Chemokine Receptor CCR9 Contributes to the Localization of Plasma Cells to the Small Intestine

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Abstract

Humoral immunity in the gut-associated lymphoid tissue is characterized by the production of immunoglobulin A (IgA) by antibody-secreting plasma cells (PCs) in the lamina propria. The chemokine CCL25 is expressed by intestinal epithelial cells and is capable of inducing chemotaxis of IgA⁺ PCs in vitro. Using a newly generated monoclonal antibody against murine CCR9, we show that IgA⁺ PCs express high levels of CCR9 in the mesenteric lymph node (MLN) and Peyer's patches (PPs), but down-regulate CCR9 once they are located in the small intestine. In CCR9-deficient mice, IgA⁺ PCs are substantially reduced in number in the lamina propria of the small intestine. In adoptive transfer experiments, CCR9-deficient IgA⁺ PCs show reduced migration into the small intestine compared with wild-type controls. Furthermore, CCR9 mutants fail to mount a regular IgA response to an orally administered antigen, although the architecture and cell type composition of PPs and MLN are unaffected and are functional for the generation of IgA PCs. These findings provide profound in vivo evidence that CCL25/CCR9 guides PCs into the small intestine.

Key words: gut • IgA • lamina propria • CCL25 • cell trafficking

Introduction

The gut acts as the port of entry for a vast array of foreign antigens, including food components, but also potentially harmful pathogens. A first line of defense against these antigens is built by neutralizing immunoglobulins directed against pathogens or toxins (1). For this purpose, antibody-secreting plasma cells (PCs) of the lamina propria produce dimeric IgA that is transported into the gut lumen by transcytosis and bound to the mucus overlying the intestinal epithelium.

In the intestine, antigens are sampled by DCs located in the epithelium or by specialized epithelia overlying Peyer's patches (PPs). In these follicle-associated epithelia, microfold cells nonspecifically sample antigens from the gut lumen and transport them to professional antigen-presenting cells located in the subepithelial dome (SED; reference 2). To elicit an immune response, these cells migrate into either the adjacent interfollicular T cell zone, the B cell–rich follicles of PPs, or even into the draining mesenteric lymph node (MLN) to activate lymphocytes (3, 4). Some of the activated B cells start to proliferate and generate germinal centers within PPs or MLN, which have been identified as the places where affinity maturation and probably isotype switch from IgM to IgA occurs. However, more recently it has been shown that isotype switch of B220⁺ IgM⁺ cells at least in part occurs in the lamina propria under the influence of local stimuli (5). Most of the fully differentiated B cells leave PPs and MLN and migrate via the lymphatics and the thoracic duct into the blood and from there to the lamina propria of the small intestine.

It has been proposed that signaling through the chemokine receptor CCR9 might be an important factor that targets cells to the intestine (6, 7). The CCR9 ligand CCL25/ TECK is expressed by epithelial cells of the small, but not the large, intestine. CCR9 is expressed on virtually all small intestinal T cells, and murine IgA-producing PCs from the spleen, PPs, and MLN have been shown to migrate toward CCL25 and CXCL12, a ligand for CXCR4 in vitro (8–10). Notably, PCs of IgG or IgM isotype do not respond to

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CCL25 but migrate toward CXCL12 and CXCL9 (9, 11, 12), suggesting that the differential expression of chemokine receptors targets PCs to their final destination depending on the isotype of immunoglobulins they produce. Furthermore, during the course of a memory response, CXCR3 and CXCR4 have been implicated in guiding plasma blasts to inflamed tissues or to the bone marrow, respectively (13). In this report, we provide in vivo evidence that CCR9 is crucial for the positioning of PCs to the small intestine.

Materials and Methods

Isolation of Lamina Propria Cells (LPCs) and Flow Cytometry. Animals were bred at the animal facility of Hannover Medical School under specific pathogen-free conditions. CCR9-deficient mice have been described previously (14). In this analysis, CCR9-deficient mice and littermates of a mixed genetic background were used. 8–10-wk-old animals were killed, and LPCs were isolated using standard procedures.

Cells were stained using the following antibodies: anti-CD3-PE (Caltag), IgA-biotin, CD19-biotin (Biosource International), CD138-PE, B220-PerCP, IgM, and IgD (BD Biosciences). To stain cytoplasmatic IgA, cells were fixed for 20 min in 2% PFA in PBS on ice, washed, and resuspended for 20 min in 0.1% saponin in PBS.

Generation of Monoclonal CCR9 Antibody. A peptide comprising amino acids 3–22 of mouse CCR9 was synthesized and coupled to KLH or OVA. Rats were immunized subcutaneously and intraperitoneally with a mixture of 50 μ g peptide-KLH, 5 nmol CPG oligonucleotide (Tib Molbiol), 500 μ l PBS and 500 μ l IFA as described previously (15). Supernatants were tested by a differential ELISA and analyzed by flow cytometry using thymocytes derived from wild-type and CCR9-deficient mice.

Immunofluorescence. Immunohistological analysis of adult PPs and MLN was done on cryosections as described previously (16). For detection of CXCR4 (clone 2B11) and CCR9 (clone 7E7, IgG2b), sections were blocked with mouse serum, incubated with hybridoma supernatants, and detected using mouse anti–rat Cy3 antibodies (Jackson ImmunoResearch Laboratories).

In Vivo Migration of BrdU-labeled Cells. To label proliferating cells in vivo, wild-type and CCR9-deficient animals were injected intraperitoneally with 120 mg/kg BrdU (Sigma-Aldrich) in PBS 1 h before killing. Cells were isolated from MLN and PPs, and a total of 10⁸ cells was injected intravenously into wild-type recipients. After 16 h, mice were killed, and the small intestine was embedded in paraffin using standard procedures. Sections were dewaxed, and BrdU-incorporated cells were detected using the BrdU staining kit (Oncogene Research Products) and Cy3-tyramid (NEN Life Science Products). IgA⁺ cells were identified using anti-IgA FITC antibody (Caltag).

Oral OVA Immunizations and Serum Ig Analysis. ELISA assays for total serum Ig levels were performed as described previously (16). Biotinylated anti-Ig antibodies were purchased from BD Biosciences (anti-IgM, anti-IgG1, anti-IgG2a, anti-IgG2b, and anti-IgG3) and Biosource International (anti-IgA).

10 mice per genotype were gavaged with 2.5 mg OVA and 10 μ g cholera toxin (CT) six times at 10-d intervals. For detection of OVA-specific IgA and IgM antibodies, plates were coated overnight with 5 μ g/ml OVA, and appropriate dilutions of serum samples were added (1:100–1:6,400). Anti–IgA-biotin and anti–IgM-biotin followed by streptavidin-peroxidase and 5-thio-2-nitrobenzoic acid were used for detection.

Results and Discussion

IgA⁺ PCs Express CCR9 Abundantly in Secondary Lymphoid Organs but Not the Small Intestine. It has been suggested that the interaction of CCL25 with CCR9 might play an important role in the establishment of an IgA⁺ PC pool in the small intestine. Therefore, we characterized the expression of CCR9 on PCs in different organs with a newly generated monoclonal antibody against CCR9. The antibody displayed high levels of CCR9 on wild-type thymocytes but not on thymocytes derived from CCR9-deficient mice (Fig. 1 A).

In cell preparations isolated from the LPCs of wild-type mice, two distinct populations of cells can be distinguished based on size and granularity. Small LPCs are predominantly CD3⁺ lamina propria lymphocytes, whereas large LPCs heavily stain with anti-IgA mAb after permeabilization (unpublished data). Additionally, the majority these cells show a CD138⁺, IgD⁻, IgM⁻, and CD19⁻ surface phenotype and, thus, can be defined as lamina propria PCs (unpublished data). CCR9 could be detected on most lamina propria lymphocytes (Fig. 1 B), but more importantly, only ~5% of all lamina propria PCs are CCR9⁺ (Fig. 1 C).

These observations were confirmed applying the CCR9 mAb in immunohistology. In contrast to IgA⁺ cells of MLN and PPs, which showed high levels of CCR9 (Fig. 1 D and not depicted), the majority of IgA⁺ PCs of the small intestine showed weak or undetectable staining with the anti-CCR9 mAb (Fig. 1 E; compare with staining intensity shown in Fig. 1 D). In addition, cells staining positive for CCR9 seem to represent T cells (Fig. 1 E). Interestingly, another chemokine receptor, CXCR4, was readily detectable on all IgA⁺ PCs of all lymphoid organs examined, including PPs, MLN, and the small intestine (Fig. 1 F and not depicted). Because IgA⁺ PCs are known to readily leave the place where they have been generated (i.e., the secondary lymphoid organs), high expression of CCR9 appears to be specific for newly generated PCs, whereas this receptor becomes down-regulated once these cells reached their final destination. These data suggest that high levels of CCR9 are required for the homing of newly generated IgA⁺ PCs to the intestine, whereas this receptor seems to be dispensable for retaining PCs within this compartment. Because CXCR4 remains expressed on resident IgA⁺ PCs within the intestine, it seems possible that CXCR4 participates in this process. Indeed, data derived from CXCR4-deficient mice suggest a role for this chemokine receptor in retaining PCs within lymphoid organs such as bone marrow (11).

Reduced Numbers of PCs in the Intestinal Lamina Propria of CCR9 Mutant Mice. Based on these findings, we compared the PC populations of wild-type and CCR9 mutant mice by counting the number of IgA⁺ PCs on cryosections of the small intestine (Fig. 2, A and B). In this paper, only villi were counted that were cut (judged on the analysis of serial sections) through the core of the villus. In wild-type animals, an average of 20.6 ± 1.2 (mean \pm SEM) IgA⁺ cells per villus section was found. In contrast,



Figure 1. CCR9 is expressed on all IgA⁺ PCs in MLN but on few lamina propria PCs. (A–C) Cells isolated from thymus (A) and small intestinal lamina propria (B and C) of wild-type mice (solid line) and CCR9-deficient animals (dashed line) were stained using a CCR9-specific monoclonal antibody or an isotype control (shaded area). The histograms shown in B were obtained by gating on small LPCs, whereas data shown in C were obtained from large LPCs. (D–F) Three color immunohistology. Sections of MLN (D) and small intestine (E and F) from wild-type mice were stained with anti-CCR9 (D' and E', red) or anti-CXCR4 (F', red). Sections were further stained with anti-IgA (D–F, green) and anti-CD3 (D'–F', blue). The three colors obtained from single sections have been separated as depicted. Arrows indicate the position of IgA⁺ PCs.

in CCR9 mutant mice, the number of PCs per villus section was severely reduced to 11 ± 1.1 (mean \pm SEM) cells per villus section (Fig. 2 C). As an internal control, the number of IgA⁻ DAPI⁺ LPCs was determined on the same sections, revealing that CCR9 deficiency does not affect cell types other than IgA⁺ PCs in this compartment (Fig. 2 C, 19 ± 0.9 cells vs. 22 ± 1.6 cells). These results could also be confirmed by flow cytometry on permeabilized LPCs using an anti-IgA mAb that revealed a reduc-



Figure 2. CCR9 mutant intestines harbor reduced numbers of IgA⁺ PC. Cryosections through the small intestine of wild-type (A) and CCR9-targeted mice (B) were stained for IgA (red), CD3 (green), and nuclei (blue). Unaltered numbers of IgA⁻ cells were present in the lamina propria of wild-type and CCR9 mutant animals, whereas numbers of IgA⁺ cells were reduced in CCR9 mutants (C, ***, P < 0.001; data were derived from five wild type and five mutants analyzing 60 villi each).

tion of IgA⁺ PC numbers by \sim 50% in CCR9 mutants (unpublished data).

Because it has been suggested that isotype switch can take place in the lamina propria (5), we also used antibodies specific for other immunoglobulin isotypes to detect differences between wild-type and CCR9 mutant mice. However, immunohistology for IgM and IgG did not reveal any significant differences between both strains (unpublished data), rendering it unlikely that impaired local isotype switching accounts for the reduced number of IgA-positive cells in CCR9 mutants. Furthermore, the phenotype of the residual PC population in CCR9 mutants as determined by expression of surface antigens (CD138, CD19, B220, IgA, IgM, and IgD) was indistinguishable from that of wild-type PCs, suggesting that loss of CCR9 does not affect the differentiation of PCs.

Interestingly, in wild-type mice, most of the PCs locate to the lower half of the villus (Fig. 2 C), which is the region where peak levels of CCL25 expression by the abutting epithelial cells have been described previously (8), further supporting the hypothesis that CCL25 signaling via CCR9 contributes considerably to the efficient homing to the small intestine. Most notably, no differences in the number of IgA⁺ PCs were identified in the large intestine of CCR9 mutants (wild type: 1 PC/4699 \pm 255 μ m²; CCR9 deficient: 1 PC/4832 \pm 331 μ m²; n = 5 animals for each group). This observation is also in line with the reported absence of CCL25 in the large intestine (8).

Reduced Homing Capacity of CCR9-deficient IgA⁺ PCs. More than two decades ago, McDermott et al. identified proliferating cells in MLN and PPs, but not in peripheral LN, that were able to migrate into mucosal tissues and give rise to IgA-secreting cells (17). To directly address the function of CCR9 in this process, we labeled proliferating cells using BrdU and isolated them from MLN and PPs of wild-type and CCR9-deficient mice. 10^8 cells were injected i.v. into wild-type recipients, and after 16 h, the



Figure 3. CCR9 mutant PCs show an impaired migration into the small intestine. (A) 16 h after adoptive transfer of BrdU-labeled cells isolated from MLN and PP of wild-type and CCR9 mutant mice, the number of BrdU⁺ IgA⁺ cells per villus was determined (*, P < 0.05; data were derived from three recipients of each group analyzing 60 villi each). CCR9 mutant mice show a reduced humoral IgA response. (B) Groups of 10 CCR9 mutants (solid line) and control animals (dashed line) were each gavaged with OVA and CT at 10-d intervals, and serum levels of OVA-specific IgA were measured. In contrast to wild-type animals showing a robust increase of OVA-specific IgA levels, CCR9 mutants barely produced OVA-specific IgAs.

numbers of $BrdU^+$ IgA⁺ PCs in the small intestine had been determined. Interestingly, PCs from wild-type mice were threefold more efficient in migrating into the small intestine compared with cells derived from CCR9-deficient mice (Fig. 3 A). These results contributed further weight to the idea that CCR9 is required for efficient migration of newly formed PCs into the small intestine.

CCR9-deficient Mice Do Not Mount a Proper Immune Response to Oral Antigens. To test whether the reduced number of IgA-secreting PCs observed in the intestine of CCR9 mutants and the reduced migration efficiency of IgA⁺ PCs are paralleled by reduced serum IgA levels, we analyzed the amount of serum Ig in wild-type and CCR9 mutant mice. CCR9-deficient and wild-type mice were not found to differ significantly with regard to normal serum levels of any immunoglobulin isotypes tested (IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA), indicating that CCR9 is dispensable for systemic immunoglobulin production (unpublished data).

Although the total amount of serum IgA was found to be unchanged between wild-type and mutant mice, we were interested to know whether differences exist between CCR9 mutants and wild-type mice in the induction of antigen-specific IgA response after oral immunization with a T celldependent antigen. To this end, wild-type mice and CCR9 mutants were gavaged with 2.5 mg OVA and 10 μg CT at 10-d intervals. Serum levels of OVA-specific IgA were determined 9 d after each single OVA application. In wild-type animals, significant OVA-specific IgA levels were detectable after three OVA applications that further increased after subsequent applications of the antigen (Fig. 3 B). Interestingly, in CCR9 mutants, a barely detectable increase in OVA-specific IgA titers could be determined within the period of time analyzed, suggesting a severe impairment in the production of OVA-specific IgA in these animals (Fig. 3 B).

PPs in CCR9-deficient Mice. Because it is assumed that the induction of an IgA-specific antibody response after oral application of antigen plus CT requires antigen presentation within morphologically intact PPs and MLN, we further analyzed both organs. We used immunohistology and flow cytometry to identify possible alterations in cellular composition or architecture of both organs in CCR9 mutants that contained normal numbers of B and T cells, and both cell types were located in their appropriate microenvironments. In addition, PPs contained normal numbers of CD11c⁺, CD11b⁻, and CD11c⁺CD11b⁺ DCs (unpublished data). Recently, it has been described that DCs of the SED can be labeled and their path subsequently followed using fluorescent latex beads (4). Because we found CCR9-expressing cells, including DC within the SED (unpublished data), we tested whether the mobilization of DCs of the SED is affected in CCR9 mutants. Wild-type and CCR9-deficient mice were deprived of water and food for 4 h and subsequently gavaged with 1012 fluorescent latex beads (200 nm diameter; Polysciences) per animal. After 24 h, the mice were gavaged with 50 µg of CT. After another 24 h, PPs and MLN were sectioned and stained for CD11c and CD3 (Fig. 4, A-D, PPs only). In wild type and CCR9 mutants, application of CT lead to reduced numbers of CD11c⁺ cells (red) present in the SED (Fig. 4, A–D). To quantify this effect, the absolute number of fluorescent beads in the SED was counted in 20 PPs derived from four wild type and four CCR9 mutants. In both wild type and CCR9 mutants, CT administration triggers an identical fivefold decrease of bead-labeled DCs localized to the SED, suggesting that no differences in DC mobilization exist and that the activation of naive B cells should proceed normally in these CCR9^{-/-} organs (unpublished data). Indeed, analysis of cryosections from MLN of wildtype and mutant mice that were immunized five times with OVA and CT revealed comparable numbers of IgA⁺ PCs in both strains (Fig. 4, E and F). Furthermore, in wild type and mutants likewise, the total numbers of IgA+ PCs strongly increased after immunization in comparison to nonimmunized mice (unpublished data) strongly supporting the idea that the generation of IgA PCs is not affected by CCR9 deficiency.

Unaltered Architecture and Cell Composition of MLN and

The data provided in the present work demonstrate that CCR9-deficient mice possess reduced numbers of IgA-secreting PCs within the lamina propria, an observation that correlates well with the reduced migration efficiency of CCR9-deficient PCs into the small intestine and the in-ability of these animals to mount a T-dependent antigen-specific IgA response after oral application of antigen.

One hypothesis accounting for these defects would postulate the existence of a rather uniform PC population inside the small intestine. In this scenario, freshly generated plasma blasts that express high levels of CCR9 gain efficient entry into the intestinal lamina propria by CCL25mediated signaling, whereas the residual PC population in CCR9-deficient mice would enter the lamina propria in-



Figure 4. Regular generation of IgA⁺ PCs in CCR9-deficient animals. Wild-type (A and C) and CCR9-deficient mice (B and D) were gavaged with fluorescent latex beads and treated after 24 h with PBS alone (A and B) or CT in PBS (C and D). After 24 h, PP were removed, sectioned, and stained for CD11c (red), CD3 (green), and nuclei (blue). Wild-type and CCR9-deficient mice showed no differences in architecture, nor mobilization of CD11c⁺ cells or mobilization of bead-loaded cells. (A, inset) Arrows point to bead-loaded CD11c⁺ cells. Sections of MLN of wild type (E) and CCR9 mutants (F) that had been immunized with OVA and CT five times at 10-d intervals were stained with anti-IgA (green), anti-IgM (red), and anti-CD3 (blue). Similar numbers of IgA⁺ PCs are present in wild type and CCR9 MLN, demonstrating that the generation of IgA PCs is not grossly altered in CCR9-deficient mice.

dependent of CCR9, probably by a time-consuming and inefficient bypass mechanism.

Alternatively, dependence on CCR9 signaling might reflect the different developmental fate and origin of PCs populating the small intestine. It has been suggested that besides conventional B2 cells, B1 cells from the peritoneal cavity can give rise to lamina propria IgA⁺ PCs (18). However, this issue remains discussed controversially because intestinal IgA production in conventionally reared animals (as performed in this work) has been shown to be the source of almost exclusively B2, but not B1, cells (19). Unfortunately, available immunological tools would not allow dissection of B1 and B2 cell–derived PCs once they settled the lamina propria. Interestingly, intraperitoneal immunization with T cell–independent antigen results in a normal antigen-specific IgM response in CCR9 mutants, suggesting that B1 cell function per se is not affected in these animals (unpublished data). Thus, a more likely explanation for the decrease of IgA⁺ PCs in CCR9 mutants is the less efficient recruitment of B2 cell–derived PCs into the lamina propria in the absence of CCR9 signaling. This idea is consistent with a strongly reduced IgA response in orally immunized mice assuming that antigen-specific PCs are generated in CCR9 mutants but fail to reach a compartment that supports secretion and long-term survival of these cells.

In any case, CCR9-independent chemokine receptor signaling would be required to guide the remaining PCs into the lamina propria in CCR9-deficient mice, resulting in serum and fecal IgA levels that do not significantly differ from those present in wild-type animals (unpublished data). Indeed, surface expression of CXCR4 can be detected on IgA⁺ PCs (Fig. 1 F) consistent with the responsiveness of these cells to CXCL12 in in vitro migration assays (9). Furthermore, in humans, CCR10 has been documented in PCs, including intestinal IgA⁺-secreting PCs (20), and we were able to detect CCR10 expression in mouse PCs using a CCL27-IgG1 fusion protein (unpublished data) suggesting that CCR9 and CCR10 might cooperate in directing PCs into the small intestine in mice. In conclusion, our findings represent the first in vivo evidence that CCR9 is critical for the homing of IgA-secreting PCs to the small intestine.

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