CCR9 is a homing receptor for plasmacytoid dendritic cells to the small intestine

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Edited by Dan R. Littman, New York University Medical Center, New York, NY, and approved February 23, 2007 (received for review October 17, 2006)

Small intestine plasmacytoid dendritic cells (pDC) are poorly characterized. Here, we demonstrate that intestinal pDC show the characteristic plasma cell-like morphology, and are recognized by antibodies against B220, Ly6c, 120G8, and PDCA-1, markers that are typically expressed by pDC. Furthermore, intestinal pDC carry high levels of CCR9 and are largely absent in the intestine, but not in lung, liver, or secondary lymphoid organs of CCR9-deficient animals. Competitive adoptive transfers reveal that CCR9-deficient pDC are impaired in homing to the small intestine after i.v. transfer. In a model of cholera toxin-induced gut inflammation, pDC are recruited to the intestine in WT but not CCR9-deficient animals. Furthermore, after oral application of a Toll-like receptor (TLR) 7/8 ligand, myeloid DC of the lamina propria are rapidly mobilized in WT but not in CCR9-deficient animals. Mobilization of myeloid DC can be completely rescued by adoptively transferred WT pDC to CCR9-deficient mice before oral challenge. Together, our data reveal an essential role for CCR9 in the homing of pDC to the intestine under homeostatic and inflammatory conditions and demonstrate an important role for intestinal pDC for the rapid mobilization of lamina propria DC.

chemokine receptor | gut | dendritic cell migration | Toll-like receptor 7 | cell mobilization

Among the different dendritic cell (DC) subsets described, a population of cells has been identified possessing a distinct morphology and secreting large amounts of type I IFN after viral infection (1) or triggering through Toll-like receptors (TLR) 7 or 9. This subpopulation has gained much attention recently, because it is believed that these cells link innate and adaptive immunity (2). Based on their morphology, some have termed these cells plasmacytoid DC (pDC; ref. 3), whereas others have referred to them as natural IFN-producing cells (IPC; ref. 4). In mice, pDC are $CD11c^{int}B220^{+}Ly6C^{+}$ (3) and, after activation, up-regulate MHC class II and costimulatory molecules (4). pDC are continuously produced in the bone marrow (BM), and fms-like tyrosine kinase3 ligand (Flt3L) has been identified as an important growth and differentiation factor for these cells (5, 6). Some have suggested that pDC, like naive B and T cells, may constitutively migrate from blood to noninflamed lymphoid organs via high endothelial venules (3, 7), whereas others have proposed that circulating pDC are preferentially recruited to inflamed lymph nodes (8, 9). In this model, L- and E-selectin mediate rolling of pDC on inflamed endothelium whereas firm attachment of pDC to the vessel wall is mediated by β1 and β2 integrins. pDC express both inflammatory and homeostatic chemokine receptors: CXCR3, CCR2, and CCR5, which all bind inflammatory chemokines, and CXCR4 and CCR7, which bind the constitutive chemokines CXCL12 and CCL19/CCL21, respectively. Although each of these chemokine receptors is capable of mediating chemotactic response of pDC *in vitro*, there is evidence that only CXCR3 (8–10) or CCR5 (7) is able to fulfill this task at the inflamed lymph node (LN) vessel *in vivo*. In contrast to the scenario described for inflamed LN there is currently virtually no information available regarding the role of chemokines in homing of pDC to nonlymphoid tissues such as mucosal tissues.

In the present study, we reveal a role for intestinal pDC in the rapid mobilization of lamina propria (LP) myeloid DC and show that the chemokine receptor CCR9 controls the migration of pDC to the small intestine under both steady-state and inflammatory conditions.

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Results

Characterization of Plasmacytoid Dendritic Cells of the Small Intestine. We applied standard procedures to isolate immune cells located in the epithelium (intraepithelial, IE) and the LP from the intestine. In both cell preparations we found a distinct population of $CD11c^{+}B220^{+}Ly6C^{+}$ cells that accounted for up to 1% of all cells. In contrast to pDC, myeloid (m)DC ($CD11c^{+}MHCII^{+}CD3^{-}B220^{-}Ly6C^{-}$) were present only at very low numbers in the IE preparation (Fig. 1*A*). Both pDC of the LP and IE preparation showed low levels of surface MHC class II expression (Fig. 1*A*). Further analysis revealed that $CD11c^{+}B220^{+}Ly6C^{+}$ cells of both preparations uniformly express the pDC markers PDCA1 as well as 120G8 (Fig. 1*B*). Cytospins from sorted pDC ($CD11c^{+}B220^{+}Ly6C^{+}$) and myeloid DC (mDC) of the IE preparation revealed a round and smooth, plasma cell-like morphology of pDC, whereas mDC showed the characteristic dendrites (Fig. 1*C*). To further characterize the localization of pDC within the intestine we applied anti-B220, anti-120G8, and anti-CD3 mAb in immunohistology. Micrographs were randomly taken from sections and, as depicted in Fig. 1*D*, the positioning of $120G8^{+}B220^{+}CD3^{-}$ cells was determined relative to epithelial cells by using image analysis (analySIS; Olympus, Hamburg, Germany). Evaluating the positioning of ≈150 pDC we observed that 4.9% of these cells were clearly located within the epithelial cell layer whereas another 6.7% were situated within a distance of 5–10 μm from the apical tip of the epithelial cells (Fig. 1*D*). These data demonstrate that a certain amount of the pDC locate within or close to the

Author contributions: O.P. and R.F. contributed equally to this work; O.P. and R.F. designed research; M.W. and N.C. performed research; N.M., B.M., and E.K. contributed new reagents/analytic tools; M.W. and N.C. analyzed data; and R.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: DC, dendritic cell; mDC, myeloid DC; pDC, plasmacytoid DC; TLR, Toll-like receptor; Flt3L, fms-like tyrosine kinase3 ligand; LN, lymph node; LP, lamina propria; IE, intraepithelial; PP, Peyer's patches; BM, bone marrow; CFSE, carboxyfluorescein diacetatesuccinimidyl ester; TAMRA, carboxytetramethylrhodamine; CT, cholera toxin.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0609180104/DC1.

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Fig. 1. Phenotype of plasmacytoid DC in the small intestine of mice. (*A*) Cells of the IE and LP preparation of the small intestine were stained with antibodies specific for CD3, CD11c, B220, MHCII, and Ly6C. $CD3^-$ cells were analyzed for the expression of B220 and Ly6C (*Left*). The expression of MHCII and CD11c is shown for $Ly6C^+$ $B220^+$ (blue box, *Center*) and $Ly6C^-$ cells (red box, *Right*). (*B*) Expression pDC-marker. pDC ($CD11c^+$, $B220^+$, and $Ly6C^+$) of the IE and LP preparation were stained for PDCA-1 or 120G8 (solid lines) or isotype controls (shaded area) as indicated. (*C*) Cytospins of flow sorted IE mDC and pDC were stained with H&E (pDC: $CD3^-CD11c^+B220^+Ly6C^+$; mDC: $CD3^-CD11c^+B220^-Ly6C^-$). (Scale bars: 10 μm.) Representative data from one of two experiments are shown. (*D*) Cryostat sections of the small intestinal villi were stained for nuclei (DAPI, white), B220 (blue), CD3 (green), and 120G8 (red). The yellow bar in the upper left micrograph indicates how the positioning of pDC ($120G8^+B220^+$) relative to the epithelial layer was determined. (*Top Right*) Distance distribution of 150 pDC analyzed relative to the epithelium. (*Middle* and *Bottom*) Examples of the positioning of individual pDC with distances as indicated. (Scale bars: 10 μm.) (*E*) Flow cytometric analysis of pDC of the IE and LP preparation. Cells were stained with antibodies as indicated (solid lines) or isotype controls (shaded area) and gated on pDC ($CD11c^+$, $B220^+$, and $Ly6C^+$). Shown are representative data from four independent experiments with cells pooled from two to six mice each.

intestinal epithelium, whereas >80% of the cells analyzed were positioned at distances >15 μm, which renders them as LP cells (Fig. 1*D*). Because similar numbers of pDC were present in the IE and LP preparation following standard isolation procedures, it is currently unclear whether pDC of the LP contaminate the IE preparation or whether pDC locating to the epithelium are more efficiently isolated. Because we hardly find any mDC within the IE preparation we favor the latter possibility. Yet it remains to be determined whether both populations serve different functions or belong to a common cell population. Because pDC of the IE preparation, in contrast to pDC of the LP preparation, can be isolated by rather gentle procedures, exclusively pDC of the IE preparation were used for functional assays in this study.

CCR9 Expression on Intestinal pDC. To identify the molecular mechanisms that allow the migration of pDC to the intestine, we analyzed the expression of homing molecules. Although it is well established that the integrin α_E (CD103) mediates lymphocyte adhesion to epithelial cells in the intestine, we failed to identify expression of this integrin on intestinal pDC. Similarly, CCR7 (Fig. 1*E*), essentially involved in homing of lymphocytes into peripheral lymphoid organs is not expressed by pDC of the intestine. In contrast, we observed high levels of CD18 (β2-integrin) and intermediate levels of $\alpha 4\beta 7$ whereas $\approx$50% of pDC express P-selectin ligands (Fig. 1*E*). Of interest, the vast majority of intestinal pDC expressed high amounts of the chemokine receptor CCR9 (Fig. 1*E*), whereas mDC of the LP expressed no, or low levels of CCR9 [supporting information (SI) Fig. 6*A*].

CCR9 Expression of pDC Isolated from Different Lymphoid Organs. Considering the uniform expression of CCR9 on intestinal pDC, we analyzed expression of CCR9 on pDC isolated from second-

Fig. 2. Expression of CCR9 on pDC. (*A*) Cells were isolated from different lymphoid organs as indicated. pDC were addressed as CD11c$^+$B220$^+$Ly6C$^+$ and analyzed for the expression of CCR9 (solid line). (*B*) CD4$^+$CD8$^+$ thymocytes served as a positive control. (*C*) Expression of chemokine receptors (solid lines) on pDC isolated from the BM (shaded area: isotype control). SPL, spleen; ALN, axillary LN; MLN, mesenteric LN; PP, Peyer's patches; Thy, thymus). Representative data from four independent experiments with cells pooled from two to six mice each (*A* and *B*) or from three mice (*C*).

ary lymphoid organs including spleen, skin-draining LN, mesenteric LN, and Peyer's patches (PP; Fig. 2*A*) and used CD4$^+$CD8$^+$ thymocytes, known to express high levels of CCR9, as a positive control (11); Fig. 2*B*). Interestingly, only a fraction of pDC present in any of these organs expressed CCR9 (Fig. 2*A*), whereas ≈95% of pDC isolated from the BM carried this receptor (Fig. 2*C*). Most BM pDC also express CCR5 and CXCR3 whereas CCR2 is present on only roughly 25% of the cells. CXCR4, known to retain cells to the BM, is only very weakly expressed (Fig. 2*C*). Together, these data demonstrate that pDC are equipped with various chemokine receptors before being released from the BM. This feature presumably allows homing not only to places of inflammation but also to the noninflamed intestine.

Chemotaxis Analysis of Flt3L-Expanded pDC. Based on the strong expression of CCR9 on BM and intestinal pDC, we compared the migration capacity of pDC and mDC toward the chemokine CCL25/TECK, which is the sole known ligand for this receptor (12). The frequency of pDC varies between different mouse strains and it is well known that, for example, C57BL/6 (B6) mice harbor much less pDC than 129SV mice (13). However, irrespective of the genetic background the number of pDC that can be isolated from mouse tissues is insufficient to conduct standard *in vitro* chemotaxis assays. To overcome this limitation we expanded the DC population *in vivo* by implanting a Flt3-L-secreting tumor cell line (B16-FL) in B6 mice for 14 days (14).

During this time period, the percentage of CD11c$^+$ cells present in the spleen increased from 3% to 30–35% (data not shown). Eighty percent of the *in vivo* expanded pDC expressed CCR9, with levels very similar to those present on BM pDC (Figs. 2*C* and Fig. 4*C*) whereas *in vivo* expanded mDC expressed only small amounts of this receptor (SI Fig. 6*B*). Spleen pDC were enriched by CD11c$^+$ MACS-sorting, resulting in 95% purity for CD11c$^+$ cells that contained ≈15% Ly6C$^+$B220$^+$ pDC. *In vitro* transwell migration assays revealed strong chemotactic response of pDC to CCL25, as well as to CXCL9, a ligand for CXCR3 and to CXCL12, which is a ligand for CXCR4. Only a weak response was observed toward CCL19, which serves as a ligand for CCR7. mDC showed little chemotactic response toward CCL25 and CXCL9 and moderate response toward CXCL12 and CCL19 (Fig. 3*A*).

Reduced Numbers of pDC in the Small Intestine of CCR9-Deficient Mice. Based on these observations, we characterized the distribution of pDC in CCR9-deficient mice. We found similar percentages of pDC in lung and liver (SI Fig. 7) as well as in inguinal and mesenteric lymph nodes whereas the number of splenic pDC was slightly increased in CCR9$^{-/-}$ mice. In contrast, we observed a >90% decrease of intestinal and a 50% reduction of PP pDC (Fig. 3*B*).

pDC Preferentially Migrate to the Small Intestine. Based on the findings described so far, it seemed likely that pDC require CCR9 for homing to the small intestine. To prove this hypothesis, we adoptively transferred cells from B6 and CCR9$^{-/-}$ donors that carried a Flt3-L-secreting tumor for 14 days. Under the influence of Flt3L pDC expanded to a similar extent in B6 and CCR9$^{-/-}$ mice (Fig. 4*A* and SI Fig. 8) and were indistinguishable regarding the expression of CCR2, CCR5, and CXCR3 whereas CCR9 was only detected on pDC derived from B6 but not CCR9-deficient donors (Fig. 4*C*). Without further purification, splenocytes of these donors were labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) and 5(6)-carboxytetramethylrhodamine *N*-succinimidyl ester (TAMRA) respectively. A mixture of WT and CCR9-deficient cells, adjusted to contain equal numbers of pDC, was i.v. transferred in B6 recipients. After 18 h of transfer, we first analyzed the composition of adoptively transferred WT cells present in the IE as well as LP preparation and noticed that 54% and 25% of all cells recovered from the IE and LP fraction respectively were pDC (Fig. 4*B*). These findings demonstrate that among the diverse cell populations transferred pDC home most efficiently to the intestine. These competitive transfers also revealed that CCR9-deficient pDC are largely impaired in their capacity to home to the intestine as reflected by the low migration ratio of CCR9-deficient vs. WT pDC found in the IE and LP preparation (Fig. 4*D*). In contrast, CCR9-deficient and B6 pDC migrated with similar efficiency to peripheral and mesenteric lymph nodes (Fig. 4*D*). The nature of the cell labeling had no impact on these experiments because interchanging the dyes between B6 and CCR9$^{-/-}$ cells yielded identical results (data not shown). Although these data clearly demonstrate that pDC require CCR9 for efficient homing to the gut, it should be mentioned that the trafficking properties of pDC from Flt3-

Fig. 3. Chemotactic response of pDC toward the CCR9 ligand CCL25. (*A*) DC were expanded *in vivo* by treating B6 mice with Flt3L-secreting tumor cells for 14 days. Chemotactic activity of splenic pDC and mDC toward different concentrations of CCL25, CXCL9, CXCL12, and CCL19 was analyzed (open columns, mDC; black columns, pDC; mean + SD; $n = 4$ independent experiments with pooled cells from two or three mice each). (*B*) Lack of intestinal pDC in CCR9-deficient mice. Shown are the percentage (*Left*) and number (*Right*) of pDC isolated from the inguinal LN (ILN), mesenteric LN (MLN), spleen (SPL), PP, and the IE and the LP preparation of the small intestine from B6 and CCR9-deficient mice. Circles represent data of individual mice ($n = 3$); bars show mean values. Similar results were obtained in four additional experiments using mice on a mixed genetic background (BALB/c 129SV; $n = 20$ mice per genotype).

ligand treated mice might be different from those present under physiologic situations.

pDC Are Recruited to the Inflamed Intestine. Because it is known that pDC play an important role in anti viral immunity (4, 10) we speculated that pDC might be recruited to the intestine during inflammatory processes to strengthen first line defense. Cholera toxin (CT) is known to induce intestinal inflammation when administered orally and it has been reported that within 2 h after oral application the number of mDC transiently recruited to the intestine increases 4-fold (15). We observed that, in addition to mDC, pDC numbers also increased 3-fold when feeding B6 mice with 10 μg of CT (Fig. 4*E*). Of interest, the identical experimental setup never resulted in any increase of pDC neither in the IE nor the LP preparation of CCR9-deficient mice after 1, 2, or 3 h of CT-treatment (Fig. 4*E* and data not shown). The specific requirement for CCR9 on pDC for their recruitment to the intestine during inflammatory processes was

Fig. 4. CCR9-dependent homing of pDC to the small intestine. (*A*–*D* and *F*) DC were expanded *in vivo* by treating B6 and CCR9-deficient mice with Flt3L-secreting tumor cells. (*A*) Splenocytes of B6 donors were analyzed for the presence of pDC ($B220^+Ly6C^+$). (*B*) Nonpurified WT Flt3L-expanded splenocytes were labeled with CFSE and i.v. injected into recipients. The occurrence of donor pDC in the recipient's IE (*Upper*) and LP (*Lower*) preparation was analyzed 18 h later (gate on $CFSE^+$ cells). (*A* and *B*) Data shown are representative for five animals of two independent experiments. (*C*) $CD11^+B220^+Ly6C^+$ pDC in Flt3L-expanded splenocytes of B6 (*Upper*) and CCR9-deficient mice (*Lower*) were stained for the expression of different chemokine receptors as indicated. (*D*) Splenocytes isolated from Flt3L-treated B6 or CCR9-deficient mice were labeled with CFSE and TAMRA, respectively. Cells were adjusted to equal numbers of pDC and injected at a ratio of 1:1 in B6 recipients. After 18 h, recipients were killed, and the ratio of donor B6 and CCR9-deficient pDC was analyzed in the recipient's IE and LP preparation of the intestine and the inguinal (ILN) and mesenteric (MLN) lymph node (mean + SD; $n = 5$–9 recipients). (*E*) WT (+/+) and CCR9-deficient (−/−) mice received 10 μg of CT or saline orally. After 1 h, animals were killed, and the number of pDC present in the small intestine IE preparation was determined. Circles represent individual mice; bars are mean values. Similar results were obtained in two additional experiments. (*F*) CFSE-labeled splenocytes isolated from Flt3L-treated B6 and TAMRA-labeled splenocytes isolated from Flt3L-treated CCR9-deficient mice were adoptively transferred to CCR9-deficient mice at a ratio of 1:1. After 18 h, recipients were gavaged orally with 10 μg of CT. One hour later, mice were killed, and the number of labeled WT (+/+) and $CCR9^{-/-}$ (−/−) pDC isolated from the IE and LP preparation was analyzed. Circles represent individual mice; bars are mean values.

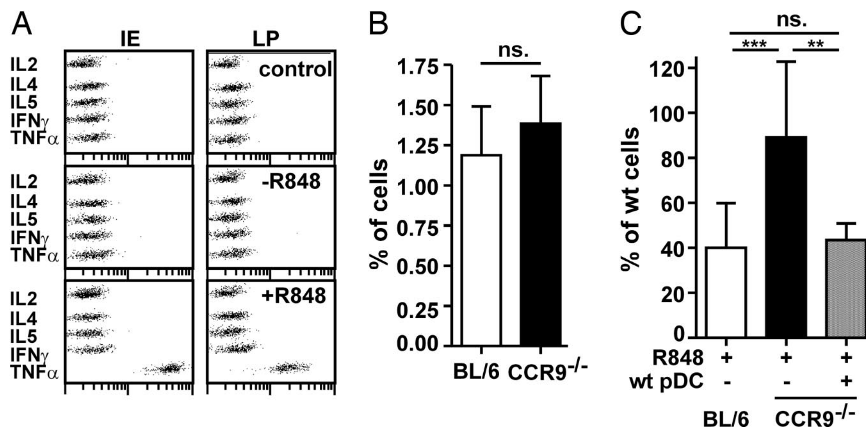

Fig. 5. Rapid mobilization of LP mDC relies on the intestinal pDC. (*A*) Cytokine bead array profile from the supernatant of sorted pDC of IE (*Left*) and LP (*Right*) preparation after 16 h *in vitro* stimulation in the absence (−R848) or presence (+R848) of R848. Control, cell culture medium. (*B*) Percentage of LP mDC ($CD45^+CD11c^+MHCII^{high}$) of untreated mice (mean +SD, $n = 6$ per group). (*C*) WT (open column) or $CCR9^{-/-}$ mice (black column) were orally gavaged with 10 μg of R848. After 2 h, the number of mDC ($CD45^+CD11c^+MHCII^{high}$) present in the LP of the small intestine was determined and expressed as percentage of untreated WT control. Gray column, $CCR9^{-/-}$ mice that i.v. received MACS-purified B6 pDC 16 h prior R848 treatment (mean + SD; $n = 6$–11 mice per group; ns, not significant; **, $P < 0.01$; ***, $P < 0.001$).

confirmed by adoptive transfer of WT and CCR9-deficient pDC to CCR9-deficient recipients followed by application of CT as described above. Whereas adoptively transferred WT pDC were amply present in the IE and LP preparation, CCR9-deficient pDC were almost completely excluded from these compartments (Fig. 4*F*). Together, these results show that during inflammatory events pDC can be recruited to the intestinal mucosa and that this mechanism relies to a large extend on CCR9.

A Role for Intestinal pDC for the Rapid Mobilization of LP Myeloid DC. It has been shown recently that oral application of the TLR7/8 ligand resiquimod (R848) results in the rapid mobilization of LP DC and that TNFα, possibly released by pDC, is involved in this process (16). We thus speculated that intestinal pDC might be the source of TNFα that potentially triggers the mobilization of neighboring mDC. To test this hypothesis, we *in vitro* stimulated B6 pDC of the IE and LP preparation for 16 h with R848. Indeed, pDC secreted considerable amounts of TNFα but failed to produce any detectable quantities of IL-2, IL-4, IL-5, or IFNγ (Fig. 5*A*). We then analyzed the mobilization of LP mDC *in vivo* after oral application of R848. Whereas untreated B6 and $CCR9^{-/-}$ mice did not differ regarding the presence of intestinal mDC (Fig. 5*B*), within 2 h oral R848 induced the mobilization of ≈60% of mDC in WT but only 10.8% in $CCR9^{-/-}$ mice. Importantly, once CCR9-deficient mice i.v. received splenic pDC of Flt3L-treated WT donors 16 h prior oral application of R848, this deficiency in intestinal mDC mobilization could be completely rescued (Fig. 5*C*). These experiments show that a CCR9-dependent homing of pDC to the intestine is involved in the rapid mobilization of intestinal mDC after oral application of a TLR7/8 ligand. Because it has been shown by others in the rat model that application of LPS also induces mobilization of LP mDC (17), we applied 50 μg of LPS i.p. to WT and CCR9-deficient animals. Of interest, under these experimental conditions we failed to observe any difference between WT and CCR9-deficient animals regarding the mobilization of LP mDC (SI Fig. 9).

Discussion

The CCR9 ligand, CCL25, is expressed by epithelial cells of the small intestine and has been suggested to target immune cells to the intestinal epithelium (18). The present study supports the idea that this chemokine attracts defined populations of immune cells to the small intestine. Agace and colleagues (19) further demonstrated that $CD8\alpha\beta^+$ T cells, activated within the mesenteric LN, selectively home to the small intestinal mucosa and that this homing depends on CCR9. Data from our group suggest a similar mechanism for plasma cells (11). Results provided here demonstrate that CCR9-deficient mice possess reduced numbers of pDC in the small intestine under steady-state conditions, an observation that correlates well with the impaired recruitment of CCR9-deficient pDC to this organ under inflammatory conditions. In accordance with the hypothesis that CCR9 is required for pDC gut homing is our finding that pDC derived from CCR9-deficient donors are impaired in homing to the intestine once adoptively transferred to WT recipients.

In addition to targeting immune cells to the epithelium, CCL25 also mediates T cell entrance into the LP across intestinal venules (20). A similar mechanism might allow homing of pDC to the small intestine. Therefore, it seems possible that CCR9 recruits pDC into the LP and, in addition, targets a fraction of these cells to the epithelium. Apart from CCR9, it is currently unclear, which adhesion molecules are involved in pDC homing to the intestine. Our data would suggest that $\alpha4\beta7$ integrin, as well as P-selectin, might also be involved in this process.

This study also reveals a previously undescribed function for intestinal pDC. We show that after oral application of a TLR7/8 ligand, intestinal pDC are required for the rapid mobilization of LP mDC, a mechanism that might involve the release of TNFα from this cell population. Although it is currently unclear how impaired mobilization might affect immunity to pathogens, it seems conceivable that the rapid mobilization of LP DC to the mesenteric LN favors the fast onset of adaptive immunity. Interestingly, pDC mediated mobilization of mDC seems restricted to distinct TLR ligands because LPS-activity bypasses the need of pDC for successful emigration of mDC from the small intestine. This finding corroborates the concept that pathogens may bias immune responses already at the early stage of their entry into the body because it is known that immature DC primed under distinct cytokine environment such as TNFα cause a shift to the subsequent $T_{helper}1/T_{helper}2$ answer. Furthermore, it is also tempting to speculate that pDC might help to enforce the armed battery of IE lymphocytes residing at the frontline of mucosal immune defense. In particular intestinal pDC might supplement mucosal protection against viral attack. However, these scenarios still await experimental approval encompassing animal models for inflammatory bowel disease and viral infections.

Materials and Methods

Mice. Animals were bred under specific pathogen-free conditions. CCR9-deficient mice, either on a mixed genetic (BALB/c × 129SV) or a C57BL/6 background (backcrosses for 5 or 9 generations) have been described elsewhere (21). Most of the experiments described in this manuscript were performed on both genetic backgrounds yielding identical results. Data depicted derive from experiments performed with mice on a C57BL/6 background except those depicted in Fig. 4*E*. All animal experiments were conducted in accordance with local and institutional guidelines.

Flow Cytometry. Immune cells of the intestine were isolated from 6- to 8-week-old mice as recently described in detail (22). Cells were stained with the following antibodies: Ly6C-FITC, $\alpha 4\beta 7$-biotin, CD103-biotin, B220-PerCP, CD11c-PE (all from BD Bioscience), CD4-PE, CD62L-PE, CD45-APC, CD18-FITC (Caltag), P-selectin-ligand (R & D Systems), CCR7-biotin (eBioscience), 120G8, (Vector Laboratories), PDCA-1-APC (Dianova). Anti CD3-Cy5 (clone 17A2) and anti CD8 (clone CD8.2) were grown in our laboratories. Anti CCR2 and CCR5 mAb were kindly provided by Matthias Mack (University of Regensburg, Regensburg, Germany) (23). The rat anti-mouse CCR9 mAb (clone 7E7) was produced in our lab and has been described (11).

Immunohistology and Cytospins. Immunhistological analysis of the small intestine of mice was done on 8-μm cryosections as described (11, 22). pDC ($CD3^-CD11c^+B220^+Ly6C^+$) and mDC ($CD3^-CD11c^+B220^-Ly6C^-$) of the IE preparation were sorted by flow cytometry (FACSAria, BD Biosciences). Acetone-fixed cytospins were prepared from sorted cells.

***In Vivo* Generation of pDC and *in Vitro* Migration Assay.** B6 and CCR9-deficient mice received s.c. 5×10^5 to 1×10^6 B16-FL cells, a murine melanoma tumor cell line engineered to stably produce murine Flt3-L (14). After 14 days, animals were killed. Flt3L-expanded, $CD11c^+$ MACS-sorted splenocytes (1×10^6), containing ≈15% pDC, were resuspended in 100 μl of RPMI medium 1640 and loaded into collagen-coated transwells (Corning BV; 5 μm pore size) that were placed in 24-well plates containing 400 μl medium or medium supplemented with various concentrations of CCL25, CXCL9, CCL19, or CXCL12 (R & D systems). After 3 h of incubation at 37°C, the migrated cells were collected, counted, and stained with mAb to determine by flow cytometry the number of migrated pDC and mDC. The ratio of the number of pDC that migrated in the presence of chemokine vs. the number of cells that migrated to PBS control was calculated and is given as the migration index.

Adoptive Transfer of Labeled Cells. Splenocytes from B16-FL tumor-carrying B6 or CCR9-deficient mice were labeled with TAMRA (red fluorescent) or CFSE (green fluorescent) or vice versa. Cell populations were adjusted to contain equal numbers of pDC. For adoptive transfers, 10^6 pDC for both colors were i.v. injected into the tail vein of recipients. After 18 h, recipients were killed and cells were isolated from the intestine as well as from mesenteric and peripheral lymph nodes.

MACS-Purification of pDC. Splenocytes from B16-FL tumor-bearing mice were negative sorted for CD3 and CD19. In a subsequent step $B220^+$ cells were enriched.

***In Vivo* Mobilization of Cells.** Ten micrograms of CT (Sigma) in 300 μl of carbonic buffer (0.1 M $NaHCO_3$) or 10 μg of R848 in 300 μl of PBS were orally administered by gavage. Fifty micrograms of LPS were i.p. injected in 150 μl of PBS. One hour to 3 h after the application of CT, mice were killed, and the number of intestinal pDC was determined. Mice that received R848 were killed 2 h after the application of this drug, and the number of mDC of the LP was determined. Mice that received LPS were killed 12 h later, and the number of LP mDC was determined. Some of the R848-treated $CCR9^{-/-}$ mice received 2–4 × 10^6 MACS-purified WT pDC i.v. 16 h prior R848 application.

***In Vitro* Stimulation of pDC.** 10^6 MACS-purified pDC were cultured in 200 μl of RPMI medium 1640/10% FCS for 14 h in the absence or presence of CpG2216 (16.5 μg/ml) or R848 (2 μg/ml). mDC ($CD11c^+MHCII^+$) of the LP preparation were sorted by flow cytometry (MoFlo, Dako-Cytomation) and activated with CpG2216 as describe above. Supernatants were collected and the amount of IFN-α determined by ELISA (Hycult). Interleukins and TNFα were detected by cytokine bead arrays (BD).

We thank Matthias Mack for providing CCR2 and CCR5 mAb and Günter Bernhardt for valuable suggestions on the manuscript. The generation and characterization of CCR9-deficient mice was supported by the Association pour la Recherche sur le Cancer and MUGEN. This work was supported by Deutsche Forschungsgemeinschaft Grant SFB621-A1 (to R.F.).

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