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Redundant Role of Chemokines CCL25/TECK and CCL28/MEC in IgA+ Plasmablast Recruitment to the Intestinal Lamina Propria After Rotavirus Infection

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Rotaviruses (RV) are the most important cause of severe childhood diarrheal disease. In suckling mice, infection with RV results in an increase in total and virus-specific IgA+ plasmablasts in the small intestinal lamina propria (LP) soon after infection, providing a unique opportunity to study the mechanism of IgA+ cell recruitment into the small intestine. In this study, we show that the increase in total and RV-specific IgA+ plasmablasts in the LP after RV infection can be blocked by the combined administration of Abs against chemokines CCL25 and CCL28, but not by the administration of either Ab alone. RV infection in CCR9 knockout mice still induced a significant accumulation of IgA+ plasmablasts in the LP, which was blocked by the addition of anti-CCL25 Ab, confirming the synergistic role of CCL25 and CCL28. The absence of IgA+ plasmablast accumulation in LP following combined anti-chemokine treatment was not due to changes in proliferation or apoptosis in these cells. We also found that coadministration of anti-CCL25 and anti-CCL28 Abs with the addition of anti-α4 Ab did not further inhibit IgA+ cell accumulation in the LP and that the CCL25 receptor, CCR9, was coexpressed with the intestinal homing receptor α4β7 on IgA+ plasmablasts. Finally, we showed that RV infection was associated with an increase in both CCL25 and CCL28 in the small intestine. Hence, our findings indicate that α4β7 along with either CCR9 or CCR10 are sufficient for mediating the intestinal migration of IgA+ plasmablasts during RV infection.

Secretory IgA in the intestinal lumen constitutes the first line of defense against a vast array of Ags (both food and pathogens) that enter through the gut. IgA is produced by plasma cells (PC) in the intestinal lamina propria (LP) as a dimeric molecule associated with J-chain. This form of IgA binds to the polymeric IgR expressed basolaterally on intestinal epithelial cells. The ligand-polymeric IgR undergoes transcytosis to the apical cell surface for luminal release by cleavage of the extracellular receptor domain, which is incorporated into the quaternary structure of secretory IgA as so-called bound secretory component (1, 2).

B cells activated in the intestine undergo expansion and differentiation predominantly in the germinal centers of the Peyer’s patches (PP) and/or mesenteric lymph nodes (MLN), where affinity maturation and isotype switching from IgM to IgA occurs (1). Most of these fully differentiated B cells, which are predominantly IgA+ plasmablasts, exit PP and MLN via the lymphatics and thoracic duct, enter the blood circulation, and then specifically traffic to the intestinal LP, where they can subsequently perform effector functions.

The tissue-specific recruitment of lymphocytes is a tightly regulated multistep process that involves the interaction between homing (integrins and selectins) and chemokine receptors on the lymphocyte surface and cell adhesion molecules and chemokines expressed on the vascular endothelium of specific target tissues (3). The requirements for multiple protein-protein interactions during lymphocyte homing ensure a high degree of specificity in targeting cells.

There is considerable evidence that the integrin α4β7 plays a critical role in lymphocyte homing to the gut. For instance, in both humans and mice, gut lymphocytes including IgA+ PC, express high levels of α4β7 (4, 5) and interact with mucosal addressin cell-adhesion molecule 1 (MADCAM-1), which is constitutively expressed in intestinal mucosa (6, 7). In addition, both oral and rectal mucosal immunizations induced B cells expressing high levels of α4β7 as compared with cells elicited after systemic immunization (8). Furthermore, β7 knockout (KO) mice have reduced numbers of IgA+ PC in the gut (9).

Among chemokines, CCL25/TECK (thymus-expressed chemokine) and CCL28/MEC (mucosa-associated epithelial chemokine), and their respective receptors CCR9 and CCR10, are in an anatomic position to play an important role in B cell homing to the gut (10–13). CCL25 is almost exclusively expressed in the small intestine (SI) and is expressed at high levels by crypt epithelial and endothelial cells. CCL25 attracts B cells that express CCR9 and IgA on their surface. Mouse IgA+ cells from PP and MLN were
highly responsive to CCL25 in contrast to cells from the upper respiratory tract, indicating that the expression of CCR9 is induced during the development of IgA+ plasmablasts/PC in the lymphoid tissues of the intestinal mucosa (10, 13, 14). In addition, CCR9+ B cells are rare in the colon and absent in other mucosal tissues (10). In contrast, the expression of CCL28 is higher in the colon than in the SI (15, 16), and it has been suggested that the differential expression of CCL25 and CCL28 along the intestine helps to compartmentalize the intestinal immune response (10). CCL28 is also found in a variety of mucosal tissues including the salivary gland, mammary gland, small and large intestine, tonsil, appendix, and trachea, and it has been previously shown that CCR10 is highly expressed on IgA+ PC (16, 17). Because of the variety of tissues in which CCL28 is expressed, it was suggested that the CCL28/CCR10 interaction may function as a unifying mucosal homing mechanism for IgA+ plasmablasts/PC (17). However, it has recently been shown that nasal-associated lymphoid tissue-derived human B cells, which also express CCR10, have limited access to the SI because they express low levels of CCR9 and αβ7 (18). Hence, the coexpression of homing and chemokine receptors contributes greatly to the compartmentalization of the mucosal immune system, with important implications for the development of effective vaccines (19).

Pabst et al. (13) demonstrated that CCR9-deficient mice had decreased numbers of IgA+ PC in the LP and an impaired mucosal immune response to orally administered OVA. In vivo treatment of cholera toxin (CT)-immunized mice with anti-CCL25 or anti-CCL28, resulted in diminished Ag-specific IgA response in the SI (12). Because these previous studies were not performed within the context of a natural intestinal infection, we examined the roles of CCL25 and CCL28, along with their interaction with αβ7, in gut homing of IgA+ plasmablasts/PC during murine rotavirus (RV) infection.

RV causes severe diarrhea in young children (20) and many mammalian and avian species including infant mice. The virus replicates to high titers in the mature epithelial cells of the SI and induces a strong intestinal IgA response, which is important for protection against reinfection (21). In mice, RV-specific B cells are responsive to CCL25 and CCL28 (10, 22), and B cells lacking β7 are defective in mediating clearance of chronic infection in immune-deficient mice (9). Acute RV infection also induces significant polyclonal activation of B cells (23). We recently observed that RV infection in suckling mice speeds up the accumulation of IgA+ PC in the intestinal LP when compared with noninfected controls. Hence, this model system provides a useful opportunity to examine the role of several trafficking molecules (CCL25, CCL28, and αβ7) in the seeding of LP of suckling mice with IgA+ PC after an enteric viral infection.

Materials and Methods

Virus and virus-like particles

The virulent wild-type (WT) murine RV strain, ECw, was used to infect mice. This virus causes diarrhea in 100% of 5-day-old suckling mice, which lasts for 7–9 days (24). The virus was propagated in suckling mice, used as a clarified intestinal homogenate, and its ID50 was determined by the Bradford method, using BSA as a standard.

Confluent microscopic images were obtained using the software Laser-sharp 2.0 and a MRC-1024 laser scanning confocal imaging system (Bio-Rad) connected to a Nikon Eclipse TE300 inverted microscope (Nikon). IgA+ secretory cells appeared as large IgA+ cells detectable in LP (Fig. 1, A, C, and D) and PP parafollicular region (data not shown) and were B220-negative (data not shown). For each section, 5–10 images were collected after scanning the entire section under the microscope. We first identified areas with IgA+ cells and adjusted fields to maximize the presence of IgA+ cells and acquired both red and green images using sequential acquisition. If there were no IgA+ cells detected in a section, 5–10 random images were acquired.

Total IgA+ cells (in red) and GFP-VLP+/IgA+ cells (in yellow) were counted, and data were expressed as number of IgA+ cells per field and

Mice and infections

BALB/c mice were originally purchased from The Jackson Laboratory and propagated and maintained at the Palo Alto Veterans Affairs Health Care System Veterinarian Medicine Unit (Palo Alto, CA). CCR9-deficient (CCR9 KO) mice on C57BL/6 and 129 hybrid background and the hybrid control mice were originally obtained from Dr. P. Love (National Institutes of Health, Bethesda, MD) and propagated and maintained in the same facility. All studies were approved by the Stanford Institutional Animal Care Committee.

Five-day-old suckling mice were orally inoculated with 3 x 101 ID50 of ECw or, in some experiments, with 2000 CFU of the aromatic aro5 mutant strain SL2207 (Salmonella typhimurium 2337-65 derivative bioG46 DEL4077[aroA:TN(Tc+)]) (B.A.D. Stocker, Stanford University, Stanford, CA). Bacterial strains were routinely grown at 37°C in Luria-Bertani broth or agar (Difco). At indicated DPI, mice were sacrificed, and SI, MLN, and spleen (SP) were collected. Lymphocytes from intestinal LP, MLN, and SP were isolated as described previously (26). Because PG in suckling mice intestines were difficult to isolate, while SI were digested to isolate lymphocytes. Isolated lymphocytes were used for flow cytometry analysis (see below). Additional SI were collected for immune fluorescent staining (see below).

In vivo treatment with Abs to CCL25, CCL28, and integrin αβ7

Five-day-old BALB/c, CCR9 KO, or WT control mice were infected with ECw and injected i.p. with rat mAbs against CCL25 (IgG2B, clone 89818) and CCL28 (IgG2B, clone 134306) (R&D Systems) either individually or in combination, or purified rat IgG2B as an isotype control Ab (R&D Systems) at days 0, 2, 4, and 6 postinfection. The first dose of Ab was 25 µg, and the subsequent doses were 50 µg. In some experiments, anti-α,β7 IgG2B, clone PS/2; American Type Culture Collection) (100 µg for the first dose, and 200 µg for the subsequent doses) was injected i.p. along with anti-CCL25 and anti-CCL28 Abs. Mice were sacrificed at day 7 postinfection. Lymphocytes from SI, MLN, and SP were collected for FACS analysis, and additional SI were collected for immunohistologic analysis. Treatment of CCR9 KO mice consisted of anti-CCL28 and/or anti-α,β7 following the same schedule and doses as for BALB/c mice.

Immunofluorescent staining and confocal microscopy of intestinal tissues

At different DPI (as indicated in Fig. 2), 5-day-old suckling mice were sacrificed, and SI were collected. At each time point at least four mice were examined. To avoid potential bias between litters, mice were collected from different litters at each time point. Collected intestinal specimens were placed in a histocryl embedding and frozen for routine histological and optimal cutting-temperature compound (Sakura Finetek). Sections (7 µm) were then fixed in mechant or acetone for 10 min and washed in PBS. Before staining, sections were treated with 2 mM CuCl2 in 10 mM Tris buffer (pH 7.5) for 1 h to reduce autofluorescence (27). After washing three times in PBs, sections were blocked with 5% normal goat serum and stained with GFP-VLP (10 µg/test) for 1 h at room temperature. Sections were then washed and stained with Texas Red-labeled goat anti-mouse IgA (Southern Biotechnology Associates) for 45 min at room temperature. Finally, after washing, the slides were mounted using aqua-poly/mount mounting medium (Polysciences).

Of note, we and others have previously demonstrated the specificity of the staining using GFP-VLPs to identify RV-specific B cells by showing that there is insignificant binding of GFP-VLPs to B cells in noninfected mice and that GFP-VLP binding can be specifically blocked using unlabelled VLPs or purified virus (28, 29). It is also important to note that after RV infection, the predominant Ab response is directed against the two viral proteins (VP2 and VP6), which are the components of the GFP-VLPs (30).
Flow cytometry analysis

Isolated SI LP, MLN, and SP lymphocytes were stained with different combinations of GFP-VLP and the following anti-mouse Abs (all obtained from BD Pharmingen unless specified): IgA FITC (Southern Biotechnologies Associates); B220 PerCP (RA3-6B2); αββ PE or allophycocyanin (DATK32.2, allophycocyanin-labeled by Chromoprobe); CCR9 allophycocyanin (242503; R&D Systems); CD11b PE (261-2); and a dump PE or biotin, which included Thy 1.2 (53-2.1), CD-11c (HL3), CD-11b (M1/7), and IgD (11–26). After staining, cells were washed once and fixed with 1% paraformaldehyde (Electron Microscopy Sciences). At least 500,000 cells were acquired and four- or five-color flow cytometry was performed using a FACScanLibur or a LSRII (BD Biosciences), and data analysis was performed with CellQuest Pro (BD Biosciences). Dead cells were excluded by forward and side scatter gating.

To measure lymphocyte proliferation in vivo, infected suckling BALB/c mice were injected i.p. with 100 μg of BrdU (BD Pharmingen) at 6 DPI. Mice were sacrificed 16–18 h after BrdU administration, and lymphocytes from LP, MLN, and SP were isolated. BrdU staining was performed using the BrdU Flow Kit (BD Pharmingen) following the manufacturer’s instructions. To compare the levels of lymphocyte apoptosis after anti-chemokine Ab treatment, LP and MLN lymphocytes from anti-chemokine or isotype control Ab-treated mice at 7 DPI were collected and stained using the annexin V-PE apoptosis detection kit I (BD Pharmingen) following the manufacturer’s instructions.

Quantification of IgA production in SI by ex vivo intestinal fragment culture

Ex vivo intestinal fragment culture for measuring IgA secretion from SI was performed as described previously (31). Five-day-old CCR9 KO suckling mice were orally infected with ECw and treated with anti-CCL28 or isotype control as described above. At 7 DPI, mice were sacrificed, and SI sections of ~0.5 cm long were collected and individually weighted. The intestinal sections were opened longitudinally, washed, and cultured in 95% O2 and 5% CO2 chamber. After 3 days of culture, the media were collected, and total and RV-specific IgA concentration were determined by ELISA as described previously (32). IgA production by intestinal fragments was expressed as micrograms of IgA per 100 mg of intestine weight.

Measurement of intestinal CCL25 and CCL28 by ELISA

ELISA kits for the detection of CCL25 and CCL28 were purchased from R&D Systems. SI from RV-infected or uninfected suckling mice were collected at 1 and 3 DPI and immediately frozen at ~70°C. Before assay, 250 μl of PBS with protease inhibitor mixture including 4-(2-aminophenyl)benzenesulfonyl fluoride HCl (1 mM), E-64 (10 μM), EST (40 μM), leupeptin hemisulfate (10 μM), pepstatin A (1 μM), Na-tosyl-lys chloromethyl ketone, HCI (40 μM), and Na-tosyl-phe chloromethyl ketone (40 μM) (Calbiochem) was added to each sample and homogenized. The homogenates were centrifuged at 10,000 rpm for 10 min. Supernatants were serially diluted before addition to ELISA plates. Mouse CCL25 and CCL28 detection was performed according to the manufacturer’s instructions. Protein concentration for each sample was determined with a BCA protein assay kit (Pierce). The amount of chemokine in each intestinal sample was expressed as picograms of CCL25 or CCL28 per milligrams of tissue protein.

Statistical analysis

Statistical analyses were performed with StatView (SAS Institute). Nonparametric Mann-Whitney U test (two-tailed) was used to analyze immunofluorescent staining data. Two-way ANOVA was used to evaluate the statistical significance of treatment factors such as RV infection, anti-chemokine Ab treatment, Ab production by ex vivo intestinal fragment culture, and quantification of chemokines by ELISA. Because it was necessary to combine several independent experiments with variable baseline results, we also included an additional factor that identified individual experiments in the ANOVA model. Therefore, the p values for treatment effects reported in Results were adjusted for interexperiment variations. In addition, the data were log transformed for the chemokine’s quantification data by ELISA to normalize the data distribution.

Results

RV infection speeds up the accumulation of total and RV-specific IgA+ PC in intestinal LP in suckling mice

Five-day-old BALB/c mice were orally infected with WT RV ECw. At various DPI, mice were sacrificed and the SI isolated and frozen in Tissue-Tek optimal cutting temperature compound. The frozen tissue sections were stained with Texas Red-labeled goat anti-IgA and GFP-VLP to detect IgA+ B cells and RV-specific B cells, respectively. IgA+ and GFP-VLP+ cells were observed by confocal microscopy. The subset of IgA+ cells that we identified were large, B220+ and primarily located in the LP and parafollicular region of PP (data not shown), indicating these cells were IgA+ plasmablasts and PC. Fig. 1 shows IgA+ cells in the intestinal LP of infected and noninfected mice at 12 days of age (equivalent to 7 DPI). IgA+ cells could be detected in the LP of RV-infected suckling mice starting 5 DPI (10 days after birth; Fig. 1A), and the numbers increased rapidly during the course of infection (Fig. 2). In contrast, IgA+ cells were virtually nondetectable in the LP of noninfected mice during the first 11 days after birth (equivalent to 6 DPI; Fig. 1B), and the accumulation of IgA+ cells occurred slowly during the first 2 wk after birth (equivalent to 9 DPI) (Fig. 2). The speed of accumulation in uninfected mice increased thereafter and reached the levels comparable to the infected mice around 3 wk of age (equivalent to 16 DPI) (Fig. 2). The amounts of IgA+ cells accumulating in the LP (expressed as number of cells per field) were significantly greater in the infected group between
RV-specific IgA⁺ cells, identified by GFP-VLP and IgA double-positive staining, could be detected in infected mice starting at 6 DPI, and the highest numbers were detected at 21 DPI (Fig. 1C). However, the proportion of RV-specific IgA⁺ cells of total IgA⁺ cells in LP of infected mice was low, especially during the first 10 days of infection (6% or less; Fig. 2). Therefore, most of the IgA⁺ cells in LP during RV infection were non-RV specific, and cells were presumably generated as the result of polyclonal B cell activation after infection, as previously shown in adult mice (23).

The rapid accumulation of IgA⁺ cells in the small intestinal LP of RV-infected suckling mice as detected by immunohistologic examination was confirmed by FACS analysis. Mice were sacrificed at 7 DPI, and lymphocytes from LP, MLN, and SP were isolated. For analysis, cells were gated on large lymphocytes, dump (Thy 1.2, CD11c, CD11b, IgD) negative, B220 intermediate or low and percentages of IgA⁺ cells were determined (Fig. 3A). The mean percentage of IgA⁺ plasmablasts in the LP of RV-infected suckling mice at 7 DPI was ~30 times higher than in age-matched noninfected mice (Fig. 3, A and B) ($p = 0.006$). The number of IgA⁺ plasmablasts was also significantly higher in the MLN (5.8 times) of infected mice as compared with the control group (Fig. 3B) ($p = 0.018$). The difference in percentages of IgA⁺ plasmablasts between infected and noninfected mice in LP and MLN remained significant at 10 DPI (data not shown). In contrast, the percentages of IgA⁺ plasmablasts in the SP of both infected and noninfected

**FIGURE 2.** Number of IgA⁺ cells and VLP⁺. RV-specific cells in the LP of infected suckling mice as a function of time. Intestines from infected and noninfected suckling mice were collected at indicated time points and analyzed for the presence of IgA⁺ and IgA⁺/VLP⁺ cells. For each section, 5–10 images were collected. Total IgA⁺ cells and GFP-VLP⁺/IgA⁺ cells were counted, and data were expressed as number of IgA⁺ cells per field and percentage of GFP-VLP⁺ cells per total IgA⁺ cells. The average of the total IgA⁺ cells for each mouse was calculated and used to compute the group mean and SEM, presented in the graph, for each time point. There were significant statistical differences in the number of total IgA⁺ cells between infected and noninfected groups from days 5 to 11 postinfection ($p < 0.05$, Mann-Whitney $U$ test).

**FIGURE 3.** Flow cytometry analysis of IgA⁺ plasmablast accumulation in the intestinal LP after RV infection. A, Lymphocytes were isolated from the intestinal LP of infected (7 DPI) and age-matched noninfected BALB/c and CCR9KO suckling mice and stained with mAbs against Thy1.2/IgD/CD11c/CD11b (dump), B220, IgA, and $\alpha_\beta_2$. For analysis, cells were gated on large, dump⁻, B220⁺/low lymphocytes. Based on this subpopulation, dot plots were created showing expression of IgA and $\alpha_\beta_2$. The percentages of large dump⁻ IgA⁺ cells expressing or not expressing $\alpha_\beta_2$ are indicated in the right quadrants. B, Percentage of large, dump⁻, B220⁺/low IgA⁺ plasmablasts in the intestinal LP and MLN from noninfected and infected BALB/c and CCR9 KO suckling mice (7 DPI). Frequencies of cells expressing IgA were obtained as described in A. Data are summarized from three independent experiments with two to four mice per group for each experiment. *, Significant differences between infected and noninfected mice ($p = 0.006$ in the LP, and $p = 0.018$ in MLN). C, Coexpression of CCR9 and $\alpha_\beta_2$ in MLN and intestinal LP large, dump⁻, B220⁺/low IgA⁺, and GFP-VLP⁺ lymphocytes.
groups were very low (~0.02–0.03%) and were not significantly different between the 2 groups (data not shown) (p > 0.05).

It is important to note that when we examined the LP by FACS analysis, we were unable to dissect out PP and isolated lymphoid follicles. However, the results of the FACS analysis are highly comparable to the results obtained by histologic examination (Figs. 2, and 3, A and B), where we were able to reliably discriminate between PP and the LP, supporting the proposition that the FACS analysis is primarily measuring cells in the LP. The striking differences in IgA + cell numbers in the intestinal LP between RV-infected and noninfected suckling mice at early time points postinfection provided a unique opportunity to study the role of homing and chemokine receptors and their ligands in the migration of IgA + plasmablasts/PC into the LP during a mucosal viral infection.

**Phenotype of RV-specific and IgA + B cells elicited during acute infection**

To better characterize the large lymphocytes, dump negative, B220 intermediate, GFP-VLP +, or IgA + cells elicited during acute RV infection in the MLN and the intestinal LP, five-color staining for FACS analysis was performed. As shown in Fig. 3C, in the MLN, 94% of the IgA + cells were αβ +, and among those 75% coexpressed CCR9. Interestingly, in the MLN, a lower percentage of the GFP-VLP + cells expressed αβ + (50%), but similar to the total IgA + population, of those, 72% coexpressed CCR9. Similarly, in the LP, a high percentage of IgA + cells expressed αβ + (80%), but this percentage was only 38% in the RV-specific cells. Moreover, the expression of CCR9 appeared to be down-regulated in the intestinal LP for both IgA + and VLP + subsets, because only 22 and 36% of the cells expressed this receptor, respectively. The VLP + population in both the LP and the MLN had a lower proportion of αβ + cells than the total IgA + population (Fig. 3C). We also looked at the expression of CD138 on total IgA + cells (data not shown) in the MLN and intestinal LP and found 73 and 62% to be CD138 +, respectively, suggesting that the majority of the IgA + cells elicited after infection are plasmablasts or PC.

**Effect of Salmonella infection in the accumulation of total IgA + cells in the MLN and intestinal LP**

To determine whether the increase in the total number of IgA + cells in the MLN and intestinal LP after RV infection in suckling mice was due to a specific effect of RV, we infected suckling mice with an unrelated intestinal pathogen, *Salmonella*. *Typhimurium*. Interestingly, up to 10 days after *Salmonella* infection, we could not detect an increase in the percentage of IgA + cells in the MLN (Fig. 4). In the intestinal LP, at 7 DPI there was a slight increase in the number of IgA + cells (1%, data not shown). However, this increase was not sustained over time, as it was during RV infection, and by 10 DPI there was no difference in the percentage of IgA + plasmablasts/PC in the LP of *Salmonella*-infected suckling mice when compared with noninfected mice (Fig. 4). We concluded from these experiments that the increase in numbers of total IgA + plasmablasts/PC seen during acute RV infection was not a nonspecific consequence of all enteric infections in suckling mice.

**Effects of anti-CCL25 and CCL28 in IgA + plasmablast accumulation in the LP after RV infection**

To study the role of chemokines CCL25 (TECK) and CCL28 (MEC) in intestinal LP accumulation of IgA + plasmablasts, we treated suckling mice with anti-CCL25 and anti-CCL28 Abs either individually or in combination at 0, 2, 4, and 6 DPI, and the presence of IgA + plasmablasts in LP, MLN, and SP was examined at 7 DPI by FACS (Fig. 5, A and B). When anti-CCL25 and CCL28 were used together, the numbers of IgA + PC in LP were reduced by 62 ± 4.2% (mean ± SEM) as compared with control mice (Fig. 5, A and B, and Table I) (p < 0.0001). Interestingly, when the Abs were used individually, there was a reduction trend in the number of IgA + PC in the LP compared with the isotype control-treated mice (11 ± 4.4 and 19 ± 7.1% for anti-CCL25 and anti-CCL28, respectively) that was not statistically significant (Fig. 5B) (p > 0.05). In addition, we observed that the reduction, when using the combination of anti-CCL25 and anti-CCL28, was higher in the αβ + subset of IgA + plasmablasts (71.4 ± 2.7%) than in the αβ + subset (54 ± 5.2%), and this difference was significant (p = 0.01) (Fig. 5A and Table I).

The reduction of IgA + plasmablasts in the LP after antichemokine treatment was also confirmed by immunofluorescent staining of intestines collected at 7 DPI (Fig. 5C). The reduction between the anti-CCL25/CCL28 and the isotype-treated groups, as measured by immunohistology, was 61.4% (p = 0.04), similar to the reduction measured by FACS. In addition, no apparent differences in the structure of germinal centers or number of cells in the parafollicular region of the PP were observed by histologic examination between groups (data not shown), suggesting that the Ab treatment had little or no histologic impact on the intestinal inductive site of the B cell response.

The administration of anti-CCL25 and anti-CCL28 not only reduced the accumulation of total IgA + plasmablasts/PC in the LP but also the numbers of RV-specific B cells (Fig. 5A and Table I). However, the reduction observed for VLP + plasmablasts was not as pronounced as for total IgA + plasmablasts/PC (41.2 ± 9 and 62 ± 4.2%, respectively), although still significant (p = 0.0035). The αβ + subset of RV-specific B cells was more efficiently blocked than the αβ + subset (68.1 ± 5.2 vs 29.4 ± 10.6%) (p = 0.007) (Table I).

As a consequence of Ab treatment, the numbers of IgA + plasmablasts were increased in the MLN and SP. This effect was consistent between experiments and considerably higher in the SP than in the MLN, where the numbers of IgA + plasmablasts increased by 133 ± 50.9% compared with the isotype control-treated group (p = 0.03) (Fig. 5D). Of note, most of the IgA + cells detected in the SP of treated mice expressed αβ +. The change in number of IgA + plasmablasts in the MLN was small after Ab treatment (23 ± 10.5%) and not significant (Fig. 5D) (p > 0.05).
The treatment with anti-chemokine Abs did not alter IgA⁺ plasmablast replication or apoptosis in RV-infected suckling mice

To exclude the possibility that the reduction of IgA⁺ cells in the LP was caused by an effect of the anti-CCL25 and anti-CCL28 treatment on cell proliferation, mice were injected with BrdU at 6 DPI to label replicating cells in vivo and sacrificed 16–18 h later.

Table I. Differential effect of combined anti-CCL25 and anti-CCL28 treatment in BALB/c mice and anti-CCL28 treatment in CCR9 KO mice on the accumulation of α⁺β⁺, and α⁺β⁻ subsets of IgA⁺ plasmablasts in the LP

<table>
<thead>
<tr>
<th></th>
<th>BALB/c</th>
<th>CCR9 KO</th>
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<tbody>
<tr>
<td></td>
<td>Total IgA⁺</td>
<td>VLP⁺</td>
</tr>
<tr>
<td>Isotype control</td>
<td>62 ± 4.2ᵇ</td>
<td>41.2 ± 9ᵇ</td>
</tr>
<tr>
<td>Anti-CCL25 + CCL28</td>
<td>71.4 ± 2.7ᵇᶜ</td>
<td>68.1 ± 5.2ᵇᶜ</td>
</tr>
<tr>
<td>Anti-CCL25</td>
<td>54 ± 5.2ᵇ</td>
<td>29.4 ± 10.6ᵇ</td>
</tr>
</tbody>
</table>

ᵇ Five-day-old mice were infected with RV and treated with Abs against CCL25 and CCL28. Total IgA⁺ and VLP⁺ cells in LP were detected at 7 DPI by FACS. Data (mean ± SEM) are expressed as percentage of reduction compared to the isotype control-treated mice. Numbers derived from three independent experiments with 2–4 mice per group for each experiment.

ᶜ Significant reduction compared to isotype control-treated mice (p < 0.05).

ᶜᶜ Significant difference between α⁺β⁺ and α⁺β⁻ subsets (p < 0.05).

The anti-chemokine treatment did not modify the levels of replication of IgA⁺ plasmablasts in either LP or MLN (p > 0.05) (Table II). In a separate set of experiments, we stained cells with annexin V and 7-aminoactinomycin D (7-AAD) to assess the effects of Ab treatment on apoptosis of IgA⁺ plasmablasts. The percentage of IgA⁺ cells stained with annexin V alone (measurement...
of an early stage of apoptosis) or contained cells for annexin V and 7-AAD (measurement of dead cells or a late stage of apoptosis) in anti-CCL25/anti-CCL28-treated mice were not different from control mice in either LP or MLN (Table II) \( (p > 0.05) \).

**Effects of RV infection and anti-CCL28 treatment on IgA⁺ plasmablast accumulation in intestinal LP of CCR9 KO**

To confirm and better dissect the roles of CCL25 and CCL28 in IgA⁺ plasmablasts migration to the intestinal LP, we repeated the experiments previously described in BALB/c pups using CCR9 KO mice. RV infection significantly increased the IgA⁺ plasmablast population in both LP and MLN of infected CCR9 KO mice compared with noninfected mice (Fig. 3, A and B). Moreover, the increase was similar to that seen in infected WT controls and BALB/c suckling mice (data not shown and Fig. 3B). These results indicated that RV infection could effectively speed up the accumulation of IgA⁺ plasmablasts in the intestinal LP in the absence of CCR9.

However, when CCR9 KO mice were infected and treated with anti-CCL28 Ab, the number of IgA⁺ plasmablasts in the LP was reduced by 71.1 ± 4.6\% \( (p = 0.02) \) (Table I). The percentage reductions of IgA⁺ \( \alpha_4\beta_7^- \) and IgA⁺ \( \alpha_4\beta_7^- \) lymphocytes were also significant \( (p < 0.01) \), but the difference between these two subsets was not significant \( (p > 0.05) \) (Table I). The blockade of RV-specific (VLP⁺) cells in the LP of CCR9 KO mice by anti-CCL28 was also significant when compared with isotype control Ab-treated mice \( (p = 0.02, 0.04, \text{and } 0.02 \text{ for VLP⁺, VLP⁺} \alpha_4\beta_7^-, \text{and VLP⁺} \alpha_4\beta_7^- , \text{respectively}) \). As a control for the CCR9 KO mice, we treated WT mice with Abs to CCL25 and CCL28 alone or in combination. Only combined anti-CCL25 and anti-CCL28 treatment could effectively reduce the IgA⁺ plasmablasts accumulation in the LP, as was observed for WT BALB/c mice (data not shown).

**Effect of anti-chemokine treatment on intestinal IgA production**

To investigate whether, as a consequence of the reduced number of IgA⁺ plasmablasts in the LP after anti-chemokine Ab treatment, there would be an impact in the intestinal IgA production, we isolated SI fragments from RV-infected and anti-CCL28-treated CCR9 KO mice at 7 DPI, cultured them in vitro for 3 days, and measured IgA by ELISA in the culture supernatant. The intestinal IgA production, expressed as micrograms of IgA per 100 mg of intestine, was significantly reduced in CCR9 KO mice treated with anti-CCL28 compared with the isotype control Ab-treated mice \( (71.7\% \text{ reduction}) \ (p = 0.01) \) (Fig. 6A). However, no reduction was observed in the amount of RV-specific IgA between groups (data not shown), which is probably due to the less efficient blocking of RV-specific plasmablasts accumulation in the LP after the anti-chemokine treatment (Table I). Intestinal IgA production was also determined from intestinal fragments of RV-infected BALB/c mice treated with anti-CCL25 and anti-CCL28 Abs, and results were comparable to the anti-chemokine-treated CCR9 KO mice (data not shown).

The interaction between the intestinal homing receptor \( \alpha_4\beta_7 \) and the chemokines CCL25 and CCL28 in IgA⁺ plasmablasts accumulation in LP

It has been shown that \( \alpha_4\beta_7 \) is crucial for lymphocyte homing to the gut. We investigated whether combined anti-\( \alpha_4\beta_7 \) and anti-chemokines CCL25 and CCL28 treatment would result in a more effective blockade of IgA⁺ plasmablast accumulation in the LP after RV infection, compared with anti-chemokine treatment alone. For this purpose, CCR9 KO mice were infected and treated with anti-\( \alpha_4 \), anti-CCL28, or anti-\( \alpha_4 \) plus CCL28. The percentage reductions of IgA⁺ plasmablasts in the LP were similar among anti-\( \alpha_4 \) and anti-CCL28-treated mice (Fig. 6B), and, interestingly, we observed that the concomitant administration of anti-\( \alpha_4 \) and...
anti-CCL28 did not enhance blockade over anti-α4 alone (Fig. 6, B and C). These results suggest that the specificity of IgA+ plasmablasts for intestinal gut homing is determined by the coexpression of the homing receptor α4β7 and either CCR9 or CCR10. Of note because PS/2 is an anti-α4 Ab, it will interact with both α4β7 and α4β1 receptors. However, we conducted similar experiments using a specific anti-α4β7 Ab (DAKT32, IgG2a) and obtained similar results (data not shown).

Effect of RV infection on CCL25 and CCL28 expression in SI of suckling mice

To measure CCL25 and CCL28 expression after RV infection, we isolated the SI of BALB/c suckling mice at 1, 3, and 5 DPI. The quantities of chemokines in the intestine were determined by ELISA, and results were expressed as picograms per milligrams of tissue protein (Fig. 7). At 1 DPI, there was no significant difference in CCL25 or CCL28 expression between infected and noninfected mice (p > 0.05). However, CCL25 was significantly increased in the SI of RV-infected mice both at 3 and 5 DPI (p < 0.05). In addition, CCL28 was also significantly increased at 3 DPI (p = 0.007). These results suggested that RV infection in suckling mice increases the quantity of CCL25 and CCL28 in the SI, hence facilitating the recruitment into the LP of the cells expressing their receptors.

Discussion

In this study, we document the impact of RV infection on the early accumulation of IgA+ plasmablast and PC in the intestinal LP of infant mice and the role that the integrin α4β7 and the chemokine receptors CCR9 and CCR10, along with their ligands, play in this process.

It has been previously shown that the number of plasmablasts/PC in the MLN, PP, and intestinal LP greatly depends on exogenous antigenic exposure (33). In fact, in these tissues hardly any IgA+ PC could be detected in the first weeks of life in uninfected suckling mice, but immediately after weaning the number of IgA+ PC increased considerably (Ref. 33 and Fig. 2). In suckling mice (5 days old), we were able to determine that RV infection not only induces a virus-specific immune response but also a massive bystander activation/proliferation of B cells in the MLN (Fig. 3B) and PP (data not shown). This effect appears to be specific to only some enteric pathogens, because we show that Salmonella infection does not lead to proliferation of B cells in the intestine (Fig. 4). We selected Salmonella for comparison because it is, like RV, an enteric pathogen of both mice and humans. Previous reports suggest that reovirus infection of suckling mice might have a similar effect as RV on B cell activation (34). It has recently been proposed, in adult mice, that the mechanism by which RV induces the polyclonal activation of B cells is likely to be TLR4 mediated (35), and future experiments will determine whether this is also the case in suckling mice. Because Salmonella also activates TLR4, it is unclear why B cell proliferation was not seen during this infection (36).

Our data also show that this polyclonal activation resulted in an enhanced accumulation of total IgA+ plasmablast/PC to the intestinal LP of suckling mice during the first 2 wk after infection (Figs. 1 and 2). Taking into account the substantial and easily quantified differences in the number of intestinal IgA+ plasmablasts/PC between RV-infected and noninfected suckling mice, RV infection provided an excellent system to study the mechanisms that control the specific B cell population of the intestinal LP after a natural viral infection, and perhaps, in the absence of infection as well.

In this study, we were able to characterize the homing phenotype of an important subset of both RV-specific and total IgA+ plasmablasts/PC in the MLN that coexpressed α4β7 and CCR9 on their surface (Fig. 3C). We believe that these cells are likely to also express CCR10, because in previous chemotaxis experiments we have shown that after RV infection a large subset of IgA+ and RV-specific B cells from the MLN migrated to both CCL28 and CCL25 (22). Of note, this result is comparable with our previous findings in humans, where we showed that during acute infection in children, RV-specific plasmablasts coexpressed α4β7, CCR9, and CCR10 and that the overall circulating plasmablast population had up-regulated the expression of these receptors on their surface (22). We hypothesized, as has been suggested for T cells (37), that because the activation of the B cells occur in the inductive sites of the intestine (i.e., PP and MLN), dendritic cells in these tissues would induce expression of high levels of α4β7, CCR9, and CCR10 (i.e., gut homing imprinting phenotype) in all the locally activated B cells, and not only the RV-specific ones. However, the intestinal LP RV-specific and total IgA+ plasmablasts/PC expressed lower levels of α4β7 (Fig. 3, A and C) compared with the

FIGURE 7. RV infection increases the quantity of CCL25 and CCL28 in the SI of suckling mice. Five-day-old BALB/c mice were infected with RV. At 1, 3, and 5 DPI, mice were sacrificed, and SI were isolated. The amount of CCL25 (A) and CCL28 (B) in small intestinal samples was determined by ELISA (see Materials and Methods). Data from three independent experiments with three to four mice per group for each experiment, expressed as the geometric mean of the amount of chemokine (in picograms) per milligrams of total intestinal protein and SEM. *, Significant difference between the infected and noninfected group (p < 0.05).
MLN, and we could not detect significant levels of expression of CCR9 (Fig. 3C). The fact that only a small percentage of RV-specific plasmablasts/PC express α4β7 in the intestinal LP of suckling mice is in agreement with previous studies in adult mice (28) and could reflect down-regulation of this receptor once the cells reach their effector site. Although prior studies indicate that β7 KO B cells fail to mediate anti-RV effects in RAG 2 KO mice (9), the β7 KO experiments were done using cells derived from the SP of immune mice, and these cells may have a different effector phenotype than the cells studied here. Concerning the low expression of CCR9 in the LP, our findings are in agreement with a previous study (13) and also suggests that upon entry into the intestinal LP, RV-specific and total IgA+ plasmablasts/PC down-regulate CCR9 expression, perhaps because they have reached their effector site. An alternative explanation could be that these cells are activated by the ongoing RV infection and down-regulate CCR9 as a result of this activation. It remains to be determined whether the expression of CCR10 is also down-regulated in the plasmablasts/PC in the intestinal LP during RV infection.

Our data suggest that the enhanced accumulation of IgA+ plasmablasts/PC in the LP observed following RV infection is the result of recruitment of lymphocytes activated in the PP and MLN. Of note, short-term homing experiments would be required to directly test this hypothesis, but such experiments have been extremely difficult to perform in suckling mice. Therefore, we provided indirect evidence to support the proposition that plasmablasts trafficking from inductive sites are responsible for the LP accumulation. First, we showed that this accumulation could be significantly inhibited by Abs directed to the chemokines CCL25 and CCL28 and the integrin α4. Interestingly, previous studies of short-term homing in adult mice using the PS/2 Ab have demonstrated the inhibitory effect of this Ab in lymphocyte homing to the intestine (38). Second, the chemokine Ab treatment did not have an effect on cell division or apoptosis of plasmablasts/PC in the LP. Third, after treating the mice with the anti-chemokines Abs, we detected a redistribution of the plasmablasts/PC to systemic sites in the SP (Fig. 5D), and these cells were predominantly α4β7+. In addition, using histologic analysis, we observed that PP in the anti-chemokine-treated mice appeared identical with those in untreated or isotype-treated control mice after RV infection. This last result led us to believe that the reduced number of plasmablasts/PC in the LP after anti-chemokine administration was not the result of an effect of the Ab treatment on the intestinal inductive site.

Finally, we were also able to show that RV infection not only had an effect on the number and homing phenotype of B cells elicited during acute infection but also lead to increased CCL25 and CCL28 expression in the intestine of suckling mice at early time points after infection (Fig. 7). The up-regulation of these chemokines would facilitate the recruitment of the cells bearing their respective receptors on their surface. Interestingly, a previous report in humans showed that CCL28 was up-regulated upon inflammation in the colon (39), and it has also been shown in mice, during influenza infection, that there is up-regulation of the expression of inflammatory chemokines in the lung (40). Whether the effect of RV infection on chemokine expression is related to inflammation as in the studies mentioned above or is mediated through other mechanisms remains to be determined.

Taken together, all of these results suggest that after RV infection there is active recruitment of RV-specific and total IgA+ plasmablasts/PC into the intestinal LP in which the chemokines CCL25 and CCL28 and the integrin α4 play an active role. It is important to clarify here that these three molecules would likely play their respective roles at different points in the multistep homing model. For instance, the interaction of the integrin α4β7 and its receptor MADCAM-1 brings the lymphocytes in contact with the endothelium, where the tethered cells have an opportunity to detect chemotactic gradients from the underlying tissue. In contrast, the CCL25/CCR9 and CCL28/CCR10 interactions lead to migration across the endothelium and potentially to the cell localization and retention in the intestinal LP. Interestingly, both CCL25 and CCL28 enhance the α4-dependent adhesion of IgA+ plasmablasts to MADCAM-1 (12).

An alternative, but less likely, explanation of our findings would be that the accumulation of IgA+ plasmablasts observed in the LP after RV infection is mainly due to sideways migration of these cells from intestinal inductive sites. However, if this was the case, the combined anti-chemokine treatment would not be expected to significantly reduce the number of plasmablasts/PC detected by FACS in the SI (which includes the inductive sites) nor would redistribution of cells to the SP and MLN be anticipated (Fig. 5D). In addition, it has been shown that many of the plasmablasts found near to the gut-associated lymphoid tissue follicles apparently belong to exhausted B cell clones with decreased J-chain expressing potential and increased class switching to IgG (reviewed in Ref. 19).

Our experiments demonstrating the inhibition of recruitment of plasmablasts/PC to the intestinal LP only by the combined effects of Abs to CCL25 and CCL28 provide, for the first time, evidence of the functionally redundant role of the chemokine receptors CCR9 and CCR10 and their ligands. Two different sets of experiments support this conclusion. First (Fig. 3, A and B), we showed that in CCR9 KO suckling mice, a normal response to RV infection was elicited because we found comparable numbers of RV-specific and total IgA+ plasmablasts/PC in the intestinal LP and MLN of these animals compared with controls. Furthermore, in these KO mice, the length and severity of diarrhea during RV infection was the same as controls (data not shown). Second, as shown in Fig. 5, A and B, only when WT BALB/c mice were treated with anti-CCL25 and CCL28 simultaneously could we significantly reduce the number of RV-specific and total IgA+ plasmablasts/PC in the LP, whereas treatment with each Ab alone had little or no effect. This result might have been expected because, as discussed previously, we have evidence to support the fact that these cells coexpress the two chemokine receptors on their surface.

Of note, these results are inconsistent with two previous reports. Hieshima et al. (12) showed that in adult mice immunized with CT, individual treatment with either anti-CCL25 or CCL28 alone had a significant effect on the number of CT IgA+ PC in the intestinal LP. Also, Pabst et al. (13) showed an impaired response to OVA plus CT in CCR9-deficient mice. The fact that these studies were conducted in adult mice, that the immunizations were not exclusively enteric, and that the nature of the Ag studied differed from the one in our study, could account for the differences observed. In addition, it is unclear what the actual homing and chemokine receptor repertoire expressed on the surface of the B cells was in the study by Hieshima et al. (12). If different from the one in our study, specifically if the Ag-specific cells do not coexpress CCR9 and CCR10, this could explain the differences observed using individual anti-chemokine treatment.

The finding of functional redundancy for chemokine receptors and their ligands does not appear to be exclusive to the gut because in skin homing, CCR4 and CCR10 are simultaneously expressed in a subset of effector T cells, can both support homing of T cells to skin, and either one or the other is required for
lymphocyte recruitment in cutaneous delayed-type hypersensitivity (41, 42).

It is also important to point out that the addition of the anti-α4 Ab to the anti-chemokine treatment did not augment the reduction of virus-specific or total IgA+ plasmablasts in the LP. Moreover, we showed (Table I) that the population that is preferentially blocked by the anti-chemokine treatment is the α4β7+ one. These two findings are in agreement with the phenotype of the B cells induced during infection, because these B cells coexpress α4β7, CCR9, and, most likely, CCR10, and treatment with the anti-α4 and the anti-chemokine Abs is apparently targeting the same cell population.

Of interest, even when using these three Abs at optimal concentrations, we were only able to reduce by ~60% the number of IgA+ cells present in the intestinal LP, compared with isotype control-treated mice. This finding suggests that other molecules might also be involved in homing of lymphocytes to the gut and that a subset of IgA+ plasmablasts/PC primed in the PP and MLN could express a different combination of integrin/chemokine receptors, allowing them to be recruited into the LP. This hypothesis is supported by preliminary experiments, where we have infected suckling β7- KO mice and have observed a delayed but significant accumulation of RV-specific and IgA+ PCs in their LP. Additional experiments in these KO mice, as well as the generation of dual CCR9/10 KO mice will allow us to better understand whether other molecules are involved in homing to the gut. Previous reports suggest that the chemokine receptors CCR4 and CCR6 might also play a role in human PC recruitment to the bone marrow and other tissues including the intestine where their respective ligands (CXCL12 and CXCL16) are expressed (43). Alternatively, the possibility remains that the plasmablast/PC population that we detect in the LP, despite the blocking treatment, arises from a residual B cell population originally present in the LP, which upon RV infection, actively proliferates in situ. Ongoing studies will determine whether the trafficking model suggested in this study can be extrapolated to the recruitment of IgA+ plasmablasts/PC into the intestinal LP of infant and adult mice during normal circumstances (i.e., noninfection).

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Disclosures

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References


