in the absence of inflammatory stimuli.


Phenotypic heterogeneity among blood monocytes is known in humans, mice, swine, and rats (1–4). In mice and humans, two major, functionally distinct subsets can be distinguished by expression of chemokine receptors and other markers (3, 4). One “inflammatory” subset, CCR2<sup>high</sup>CX3CR1<sup>int/low</sup>, enters inflamed tissues in response to MCP-1 (5) while the other, CCR2<sup>low</sup>CX3CR1<sup>high</sup>, migrates toward fractalkine (CX3CL1) rather than MCP-1 (6). Murine CCR2<sup>low</sup>CX3CR1<sup>high</sup> M<sub>0</sub>s selectively express high levels of CD43 but low levels of Ly6C (7). In rats, M<sub>0</sub>s subsets have been defined by CD43 expression (1) but the functions of these subsets and their relationship to their mouse and human counterparts are unknown. Moreover, the developmental relationships between M<sub>0</sub>s subsets have not been fully defined in any species.

Dendritic cells (DCs) migrate constitutively from peripheral tissues to draining LNs (DLNs) in lymph and thus need continual replenishment. During inflammation, numbers of tissue DCs increase markedly, and at least some are derived from blood M<sub>0</sub>s. The origins of steady-state (SS) DCs are much less clear; murine Langerhans cells arise from self-replenishing peripheral pool in SS but from blood precursors during inflammation (8). It has been shown that after intracutaneous injection of latex beads CCR2<sup>high</sup> M<sub>0</sub>s are recruited to murine skin (9). Most bead-containing cells remain locally and become macrophages but in addition MHC-ll<sup>high</sup> latex<sup>+</sup> cells with DC characteristics can be recovered from the DLN (9–11). Beads are carried preferentially to the DLN by beads with the small proportion of recruited CCR2<sup>high</sup>CX3CR1<sup>low</sup> M<sub>0</sub>s that enter lungs after i.v. transfer (3). Importantly, the fate of CCR2<sup>low</sup>CX3CR1<sup>high</sup> blood M<sub>0</sub>s in tissues remains uncertain.

In this study, we examine the origins and fates of M<sub>0</sub>s using approaches available in rats but not in mice or humans. To maximize blood M<sub>0</sub>s recovery, including the sizeable marginating pool (not recovered by bleeding), we used vascular perfusion (12). To trace the fate M<sub>0</sub>s in blood and inflamed tissues, CFSE-labeled, congenic blood M<sub>0</sub>s subsets were transferred into normal rats and rats with peritonitis. To examine migrating DCs, mesenteric LNs from young rats were removed and the afferent and efferent lymphatics were allowed to anastomose. Subsequent thoracic duct cannulation of these mesenteric-lymphadenectomized (MLNX) rats permits collection of intestinal lymph DCs (iL-DC) that have just left the gut wall (13, 14). To investigate the relationship between M<sub>0</sub>s and migrating DCs, we adoptively transferred labeled congenic M<sub>0</sub>s into cannulated MLNX rats and analyzed the frequency and phenotype of donor-derived il-DCs. The MLNX rats were not irradiated before the transfer to allow for examination of the M<sub>0</sub>s contribution to il-DCs under SS conditions.

Using this unique system, we show that CCR2<sup>low</sup>CX3CR1<sup>int/low</sup> and CCR2<sup>high</sup>CX3CR1<sup>high</sup> rat M<sub>0</sub>s correspond to the CCR2<sup>high</sup>CX3CR1<sup>low</sup> and CCR2<sup>low</sup>CX3CR1<sup>high</sup> functionally distinct subsets identified in mice and humans, and importantly that CD43<sup>low</sup> M<sub>0</sub>s are able to differentiate into CD43<sup>high</sup> M<sub>0</sub>s in the blood stream without cell division. Finally, we show in vivo that CCR2<sup>low</sup>CX3CR1<sup>high</sup> blood M<sub>0</sub>s can differentiate into a small proportion of iL-DCs in the absence of added phagocytic or inflammatory stimuli. These results define one origin and differentiation pathway of the CCR2<sup>low</sup>CX3CR1<sup>high</sup> subset of blood M<sub>0</sub>s in vivo under SS conditions.

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

Received for publication December 9, 2005. Accepted for publication January 24, 2006.

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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

2 Abbreviations used in this paper: M<sub>0</sub>: 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.

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0022-1767/06/$02.00
Materials and Methods

Rats and surgical procedures

PVG RT1<sup>a</sup> and congenic RT1<sup>R</sup>R<sup>T</sup> (RT<sup>T</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% thioglycolate, followed by goat anti-mouse Dynabeads (Dynal). This gave 96–98% pure CD11b<sup>-</sup> cells. CD32, -CD200R, and -CD62L resulting in 4156 MONOCYTE SUBSETS AND iL-DCs purified from cell culture supernatants were used for depletions or conjugated to biotin, FITC, or Alexa-647. Anti-CD32 (D3-485), later biotinylated, anti-PMN (RP-1-PE), anti-MHC-II (OX6-PerCP), and streptavidin-PE and -allophycocyanin were all purchased from BD Pharmingen. Anti-CD11c (8A2), later biotinylated and anti-CD86 (24F-PE) was from Cedarlane Laboratories. Polyclonal rabbit anti-rat CX3CR1 was purchased from AMS Biotechnology, goat anti-rat CCR2 (sc-6226) and CCR7 (A-19) from Santa Cruz Biotechnology, and all three were later biotinylated in house.

Isolation of cells

**Blood M<sub>B</sub>.** The vascular perfusate was collected and spun on a Percoll gradient (Amersham Bioscience) as described (12). Mononuclear cells were depleted of B, T, and NK cells using anti-CD6, -CD8, and -CD45RA Abs, followed by goat anti-mouse Dynabeads (Dynal). This gave 96–98% pure CD172a<sup>-</sup>CD103<sup>-high</sup> M<sub>B</sub>. In adoptive transfer experiments followed by cannulation, enriched M<sub>B</sub> were depleted of CD34<sup>+</sup> M<sub>B</sub> using anti-CD32, -CD200R, and -CD62L resulting in >93% pure CD43<sup>low</sup> M<sub>B</sub> (contaminating cells were exclusively CD43<sup>high</sup> M<sub>B</sub>).

**Bone marrow (BM) M<sub>B</sub>.** BM cells were flushed from femurs and tibias and RBC lysed with ACK lysis buffer. The cells were depleted of lymphocytes and precursors using anti-CD5, -CD6, -CD8, and -CD45RA, and -CD90. In adoptive transfer experiments followed by cannulation, CD43<sup>high</sup> M<sub>B</sub> and polymorphonuclear leukocytes (PMNs) were depleted using anti-CD4 and -CD45 giving >93% pure CD172a<sup>-</sup>CD103<sup>-low</sup> M<sub>B</sub>. In cannulation experiments, involving R-848 injection the CD43<sup>low</sup> M<sub>B</sub> were further purified using anti-CD11b-biotin and anti-biotin MACs beads (Miltenyi Biotec) generating >80% pure CD43<sup>low</sup>CD11b<sup>+</sup> M<sub>B</sub> (contaminating cells were exclusively PMNs).

In experiments where blood and BM M<sub>B</sub> subset recruitment to inflamed tissues and subset differentiation in blood was studied, enriched M<sub>B</sub> were sorted into CD43<sup>high</sup>, CD43<sup>low</sup>, CD43<sup>low</sup>CD11b<sup>-</sup>, or - subsets using a MoFlo (Cytomation). The purity was then >98%.

Thoracic duct leukocytes (TDLs) were collected on ice and depleted of lymphocytes as above. Parathymic LN (PLNs) were washed 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>B</sub> were labeled with 5 μM CFSE (Molecular Probes) by incubating them without serum for 10 min at 37°C at 5 × 10<sup>5</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>B</sub> comprise two subsets with distinct migratory properties

Rat blood M<sub>B</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>B</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>CS</sub> (7), rat M<sub>B</sub> comprise two subsets differing in CD43 expression (1, 15).

To obtain all M<sub>CS</sub>, including the marginating pool, rats were perfused with 1 L of buffer. M<sub>B</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>B</sub> and additionally identified M<sub>0</sub> populations in BM (Fig. 1A). The main M<sub>B</sub> subsets defined by CD43 expression were then further characterized (Fig. 1A). In comparison with CD43<sup>high</sup> M<sub>B</sub>, CD43<sup>low</sup> M<sub>B</sub> are larger, express more CD32, CD200R, and CD62L but less CD4 and CD11c. Moreover, CD43<sup>high</sup> M<sub>B</sub> express CX<sub>C</sub>CR1 but are CCR2<sup>-high</sup> while CD43<sup>low</sup> M<sub>B</sub> are CX<sub>C</sub>CR1<sup>-low</sup> but CCR2<sup>-low</sup>. The ratio of CD43<sup>low</sup> M<sub>B</sub> in blood is 1:8 but in BM is ~8:1. The only consistent phenotypic difference between M<sub>B</sub> isolated from blood and BM is that some CD43<sup>low</sup> BM M<sub>B</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>B</sub> were sorted by FACS. Given the low CD11b levels on some CD43<sup>low</sup> BM M<sub>B</sub>, these were subsetted into CD43<sup>low</sup>CD11b<sup>-</sup> and CD43<sup>low</sup>CD11b<sup>+</sup> subsets. Sorted M<sub>B</sub> from RT1<sup+a</sup> rats were given i.v. to RT1<sup+a</sup> rats with peritoneitis and peritoneal cells examined 21 h later. Strikingly, no CD43<sup>high</sup> M<sub>B</sub> were detected in the peritoneum but significant and similar numbers of CD43<sup>low</sup> blood M<sub>B</sub> and CD43<sup>low</sup>CD11b<sup>-</sup> and – BM M<sub>B</sub> were present (Fig. 1B). Even after giving five times as many CD43<sup>high</sup> blood M<sub>B</sub>, very few were recovered from the cavity (Fig. 1C). In addition, only CD43<sup>low</sup> BM M<sub>B</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>B</sub>; CCR<sub>2</sub><sup>high</sup>CX<sub>C</sub>CR1<sup>low</sup>CD43<sup>high</sup> M<sub>B</sub> are recruited to inflamed tissues while CCR<sub>2</sub><sup>low</sup>CX<sub>C</sub>CR1<sup>high</sup>CD43<sup>low</sup> M<sub>B</sub> are not.

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

The developmental relationships between subsets of M<sub>B</sub> are uncertain, particularly in the SS. To examine M<sub>B</sub> differentiation in vivo, CFSE-labeled M<sub>B</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>B</sub> had up-regulated CD43 to intermediate levels whereas the majority of transferred CD43<sup>low</sup> BM M<sub>B</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>B</sub> were analyzed for CD43 and CFSE. All donor CD43<sup>high</sup> and low blood M<sub>B</sub> and CD43<sup>low</sup>CD11b<sup>+</sup> BM M<sub>B</sub> were now CD43<sup>high</sup> (Fig. 2C). Importantly, their CFSE content showed that none of these M<sub>B</sub> had divided. In contrast, some donor CD43<sup>low</sup>CD11b<sup>-</sup> BM M<sub>B</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>B</sub> were sorted by CD43 expression and BM M<sub>B</sub> were sorted into CD11b<sup>-</sup>CD43<sup>high</sup> to avoid the dividing cell population. Sorted congenic M<sub>B</sub> subsets were transferred i.v. into naive recipients. After 40 h, recipients were perfused and donor M<sub>B</sub> numbers and phenotypes were assessed. CD43<sup>high</sup> M<sub>B</sub> retained their original phenotype (Fig. 3A), whereas all donor CD43<sup>low</sup> M<sub>B</sub>, independent of origin (blood or BM), had become CD43<sup>high</sup> expressed uniform high levels of CD4, and had up-regulated CX<sub>C</sub>CR1 (Fig. 3, B and C). In addition, the adoptively transferred CD43<sup>low</sup> M<sub>B</sub> had down-regulated CD62L, CCR7, and to a lesser extent CCR2.
These experiments show that after adoptive transfer under SS conditions, CD43low MOs gradually acquire the phenotype of CD43high MOs without cell division.

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

The fate of murine CCR2highCX3CR1low MOs in tissues has been examined recently (3, 9, 11) but the fate of CCR2 lowCX3CR1high in tissues is less understood. Immature CX3CR1+ 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 MOs can become lymph-borne migratory intestinal DC, we transferred CFSE-labeled CD43high blood MOs or CD43 low BM MOs, into thoracic duct-cannulated MLNX rats (Fig. 4A). Eighteen hours after transfer of 4.5 × 10⁶ MOs, donor MOs 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 MOs (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 MOs (Fig. 4B, top row of panels).

Donor-derived cells in lymph with a MO phenotype (SSClowCD103low MHC-IIlow) were also detected and as noted with the monocyte-derived iL-DCs, only the lymph MOs originating from the CD43low BM MOs had divided (Fig. 4B, middle row of plots). No transferred CD43high blood MOs and very few, if any, CD43low BM MOs developed into PMNs in lymph (Fig. 4B, bottom row of plots).

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

Blood MOs can differentiate into both CD172ahigh and CD172alow 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 MOs. As numbers of recovered iL-DCs were limiting for analysis, to maximize cell recovery and to minimize changes induced by in vitro handling, MOs were enriched by a single depletion from blood (Fig. 5A). CFSE-labeled blood MOs were then transferred i.v. into MLNX rats. After 3 days, 4.2% of the recipient blood MOs were CFSE+ (Fig. 5B). At this time, the rats were cannulated and iL-DCs were analyzed by FACS. Since we have previously shown that all large CD103high cells in lymph are also MHC class IIhigh (Ref. 14 and Fig. 4B), MHC class II staining was replaced with 7AAD to ensure that only viable CD103 high cells were analyzed. A distinct population of MO-derived iL-DCs constituting 0.12% of total CD103high blood MOs was detected (Fig. 5C, top density plot). These MOs-derived iL-DCs were then transferred i.v. into MLNX rats. After 3 days, 4.2% of the recipient blood MOs were CFSE+ (Fig. 5B). At this time, the rats were cannulated and iL-DCs were analyzed by FACS. Since we have previously shown that all large CD103high cells in lymph are also MHC class IIhigh (Ref. 14 and Fig. 4B), MHC class II staining was replaced with 7AAD to ensure that only viable CD103high cells were analyzed. A distinct population of MO-derived iL-DCs constituting 0.12% of total CD103high blood MOs was detected (Fig. 5C, top density plot). When these MOs-derived iL-DCs were further analyzed, both CD172a high and CD172a low CFSE+ cells were detected (Fig. 5C, histograms).

To obtain enough iL-DCs to permit comparison of subset frequencies between donor and host-derived DCs, we took advantage of the availability of congenic rats. We transferred CFSE-labeled blood MOs or BM MOs into normal R1c rats. Eighteen hours after transfer, the rats were cannulated and iL-DCs were analyzed by FACS. 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 × 10^6 CD43 low/CD11b+ MOs when total blood MOs were transferred (Fig. 5C). We repeated the experiment transferring 18 × 10^6 CD43 low/CD11b+ BM MOs and 70 × 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}CR\textsubscript{1} and CD11c but low levels of CD62L and CCR2 whereas in contrast the CD43\textsuperscript{low} subset expresses low levels of CX\textsubscript{3}CR\textsubscript{1} 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}CR\textsubscript{1}\textsuperscript{high} and “inflammatory” CCR2\textsuperscript{high}CX\textsubscript{3}CR\textsubscript{1}\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}CR\textsubscript{1}\textsuperscript{high}(Ly6\textsubscript{C}\textsuperscript{low}) MOs compared with the Ly6\textsubscript{C}\textsuperscript{high} subset (7). Moreover, we show that CD32 is expressed at higher levels by the CCR2\textsuperscript{high}CX\textsubscript{3}CR\textsubscript{1}\textsuperscript{low} (CD43\textsuperscript{low}) which correlates with human peripheral blood MOs where CCR2\textsuperscript{low}CX\textsubscript{3}CR\textsubscript{1}\textsuperscript{low}(CD16\textsuperscript{low}) MOs have been shown to express high levels of this FcR (4). In rats, the proportion of CCR2\textsuperscript{low}CX\textsubscript{3}CR\textsubscript{1}\textsuperscript{high}(CD43\textsuperscript{high}) MOs 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}CR\textsubscript{1}\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}CR\textsubscript{1}\textsuperscript{low}(CD43\textsuperscript{low}) subset. This observation, together with finding that the CCR2\textsuperscript{high}CX\textsubscript{3}CR\textsubscript{1}\textsuperscript{low}(Ly6\textsubscript{C}\textsuperscript{high}) subset appears before CCR2\textsuperscript{low}CX\textsubscript{3}CR\textsubscript{1}\textsuperscript{high}(Ly6\textsubscript{C}\textsuperscript{low}) cells during repopulation of blood MOs after depletion by clodronate, has led to the suggestion that CCR2\textsuperscript{high}CX\textsubscript{3}CR\textsubscript{1}\textsuperscript{low} murine MOs may be precursors of CCR2\textsuperscript{low}CX\textsubscript{3}CR\textsubscript{1}\textsuperscript{high} MOs (7). Additionally, human CCR2\textsuperscript{high}CX\textsubscript{3}CR\textsubscript{1}\textsuperscript{low}(CD16\textsuperscript{high}) MOs come to resemble CD16\textsuperscript{low} 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}CR\textsubscript{1}, 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...
CD43low M₀ with CD43high M₀ was 0.5–2%. Given a similar recovery of contaminating CD43high M₀, these cells cannot account for >8% of the recovered CD43high M₀. Second, the increase in CD43 expression is gradual–18 h after transfer of CD43low M₀, the recovered M₀ are CD43int but at 60 h they are CD43high in the same rats.

MOs 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 M₀s 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)M₀s are selectively recruited to the peritoneum during induced sterile peritonitis. Very few, if any, transferred CCR2lowCX3CR1high (CD43high)M₀s were recruited to the site of inflammation. Some CD43low M₀s also gave rise to CD11b/c−MHC-IIint cells in the draining PLNs. A similar subset-specific appearance of CD11c−MHC-II+ cells derived from transferred CCR2highCX3CR1low M₀s in the PLNs of mice with peritonitis has been reported previously (3). In both species, the transferred CCR2highCX3CR1low M₀s could have arrived via lymph as at least in rats this subset of M₀s express CCR7. However, these cells could also have entered the LN from blood via high endothelial venules as in both species the M₀s express CD62L. In addition, murine CCR2highCX3CR1low M₀s are recruited from the blood to inflamed LNs via high endothelial venules in a CCL2-dependent manner (5).

M₀s 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 naive mice, both subsets of blood M₀s could enter the spleen, where some CCR2lowCX3CR1−/8 (Ly6C−/8) M₀s up-regulated CD11c and MHC-II (3). In the skin, some murine CCR2highCX3CR1low (Ly6Chigh) M₀s, 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− M₀s (9). These studies could not however determine the fate of CCR2 lowCX3CR1high M₀s in vivo after entering nonlymphoid tissues as such cells were not recruited. To determine directly whether M₀s, and in particular the CCR2 lowCX3CR1low M₀s, can enter tissues and differentiate into migratory DCs under SS conditions, we transferred labeled congenic subsets of M₀s into cannulated MLNX rats. Using this model, we show for the first time that CCR2 lowCX3CR1high M₀s 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 M₀s gives rise to lymph DCs. iL-DCs that derive from CCR2lowCX3CR1high M₀s could also be identified, some of which had gone through several rounds of cell division. We do not know whether these transferred CCR2lowCX3CR1high M₀s 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 M₀s that had divided probably represent the CD11b− subset as in blood these cells divide and up-regulate CD11b. This suggests that

**FIGURE 4.** M₀s differentiate into iL-DCs in the SS. A, RT7CD43high blood or CD43low BM M₀s were enriched and analyzed for the expression of surface markers. B, The M₀s 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, -M₀s, and -PMNs were gated out as indicated by the gates and arrows and analyzed for CFSE content. The frequency of donor M₀-derived cells in each gated population is indicated. The control rat received no M₀s. The results are representative of three independent experiments.
CD11b<sup>−</sup>CD43<sup>low</sup> cells are at an earlier state of maturation than the CD11b<sup>+</sup>CD43<sup>low</sup> M<sub>0</sub>S.

After transfer of approximately the number of CD43<sup>high</sup> M<sub>0</sub>S obtained by perfusing one rat into a MLNX rat, the frequency of M<sub>0</sub>-derived DCs was 0.02–0.04%. However, 18 h after transfer, the frequency of donor cells among total recipient blood M<sub>0</sub>S 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 M<sub>0</sub>S as when this was minimized, higher frequencies of both donor-derived M<sub>0</sub>S present in blood at the time of cannulation and M<sub>0</sub>-derived iL-DCs were detected. Additionally, it may reflect the normal proportion of CD43<sup>high</sup> M<sub>0</sub>S that gives rise to DCs rather than macrophages. Moreover, we are sampling the number of M<sub>0</sub>-derived DC exiting only the gut and for a limited amount of time. Most likely, M<sub>0</sub>S 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 M<sub>0</sub>S but from an as yet unidentified blood-borne precursor. What this study does however demonstrate is that iL-DCs can derive from M<sub>0</sub>S under SS.

After transfer of both subsets of M<sub>0</sub>S, we could detect cells with a M<sub>1</sub> phenotype in lymph. These cells were not iL-DCs or PMNs as they were CD103<sup>+</sup> MHC-II<sup>−</sup> and SSC<sup>low</sup>. This is an interesting population of M<sub>0</sub>S as their existence in pseudoafferent lymph implies that at least some M<sub>0</sub>S migrate via afferent lymph under SS conditions. Importantly, we did not detect any donor-derived PMNs in lymph and the M<sub>0</sub>S we identified were not contaminating cells from blood as they were found in lymph free of RBC. In addition, these lymph- M<sub>0</sub>S were in contrast to blood M<sub>0</sub>S all CD43<sup>high</sup> (Y. Yrlid and G. G. MacPherson, unpublished observation). We are currently further investigating the migration pattern of lymph M<sub>0</sub>S.

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 individual rats then received 50 µg of LPS or 25 µg of R-848 i.v. and followed for an additional 18 h, at which time point blood samples were collected from the individual rats. B, FACS analysis of the frequency of CD103<sup>+</sup> M<sub>0</sub>S among total CD172a<sup>+</sup> 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 (Post LPS and Post R-848). C, FACS analysis of iL-DC subsets in intestinal lymph collected from SS rats (PBS) or 0–18 h after LPS or R-848 injection. The histogram in the top left corner shows the expression of CD103 by collected 7AAD<sup>−</sup> TDLs (after partial depletion of B and T cells). Indicated is also the gate used to identify the host (CFSE<sup>−</sup>) and donor M<sub>0</sub>-derived (CFSE<sup>+</sup>) iL-DCs shown in the large density plot. The two right histograms show the expression of CD172a by the CFSE<sup>−</sup> and CD172a<sup>+</sup> iL-DCs gated out as indicated by the gates and arrows (note that the CFSE labeling of M<sub>0</sub>S already in blood in this experiment was lower but homogeneous and does not reflect any cell divisions). D, A total of 10<sup>5</sup> CD43<sup>low</sup> sorted congenic blood M<sub>0</sub>S were injected i.v. into RT1<sup>+</sup> rats. Forty-two hours later, recipients were bled (Post R-848), then given 25 µg of R-848 i.v. and 1 h later perfused (Post R-848). CD172a<sup>+</sup> mononuclear cells were gated out and CD43 expression by either (all M<sub>0</sub>S) or (transferred M<sub>0</sub>S) was assessed by FACS. E, RT1<sup>−</sup>CD43<sup>high</sup> blood M<sub>0</sub>S (70 × 10<sup>5</sup>) and RT1<sup>−</sup>CD43<sup>low</sup> BM M<sub>0</sub>S (18 × 10<sup>5</sup>) were enriched, CFSE-labeled and injected i.v. into individual RT1<sup>+</sup> MLNX rats. The MLNX rats were then given R-848 i.v., cannulated as in C, and CD103<sup>+</sup> MHC-II<sup>−</sup> TDLs were analyzed by FACS. Plots represent individual rats when not stated otherwise and control rats received no M<sub>0</sub>S. 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 CD8<sup>α</sup>, but not CD8<sup>β</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 either of the subsets under SS conditions. To be able to analyze iL-DC infiltration and differentiation into the two main tissues where CD8<sup>α</sup> and CD8<sup>β</sup> donor-derived CD11c<sup>+</sup> splenic DCs (20). This study however used irradiated recipients and the pig.

Acknowledgments

We are grateful for excellent assistance with the cell sorting from Nigel Rust and helpful advice from Prof. B. Steiner. We also thank Drs. Philip Taylor and Gwendalyn J. Randolph for critically reviewing the manuscript.

Disclosures

The authors have no financial conflict of interest.

References