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Intrarectal Immunization and IgA Antibody-Secreting Cell Homing to the Small Intestine

Davide Agnello,* Damien Denimal,* Amandine Lavaux,* Leslie Blondeau-Germe,* Bao Lu,†‡ Norma P. Gerard,†‡ Craig Gerard,†‡ and Pierre Pothier*

According to the current paradigm, lymphocyte homing to the small intestine requires the expression of two tissue-specific homing receptors, the integrin α4β7 and the CCL25 receptor CCR9. In this study, we investigated the organ distribution and the homing molecule expression of IgA Ab-secreting cells (ASCs) induced by intrarectal immunization with a particulate Ag, in comparison with other mucosal immunization routes. Intrarectal immunization induces gut-homing IgA ASCs that localize not only in the colon but also in the small intestine, although they are not responsive to CCL25, unlike IgA ASCs induced by oral immunization. The mucosal epithelial chemokine CCL28, known to attract all IgA ASCs, does not compensate for the lack of CCL25 responsiveness, because the number of Ag-specific cells is not decreased in the gut of CCR10-deficient mice immunized by the intrarectal route. However, Ag-specific IgA ASCs induced by intrarectal immunization express the integrin α4β7, and their number is considerably decreased in the gut of β7-deficient mice immunized by the intrarectal route, indicating that α4β7 enables these cells to migrate into the small intestine, even without CCL25 responsiveness. In contrast, IgA ASCs induced by intranasal immunization express low α4β7 levels and are usually excluded from the gut. Paradoxically, after intranasal immunization, Ag-specific IgA ASCs are significantly increased in the small intestine of β7-deficient mice, demonstrating that lymphocyte homing is a competitive process and that integrin α4β7 determines not only the intestinal tropism of IgA ASCs elicited in GALTs but also the intestinal exclusion of lymphocytes primed in other inductive sites. 

The online version of this article contains supplemental material.

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Abbreviations used in this article: ASC, Ab-secreting cell; CLN, cervical lymph node; CT, cholera toxin; dLN, draining lymph node; D-PBS, Dulbecco’s PBS; i.n., intranasal; i.r., intrarectal; LN, lymph node; LP, lamina propria; MAECAM-1, mucosal addressin cell adhesion molecule-1; MLN, (superior) mesenteric lymph node; PC, plasma cell; pIgR, polymeric Ig receptor; PP, Peyer’s patch; rm, recombinant mouse; r.t., room temperature; RV, rotavirus; sIgA, surface IgA; VLP, viral-like particle; VP, viral protein.

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dition, only lymphocytes that have been primed in the MALT are able to migrate into mucosal organs (although not exclusively into the mucosa of origin), whereas lymphocytes primed in systemic lymphoid tissues are mostly excluded from mucosal sites (10). For instance, immunization by the oral route induces Ab production not only in the gut but also in the salivary and mammary glands, whereas intranasal (i.n.) immunization stimulates a strong immune response in the respiratory and cervicovaginal mucosa (10). However, i.n. immunization does not induce an effective immune response in the intestine, and oral immunization fails to elicit Ab production in the female genital tract (10). This preferential dissemination of effector lymphocytes to privileged mucosal sites seems to be largely determined by particular “homing receptors,” mainly integrins and chemokine receptors, which recognize tissue-specific adhesion molecules and chemokines on endothelial cells (13, 14). The different expression of these receptors on the surface of T and B lymphocytes is known to depend on the priming site. For instance, the vast majority of IgA ASCs and T cells activated in the GALT express the integrin α4β7, which binds specifically to the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on postcapillary venules of the small and large bowel (13, 14). Moreover, lymphocytes destined to home to the small intestine also express the CCR9 receptor for the chemokine CCL25. This chemokine is expressed by crypt epithelial and endothelial cells in the small intestine, where it plays an important role in the recruitment of T cells and IgA ASCs, but not in the colon or other mucosal tissues (13–21).

Immunization by the intra nasal (i.r.) route in humans has been mainly investigated for its ability to elicit a local Ab response in the colorectal mucosa, with the aim of protecting against sexually transmitted diseases (22–24). Other studies showed that in mice and nonhuman primates i.r. immunization with an HIV peptide also induced cellular immune responses in the small intestine, and protected macaques against simian/HIV infection, because it cleared the virus from its major intestinal reservoir (25, 26). In a previous study, we showed that in mice i.r. immunization with RV recombinant viral-like particles (VLPs) composed of VP2 and VP6, provided better protection against RV infection than that achieved by immunization with VLPs administered via the i.n. route (27). We also reported that although i.r. immunization with these VLPs induced lower anti-RV Ab levels in serum than those achieved with i.n. immunization, intestinal IgA production after RV infection was higher in mice immunized by the i.r. route (27). Taken together, these results suggest that i.r. immunization might be a suitable alternative to oral immunization for inducing an immune response in the small intestine, while avoiding immunogen degradation by gastrointestinal enzymes. In the present work, we investigate the homing properties of Ag-specific IgA ASCs induced by i.r. immunization. We report that IgA ASCs primed by i.r. immunization are not responsive to the small intestinal chemokine CCL25. However, i.r. immunization imparts IgA ASCs with the expression of the integrin α4β7, which allows them to migrate not only to the colon but also to the small intestine, even in the absence of CCL25 responsiveness. Finally, we also showed that the CCR10 receptor for the chemokine CCL28, which is produced by epithelial cells in several mucosal organs and attracts virtually all IgA ASCs (20, 28–30), was not required for the intestinal localization of Ag-specific IgA ASCs induced by i.r. immunization.

Materials and Methods

Mice and immunizations

Ten-week-old female BALB/cByJ mice (purchased from Charles River Laboratories, L’Arbresle, France) were used throughout the study, unless otherwise indicated. Igfb7-deficient mice on the C57BL/6 background (C57BL/6-Igfb7tm1[NK])(31) were originally purchased from The Jackson Laboratory (Bar Harbor, ME). The Ccr10-deficient mice were generated as previously described (32) and crossed to the C57BL/6 background for >10 generations. Both strains were bred under specific pathogen–free conditions at the Central Animal Facility of the University of Burgundy (Dijon, France). Female offspring derived from homozygous × heterozygous breeding pairs were used for the experiments, when they reached 8 to 12 wk of age. The genotypes of each mouse was determined by PCR analysis of blood genomic DNA, and only homozygous littersmates were used as experimental controls for homologous Ccr10-deficient mice. Procedures involving animals and their care were conducted in accordance with national and international laws and policies, and were approved by the Ethics Committee for Animal Experimentation of the University of Burgundy.

VLPs were produced by coinfected High Five insect cells (Invitrogen, Carlsbad, CA) with two recombinant baculoviruses expressing the VLPs of the RF strain of bovine RV, and purified by ultracentrifugation on a CsCl gradient, as previously described (33). To eliminate CsCl, the recombinant particles were subjected to several cycles of concentration on centrifugal filter units (Vivaspin 500; Sartorius Stedim, Goettingen, Germany; cut-off: 30 kDa) and dilution in Dulbecco’s PBS (D-PBS; Lonza, Basel, Switzerland). The mice were deprived of food for 24 h before immunization, and during this time a grid was placed on the bottom of the cage to prevent the animals from ingesting the litter. Immediately prior to the procedure, the mice were anesthetized by i.p. administration of 80 mg/kg of ketamine (Imalgene; Merial, Lyon, France) and 16 mg/kg of xylazine (Rompun; Bayer, Puteaux, France), diluted in 200 μl of D-PBS. The mice were immunized by the i.r. route with 5, 10, or 20 μg of VLPs, depending on the experiment, mixed with 3 μg of CT (Sigma-Aldrich, St. Louis, MO), diluted in 50 μl of D-PBS; that is, 5 μg of CT in 5 μl of D-PBS. The mice were immunized three to four times every other week and killed either 3 wk after the last immunization, to perform experiments with VLP-specific IgA ASCs in peripheral organs, or 5 d after the last immunization, to perform experiments with VLP-specific IgA ASCs in draining lymph nodes (dLNs), spleen, or blood (unless otherwise indicated).

Cell isolation from intestinal LP

The small intestines, carefully cleaned of any visible PP, the cecae, and the colons were cut longitudinally to expose the epithelium. Intestinal contents were removed by rinsing the opened organs with D-PBS. The resulting tissue strips were then cut in 0.5- to 1-cm segments that were washed twice in 20 ml of divalent cation-free HBSS (Invitrogen) with 10 mM HEPES (HBSS/HEPES) and 1% FCS. The epithelial cell layer was removed by incubating intestinal segments three times (colons and ceca) or four times (small intestines) for 15 min at room temperature (r.t.), with stirring, in 20 ml of HBSS/HEPES containing 5 mM EDTA (Sigma-Aldrich). The tubes were vigorously agitated at the end of each incubation, and supernatants with released cells were discarded. To neutralize and remove residual EDTA, the remaining tissues were then washed twice in 20 ml of bicarbonate-free RPMI 1640 medium containing 25 mM HEPES (Eurobio, Courtaboeuf, France) and supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 2.5 μg/ml Fungizone, 1 mM CaCl2, 1 mM MgCl2, and 10% heat-inactivated FCS (hereafter referred to as “digestion medium”). Segments were then minced into small pieces and incubated twice (colons and ceca) or three times (small intestines) for 50 min at 37°C, with stirring, in 20 ml of digestion medium containing 300 collagen digestion units per milliliter collagenase type VIII (Sigma-Aldrich) and 50 μg/ml DNase I (Roche, Mannheim, Germany). Supernatants containing LP lymphocytes were collected after each incubation session and filtered through a 100-μm nylon mesh. Released cells were immediately washed, resuspended in ice-cold RPMI/HEPES (RPMI 1640 medium containing 2 g/l sodium bicarbonate [Invitrogen] and supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 10% heat-inactivated FCS), and resuspended in ice. The digestion sessions of isolated LP cells were pooled and washed again, resuspended in 5 ml of 44% isotonic Percoll (GE Healthcare, Uppsala, Sweden) in D-PBS, and overlaid onto 5 ml of 67.5% isotonic Percoll, in 15-ml tubes precoated with FCS. After gradient centrifugation, the cells were recovered from the interface of the Percoll layers and then washed three times in 50 ml of cold HBSS/HEPES supplemented with 100 μg/ml gentamycin and 10 μg/ml streptomycin, and finally resuspended in 1 ml of cold HBSS/HEPES at a concentration of ~10^6 cells/ml. The cells were then immediately transferred to 2 ml of ice-cold RPMI/HEPES (RPMI 1640 medium containing 2 g/l sodium bicarbonate [Invitrogen] and supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 10% heat-inactivated FCS), and resuspended in ice. The digestion sessions of isolated LP cells were pooled and washed again, resuspended in 5 ml of 44% isotonic Percoll (GE Healthcare, Uppsala, Sweden) in D-PBS, and overlaid onto 5 ml of 67.5% isotonic Percoll, in 15-ml tubes precoated with FCS. After gradient
centrifugation (750 × g, 30 min, 4°C), banded cells at the interface between the two Percoll layers were collected, washed twice with ice-cold RPMI/FCS medium, and used for ELISPOT assay.

When small intestinal LP cells were prepared for the chemotaxis assay, the mice were killed 5 d after the last immunization and perfused before tissue removal with 20 ml of D-PBS containing 75 U/ml heparin through the left ventricle, to exclude from the analysis the significant number of Ag-specific IgA ASCs present in the blood of immunized mice at this time point (Fig. 2C). Intestinal lymphocytes were then isolated as described above, except that tissue dissociation was performed in digestion medium containing 0.5% low-endotoxin BSA (A9191; Sigma-Alrich) instead of FCS. Released cells were washed twice in r.t. RPMI/FCS medium (RPMI 1640 medium containing 2 g/l sodium bicarbonate, supplemented with 2 mM l-glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES, and 0.5% low-endotoxin FCS). The lymphocytes were washed and resuspended in a humidified 5% CO2 incubator and used for chemotaxis assay, without Percoll purification, after 1-h incubation at 37°C and 5% CO2.

Cell isolation from lung and bone marrow

The lungs were collected from the thoracic cavities, rinsed in HBSS/HEPES with 1% FCS, minced into small pieces, and incubated twice for 50 min at 37°C, with stirring, in 20 ml of digestion medium with 0.05% collagen digi- stions per milliliter collagenase type VIII and 50 μg/ml DNase I. Supernatants containing lymphocytes were filtered through a nylon mesh, and released cells were pooled, washed, and purified by Percoll gradient centrifugation, as described for intestinal lymphocytes (see above).

Bone marrow was obtained from the mouse femora by cutting the two ends and flushing the bone canal with ~5 ml of RPMI/FCS medium with a 21-gauge needle. Bone marrow cells were washed with HBSS/HEPES, then incubated for 10 min at 37°C in 5 ml of RPMI/FCS medium. The cells were then resuspended in 10 ml of ACK buffer (Lonza) to remove erythrocytes. Lysis was stopped by adding 35 ml of D-PBS with 2 mM CaCl2 and 0.5 mM MgCl2 (D-PBS + Ca2+/Mg2+), following overnight incubation at 4°C with 50 μg/ml mouse FcγRIIb–specific IgG Fc chimera (both from R&D Systems) in D-PBS containing 0.9 mM CaCl2 and 0.5 mM MgCl2 (D-PBS + Ca2+/Mg2+), followed overnight incubation at 4°C with 50 μg/ml goat anti-human IgG (y-chain–specific; Sigma-Alrich) in D-PBS + Ca2+/Mg2+. After washing with D-PBS, 50 μl of 2% endotoxin-free BSA in D-PBS + Ca2+/Mg2+ were added to each well to block any remaining nonspecific binding site. Plates were incubated for 2 h at 37°C, and then wells were washed again with D-PBS. LN or spleen cells were rested for 1 h at 37°C and 5% CO2 in RPMI/FCS medium at 2 × 105 cells/ml. Blood lymphocytes were resus- pend in r.t. RPMI/FCS medium and used for chemotaxis assay, after 1-h incubation at 37°C.

Spleens were mechanically dissociated into single-cell sus- pensions by pressing the tissues through a wire mesh with a rubber syringe plunger, in ice-cold RPMI/FCS medium. Lymphocytes were subsequently washed in RPMI/FCS medium and used for the assays after 1-h incubation at 37°C.

100 chemotaxis experiments performed with MLN cells, ASCs were enriched by magnetic depletion of CD90+ T cells and sIgD+ naive B cells. Equal numbers of streptavidin-conjugated magnetic beads (Dynabeads M-280 streptavidin; Invitrogen) were precoated, separately, with 1.5 × 107 beads/ml and 8 × 107 beads/ml of biotinylated rat anti-mouse CD90.2 Ab (clone 53-2.1; BD Biosciences) and 1 × 107 beads/ml of biotinylated rat anti-mouse CD90.2 Ab (clone 53-2.1; BD Biosciences), according to the manufacturer’s instructions. Coated beads were then washed in D-PBS with 0.1% endotoxin-free BSA and 2 mM EDTA (D-PBS/BSA/EDTA), and combined. MLNs were dissociated in RPMI/FCS medium; then lymphocytes were resuspended in D-PBS/BSA/EDTA, mixed with the beads, and in- cubated for 45 min at 4°C, with tilting, at final concentrations of 1 × 107 cells/ml and 8 × 107 beads/ml. CD90+ and sIgD+ lymphocytes were re- moved with a magnet, and the unbound cells, enriched for ASCs, were washed in RPMI/BSA medium and used for chemotaxis assay, after 1-h incubation at 37°C.

Blood cells were washed and resus- pended in RPMI/FCS medium, when used for ELISPOT assay, or in RPMI/ BSA medium, when used for adhesion assay.

In other experiments, blood lymphocytes were separated from RBCs by Percoll gradient centrifugation. Cells contained in ~700 μl of blood were washed in 10 ml of serum-free medium at r.t., resuspended in 5 ml of 44% Percoll, and layered onto 5 ml of 67.5% Percoll, in 15-ml tubes precoated with FCS. After centrifugation (750 × g, 30 min, r.t.), WBCs at the interface between the two Percoll layers were collected and washed twice in RPMI/FCS medium. The cells were then resuspended either in RPMI/FCS medium, when used for ELISPOT assay, or in staining buffer, when used for FACS analysis. Density gradient purification was also adopted in the separation of blood cells used for chemotaxis experiments: In this case, Percoll PLUS (GE Healthcare) was used instead of Percoll; tubes were precoated with 10% low-endotoxin BSA in D-PBS, instead of FCS; and blood cells were washed and resuspended in r.t. RPMI/BSA, instead of RPMI/FCS medium.

Chemotaxis assay

Chemotaxis was performed in 24-well plates with polycarbonate Transwell inserts (membrane pore size: 5 μm; Corning, Costar, Lowell, MA), according to the method of Bowman et al. (18). Lower wells were prepared ahead of time with 600 μl of RPMI/BSA medium containing 300 nM recombinant mouse (r) CCL25 or 300 nM rmCCL28 (both purchased in “carrier free” form from R & D Systems, Minneapolis, MN) and equi- libriated overnight at 5°C. Supernatants in the upper well of each well of D-PBS + Ca2+/Mg2+, carefully flushing the liquid along the side of the well, were added to each well. The inserts (membrane pore size: 5 μm) were placed into the wells and washed in 10 ml of serum-free medium at r.t., resuspended in 5 ml of 44% Percoll, and layered onto 5 ml of 67.5% Percoll, in 15-ml tubes precoated with FCS. After centrifugation (750 × g, 30 min, r.t.), WBCs at the interface between the two Percoll layers were collected and washed twice in RPMI/FCS medium. The cells were then resuspended either in RPMI/FCS medium, when used for ELISPOT assay, or in staining buffer, when used for FACS analysis. Density gradient purification was also adopted in the separation of blood cells used for chemotaxis experiments: In this case, Percoll PLUS (GE Healthcare) was used instead of Percoll; tubes were precoated with 10% low-endotoxin BSA in D-PBS, instead of FCS; and blood cells were washed and resuspended in r.t. RPMI/BSA, instead of RPMI/FCS medium.
nonadherent cells were aspirated through a straight manifold, modified to leave always 50 µl of liquid at the bottom of the wells. To release adherent cells, 150 µl of HBSS/HEPES containing 2 mM EDTA were dispensed to each well, plates were incubated for 5 min at r.t., and then bound cells were dislodged from the bottom of the wells with vigorous pipetting. Cells from three replicate wells were combined and transferred to a 1.5-ml pointed bottom tube containing 400 µl of adhesion medium to neutralize EDTA. The tubes were centrifuged for 10 min at 350 × g in a swinging bucket rotor, supernatant was removed, and cells were resuspended in RPMI/FCS medium for ELISPOT analysis of VLP-specific IgA ASCs. In preliminary experiments, we found that 5-min stimulation with PMA did not significantly alter the number of VLP-specific IgA ASCs present in dLNs of immunized mice (data not shown). No Ag-specific IgA ASCs were found to bind to uncoated wells (data not shown).

**ELISPOT assay**

ELISPOT assay was performed in MultiScreen HTS 96-well plates with Immobilon-P polyvinylidene fluoride membranes (MSIPS4W10; Millipore, Billerica, MA). Wells were pretreated with 20 µl of 50% (vol/vol) ethanol, rinsed three times with D-PBS, and coated with 50 µl of 35 µg/ml VLPs in D-PBS, for Ag-specific IgA ASC evaluation, or with 50 µl of 10 µg/ml purified rat anti-mouse IgA Ab (C10-3; BD Biosciences), for total IgA ASC evaluation. After 48 h at 4°C, the plates were washed three times with D-PBS and membranes were blocked for 4 h at 37°C with 200 µl/well of RPMI/FCS medium. 

Lymphocytes were then added to the plates at serial 3-fold dilutions in 200 µl of RPMI/FCS medium per well and cultured overnight at 37°C in a humidified vibration-free 5% CO2 incubator. The wells were washed six times with D-PBS containing 0.05% (vol/vol) Tween 20 (D-PBS/Tween) and once with distilled water, to lyse any remaining cells. The plates were then incubated for 2.5 h at 37°C with 100 µl/well of biotin-conjugated goat anti-mouse IgA (Southern Biologicals), peroxidase-conjugated streptavidin (Santa Cruz Biotechnology), or isotype control (Southern Biologicals). After three washes with D-PBS/Tween, 100 µl of streptavidin–alkaline phosphatase enzyme conjugate (Mabtech, Nacka Strand, Sweden), diluted 1:1000 in D-PBS, were added to each well and the plates were incubated for 45 min at r.t. After three washes with D-PBS/Tween, followed by three washes with D-PBS, spots were developed by adding 100 µl/well of one-component BCIP/NBT phosphatase substrate solution (KPL, Gaithersburg, MD). After 15 min at r.t., the reaction was stopped by extensively washing the wells with distilled water. The plates were left to dry overnight in the dark, and spots were counted with a KS ELISPOT imaging system (Carl Zeiss, Jena, Germany). As a specificity control for the assay, cells were also incubated in uncoated wells and resulted in no detectable spots (data not shown).

**FACS analysis**

Blood leukocytes were purified by Percoll gradient centrifugation, washed in RPMI/FCS medium, and resuspended in half volume of ice-cold staining buffer (D-PBS containing 0.9 mM CaCl2, 0.5 mM MgCl2, 0.1% NaN3, and 0.1% BSA) with respect to the initial volume of blood. Then 200 µl of cell suspension were incubated for 10 min on ice with 40 µl of Fc Receptor Saturation Reagent (Beckman Coulter) and 3 µg of purified rat anti-mouse CD16/CD32 (2.4G2; BD Biosciences). The cells were next immunostained for 20 min on ice with 20 µg of biotinylated VLPs (conjugated with the FluoroReporter Mini-biotin-XX Protein Labeling Kit; Invitrogen); 5 µg of anti-mouse IgA-FTTC (C10-3; BD Biosciences); 0.8 µg of anti-mouse CD45R/B220-PerCP-Cy5.5 (RA3-6B2; BD Biosciences); 1.2 µg of anti-mouse CD138 (Syndecan-1)–APC (281-2; BD Biosciences); 0.8 µg of anti-mouse IgG–eFluor 450 (11-26; eBioscience, San Diego, CA); 0.4 µg of anti-mouse CD90.2–eFluor 450 (53-2.1; eBioscience); and 2 µg of anti-mouse αβ (LPAM-1)–PE (DATK32; BD Biosciences); 2 µg of anti-mouse L-selectin (CD62L)–PE (ME14; BD Biosciences); or 2 µg of isotype control–PE (R35-95; BD Biosciences). Samples were washed with 6.4 ml of CellWASH buffer (BD Biosciences) in a FACS Lyse/Wash Assistant (BD Biosciences), then incubated for 10 min with 20 µl of PE-Cy7–conjugated streptavidin (streptavidin–PC7; Beckman Coulter) in the residual volume (~350 µl) of washing buffer. The cells were washed again and analyzed on a FACS Canto II flow cytometry system (BD Biosciences) with FACS Diva software (version 6.1.2; BD Biosciences).

**Statistics**

We used the unpaired two-tailed Student t test to analyze differences between two groups and the one-way ANOVA followed by Duncan’s new multiple range test to compare more than two groups. A P value <0.05 was considered statistically significant. Data are presented as mean ± SEM.

**Results**

**Immunization by the i.r. route induces Ag-specific IgA production in the small intestinal LP**

To evaluate the distribution of Ag-specific IgA ASCs induced by i.r. immunization in the gut and other organs, mice were immunized three times by the i.r. route with RV VLPs as Ag, together with CT as adjuvant. Three weeks after the last immunization, the number of VLP-specific IgA ASCs in the different organs was quantified by ELISPOT and compared with the number of Ag-specific IgA ASCs induced in mice immunized with the same Ag and adjuvant administered by the i.n. route. We found that, as expected, i.n. immunization induced a large number of VLP-specific IgA ASCs in the lungs and also twice as many VLP-specific IgA ASCs in the bone marrow, in comparison with i.r. immunization (Fig. 1D, 1E). In contrast, mice immunized by the i.r. route displayed 15 times as many Ag-specific IgA ASCs in the colon and 20 times as many in the cecum than mice immunized by the i.n. route (Fig. 1B, 1C). Most importantly, i.r. immunization induced 10 times as many VLP-specific IgA ASCs in the small intestinal LP in comparison with i.n. immunization (Fig. 1A), supporting the idea that this route of Ag administration can elicit a mucosal immune response not only locally, in the colorectal mucosa, but also in other intestinal segments. Small intestinal IgA production was restricted to the LP, because no VLP-specific IgA ASCs were detectable in the PP of the majority of mice immunized by either the i.r. or the i.n. route and, when present, their numbers were much lower than those measured in the LP (≤27 spots per 1 × 106 PP cells in mice immunized by the i.r. route and ≤4 spots per 1 × 106 PP cells in mice immunized by the i.n. route). No significant differences in the number of total IgA ASCs were observed between mice immunized by the i.r. route and those immunized by the i.n. route in any organ tested, with the exception of the lungs, where i.n. immunization significantly increased the number of total IgA ASCs (Supplemental Table I).

**IgA ASCs induced by i.r. immunization are not responsive to the small intestinal chemokine CCL25**

To investigate whether Ag-specific IgA ASCs induced by i.r. immunization were responsive to the small intestinal chemokine CCL25, mice were immunized with VLPs by the i.r. route, and LNs draining the colorectal mucosa—namely, the sacral LNs and the inferior MLNs—were collected at day +5 after the last immunization, when the number of Ag-specific IgA ASCs reaches its maximum (Fig. 2). Lymphocytes were allowed to migrate in vitro toward gradients of CCL25 in chemotaxis assays, and then migrated cells were analyzed by ELISPOT to evaluate the numbers of VLP-specific IgA ASCs responding to the chemokine. We found that Ag-specific IgA ASCs collected from dLNs of mice immunized by the i.r. route were unable to migrate toward CCL25, suggesting that they lacked the intestinal homing receptor CCR9 (Fig. 3A). As expected, Ag-specific IgA ASCs induced in cervical lymph nodes (CLNs) of mice immunized with VLPs by the i.n. route were also unable to migrate toward CCL25, whereas VLP-specific IgA ASCs induced by both immunization routes were, in contrast, highly responsive to the chemokine CCL28 (Fig. 3A). Because at day +5 after the last immunization some Ag-specific IgA ASCs were also detectable in (superior) MLNs of mice immunized by the i.r. route (Fig. 2B), and because several groups have previously reported that CCR9 is selectively induced on lymphocytes primed in mesenteric but not in other LNs (36–39), we tested the migratory properties of VLP-specific IgA ASCs enriched from MLNs of immunized mice. However, even in MLNs, VLP-specific IgA ASCs induced by i.r. immunization displayed only low and nonsignificant migration toward CCL25.
whereas they were highly responsive to CCL28 (Fig. 3B, 3C). In contrast, Ag-specific cells enriched from MLNs of mice immunized with VLPs by the oral route were found to migrate at significant levels to both CCL25 and CCL28 (Fig. 3B, 3C), suggesting that in MLNs IgA ASCs may be imprinted with different homing receptor patterns that depend on the route of Ag administration.

To exclude the possibility that, after i.r. immunization, some CCL25-responsive IgA ASCs were induced in other LNs we failed to identify, we tested the chemotactic activity of VLP-specific IgA ASCs circulating in the blood of immunized mice, which collects lymphocytes derived from every lymphoid organ. Nevertheless, Ag-specific IgA ASCs present in the blood of mice immunized by the i.r. route at day +5 after the last immunization also failed to migrate toward CCL25, whereas they significantly responded to CCL28 (Fig. 3D). Because these results still did not exclude the possibility that a small minority of CCL25-responsive IgA ASCs, not revealed in our chemotaxis assay, may circulate in the blood of mice immunized by the i.r. route, we determined whether they expressed the gut-homing integrin \( \alpha_4 \beta_7 \) and were able to bind to MAdCAM-1. We found that Ag-specific IgA ASCs present in the dLNs, blood, and spleen of mice immunized with VLPs by the i.r. route, at day +5 after the last immunization, bound to MAdCAM-1-coated wells in adhesion assays after stimulation with phorbol esters (Fig. 4A, 4B, 4D, 4E). As expected, VLP-specific IgA ASCs collected from the LP at day +11 after the last immunization were no longer able to migrate toward any chemokine gradient, regardless of the route of Ag administration (Fig. 3E, 3F).

IgA ASCs induced by i.r. immunization express the integrin \( \alpha_4 \beta_7 \) and adhere to MAdCAM-1

Because Ag-specific IgA ASCs induced by i.r. immunization were found to localize in the small intestine, even in the absence of CCL25 responsiveness, we determined whether they expressed the gut-homing integrin \( \alpha_4 \beta_7 \) and were able to bind to MAdCAM-1. We found that Ag-specific IgA ASCs present in the dLNs, blood, and spleen of mice immunized with VLPs by the i.r. route, at day +5 after the last immunization, bound to MAdCAM-1-coated wells in adhesion assays after stimulation with phorbol esters (Fig. 4A, 4B, 4D, 4E). As expected, VLP-specific IgA ASCs collected from the LP at day +11 after the last immunization were no longer able to migrate toward any chemokine gradient, regardless of the route of Ag administration (Fig. 3E, 3F).

FIGURE 1. Immunization by the i.r. route induces Ag-specific IgA ASCs in the small intestinal mucosa. (A-E) BALB/c mice were immunized three times every other week, by either the i.n. or the i.r. route, with 5 \( \mu \)g of VLPs together with 3 \( \mu \)g of CT. Three weeks after the last immunization, VLP-specific IgA ASCs were measured by ELISPOT in the small intestine (A), cecum (B), proximal colon (C), lung (D), and bone marrow (E). Data in the columns are mean \( \pm \) SEM of six mice per group, whose organs were pooled two by two for analysis. For colons, 12 mice per group were used. Each dot represents a pool of two mice. No VLP-specific IgA ASCs were detectable in mice that were mock immunized with CT only (data not shown). The numbers of total IgA ASCs measured in each organ are reported in Supplemental Table I. Analyses of large intestinal IgA ASCs were performed only in the proximal (ascending) colon segments, to exclude the immunization site. \(* p < 0.05, ** p < 0.01.\)
stimulated adhesion of Ag-specific cells to MadCAM-1 in normal mice (Fig. 4F, 4G), but not in mice that lacked the CCL28 receptor Ccr10 (Ccr10<sup>−/−</sup>, Fig. 4H). MadCAM-1 adhesion was also observed with Ag-specific IgA ASCs induced oral immunization in MLNs (Fig. 4A), whereas very few VLP-specific IgA ASCs present in the dLNs, blood, and spleen of mice immunized by the i.r. route were found to bind to MadCAM-1, indicating that they might not express high levels of α<sub>4</sub>β<sub>7</sub> (Fig. 4A, 4B, 4D, 4E). Accordingly, we found that in the blood of mice immunized by the i.r. route, collected 5 d after the last immunization, the large majority of B220<sup>−</sup>CD138<sup>+</sup> surface IgA (sIgA) lymphocytes immunostained by fluorescent VLPs, and therefore corresponding to VLP-specific IgA ASCs (40, 41), expressed high levels of α<sub>4</sub>β<sub>7</sub> (Fig. 4I, right panel; see Supplemental Fig. 1 for gating strategy), whereas in the blood of mice immunized by the i.n. route, the same population of cells was mostly α<sub>4</sub>β<sub>7</sub> low<sup>−</sup> (Fig. 4I, left panel).

As a control, we also performed adhesion assay experiments in wells coated with VCAM-1, and we found that VLP-specific IgA ASCs induced through every immunization route were able to bind to this adhesion molecule (Fig. 5), suggesting that integrin α<sub>4</sub>β<sub>1</sub> expression on IgA ASCs is largely independent of the route of Ag administration. Finally, we investigated whether IgA ASCs expressed the peripheral LN addressin receptor L-selectin, but we found that this molecule was essentially absent on blood CD138<sup>+</sup> sIgA<sup>+</sup> VLP<sup>+</sup> lymphocytes induced by either i.n. or i.r. immunization (Supplemental Fig. 1).

The integrin β<sub>7</sub> plays a key role in intestinal localization of IgA ASCs induced by i.n. immunization, but also in intestinal exclusion of IgA ASCs induced by i.n. immunization

To prove that the integrin α<sub>4</sub>β<sub>7</sub> was the homing molecule that determines the intestinal tropism of the IgA ASCs induced by i.r. immunization, we immunized Igb7<sup>−/−</sup> mice with VLPs by the i.r. route and measured the number of Ag-specific IgA ASCs in the gut. We found that the number of VLP-specific IgA ASCs was >80% lower in the small intestinal LP and ∼50% lower in the cecum of Igb7<sup>−/−</sup> mice than in control (Igb7<sup>+/+</sup>) mice (Fig. 6A, 6B). Surprisingly, total IgA ASCs were reduced only by ∼50% in the small intestine, whereas we found no significant reduction in the cecum (Supplemental Table II), suggesting that IgA ASC recruitment in the gut is not completely inhibited in the absence of integrin α<sub>4</sub>β<sub>7</sub>. In the colon, the number of VLP-specific IgA ASCs was reduced by 70%, but the difference was at the limits of statistical significance because of the high variability among individual mice (Fig. 6C).

As shown in Fig. 6E, the numbers of VLP-specific IgA ASCs released into the bloodstream of Igb7<sup>−/−</sup> mice 5 d after the last immunization were similar to those measured in control mice, indicating that in our model the genetic absence of integrin β<sub>7</sub> does not impair the generation of Ag-specific lymphocytes in the inductive sites. Furthermore, we did not find any difference between knockout and control mice in the numbers of VLP-specific (total) IgA ASCs in bone marrow (Fig. 6D, Supplemental Table II), and essentially no Ag-specific IgA ASCs were found in the lungs, in either Igb7<sup>−/−</sup> or Igb7<sup>+/+</sup> mice (∼3 spots/1 × 10<sup>6</sup> lung mononuclear cells), indicating that in Igb7-deficient mice the VLP-specific IgA ASCs excluded from the gut are not redirected toward these organs.

Because mice immunized by the i.n. route also display a small but detectable number of Ag-specific IgA ASCs in the small intestine (Fig. 1A) and several CD138<sup>+</sup> sIgA<sup>+</sup> VLP<sup>+</sup> lymphocytes were found to express α<sub>4</sub>β<sub>7</sub>, although at low levels (Fig. 4I), we evaluated whether IgA ASCs induced by i.n. immunization presented impaired gut localization in the absence of integrin β<sub>7</sub>. However, we found that the number of VLP-specific IgA ASCs in

![FIGURE 2.](http://www.jimmunol.org/Downloadedfrom/10.4049/jimmunol.1700415/Figure2)
FIGURE 3. IgA ASCs induced by i.r. immunization do not respond to the small intestinal chemokine CCL25. (A and D) BALB/c mice were immunized three times every other week, by either the i.n. or the i.r. route, with 5 μg of VLPs together with 3 μg of CT. Five days after the last immunization, dLNs (A) and blood (D) were collected. Lymphocytes were allowed to migrate in vitro toward gradients of CCL25 or CCL28, and Ag-specific IgA ASCs in the migrated population were identified by ELISPOT. (B and C) BALB/c mice were immunized four times every other week with either 20 μg of VLPs and 3 μg of CT by the i.r. route or 100 μg of VLPs and 10 μg of CT by the oral route. Five days after the last immunization, MLNs were collected. ASCs were enriched by magnetic depletion of CD90+ and sIgD+ cells, and migration of Ag-specific IgA ASCs was evaluated as described above. (E and F) BALB/c mice were immunized three times every other week with 5 μg of VLPs and 3 μg of CT by the i.r. route or 50 μg of VLPs and 10 μg of CT by the oral route. Two weeks later, the first group was immunized a fourth time with 20 μg of VLPs and 3 μg of CT by the i.r. route and the latter with 100 μg of VLPs and 10 μg of CT by the oral route. Chemokine responsiveness of Ag-specific IgA ASCs was evaluated in the small intestine at days +5 and +11 after the last immunization (mice were perfused before tissue collection to avoid contamination of LP cells by blood-derived VLP-specific IgA ASCs). Data in (A), (B), (D), and (E) show the percentage of Ag-specific IgA ASCs that migrated either spontaneously (medium alone) or in response to CCL25 or CCL28, calculated by dividing the number of spots present in the migrated population by the number of spots in the starting population. (C) and (F) are representative ELISPOT assays used to measure the numbers of VLP-specific IgA ASCs in the starting population (input) and among migrated cells, in MLNs (C) and small intestinal LP (F) of immunized mice. Experiments were repeated at least twice, with similar results. *p < 0.05, **p < 0.01. IMLN, Inferior mesenteric lymph node; SLN, sacral lymph node.
the small intestinal LP of Itgb7-deficient mice immunized by the i.n. route was, on the contrary, significantly increased in comparison with control mice (Fig. 6F), suggesting that IgA ASCs are able to enter the gut even in the absence of integrin β7, although in normal mice, lymphocytes primed in extraintestinal sites, and expressing low α4β7 levels, might be regularly excluded from the gut because they cannot efficiently compete with GALT-derived α4β7high cells.
immunization, dLNs (input) and among adherent cells in dLNs of immunized mice. *Itgb7 and Ccr10-deficient mice were immunized three times every other week by the i.r. route with 10 μg of VLPs together with 3 μg of CT. Five days after the last immunization, dLNs (A–C), blood (D), and spleen (E) were collected. Lymphocytes were stimulated with PMA in wells coated with VCAM-1, and the numbers of VLP-specific IgA ASCs among adherent cells were measured by ELISPOT. The percentage of VLP-specific IgA ASCs that bound to VCAM-1 either spontaneously (medium) or after stimulation with PMA is shown in (C). Data (mean ± SEM) were derived from 9 to 15 replicate wells per group, pooled three by three before ELISPOT analysis. (B) is a representative ELISPOT assay used to evaluate the number of VLP-specific IgA ASCs in the starting populations (input) and among adherent cells in dLNs of immunized mice. *p < 0.05, **p < 0.01. IMLN, Inferior mesenteric lymph node; SLN, sacral lymph node.

**FIGURE 5.** IgA ASCs induced by every immunization route adhere to VCAM-1. (A, B, D, and E) BALB/c mice were immunized three times every other week, by either the i.n. or the i.r. route, with 5 μg of VLPs together with 3 μg of CT, or four times, by the oral route, with 100 μg of VLPs together with 10 μg of CT. (C) Itgb7+/− and Itgb7−/− mice were immunized three times every other week by the i.r. route with 10 μg of VLPs together with 3 μg of CT. Five days after the last immunization, dLNs (A–C), blood (D), and spleen (E) were collected. Lymphocytes were stimulated with PMA in wells coated with VCAM-1, and the numbers of VLP-specific IgA ASCs among adherent cells were measured by ELISPOT. The percentage of VLP-specific IgA ASCs that bound to VCAM-1 either spontaneously (medium) or after stimulation with PMA is shown in (C). Data (mean ± SEM) were derived from 9 to 15 replicate wells per group, pooled three by three before ELISPOT analysis. (B) is a representative ELISPOT assay used to evaluate the number of VLP-specific IgA ASCs in the starting populations (input) and among adherent cells in dLNs of immunized mice. *p < 0.05, **p < 0.01. IMLN, Inferior mesenteric lymph node; SLN, sacral lymph node.

The chemokine receptor CCR10 is not required for the intestinal tropism of IgA ASCs induced by i.r. immunization

Because the chemokine CCL28 attracts IgA ASCs induced by i.r. immunization and stimulates their adhesion to MAAdCAM-1, we investigated whether the CCL28–CCR10 axis was important for the intestinal localization of these lymphocytes. However, when Ccr10−/− mice were immunized with VLPs by the i.r. route, the numbers of Ag-specific IgA ASCs in the small and large intestine did not differ from those measured in control Ccr10+/− mice (Fig. 7A, 7B), indicating that CCL28 is dispensable for IgA ASC homing into the gut. In addition and as previously reported (32, 42), the numbers of total IgA ASCs in the gut of Ccr10-deficient mice were no different from those of control mice (Supplemental Table III), whereas a small but significant increase in Ag-specific and total IgA ASCs was observed in the bone marrow of Ccr10−/− mice (Fig. 7C, Supplemental Table III).

Discussion

The experimental observations described above underscore the high degree of compartmentalization of mucosal effector immune responses, and in particular of IgA production, induced by immunization through different routes. Indeed, when we compared the distribution of IgA ASCs after immunization with VLPs, a particulate protein Ag, administered by the i.r. or i.n. route, we found that whereas i.n. immunization induces more Ag-specific IgA ASCs in the lungs and bone marrow, immunization by the i.r. route induces a higher number of Ag-specific IgA ASCs in the gut, not only in the colon but, importantly, also in the cecum and the small intestine. Conversely, low numbers of Ag-specific IgA ASCs were observed in the gut of mice immunized by the i.n. route, and virtually no Ag-specific IgA ASCs were detectable in the lungs of mice immunized by the i.r. route. To explain why Ag-specific IgA ASCs induced by i.r. immunization migrate into the small intestine, we compared their homing receptor pattern with that expressed by IgA ASCs induced by i.n. or oral immunization. Our experiments revealed that each of these three routes of Ag administration imprints IgA ASCs with a different array of chemokine and adhesion molecule receptors. As expected, IgA ASCs induced by oral immunization express both of the two known gut-homing receptors, the integrin α4β7 and the chemokine receptor CCR9, because they adhere to MAAdCAM-1 and migrate toward CCL25 gradients. On the contrary, Ag-specific IgA ASCs induced by i.n. immunization do not express any of these gut-homing receptors, whereas immunization by the i.r. route imprints an intermediate pattern, in which α4β7 is expressed in the absence of CCR9, demonstrating that the homing machinery displayed by IgA ASCs is extremely fine-tuned and depends on the route of Ag administration. This model strikingly contrasts with the trafficking program described for effector CD8+ T cells induced by viral or bacterial infections, which have been reported to upregulate α4β7 and CCR9 regardless of the route of pathogen entry and broadly disseminate into multiple peripheral tissues, including the gut, independently of their original activation site (43, 44).

Previous studies in human volunteers have reported the expression of α4β7 on Ag-specific IgA ASCs induced by immunization through the i.r., oral, and i.n. route; however, IgA ASCs induced by i.n. immunization expressed α4β7 together with the LN-homing receptor L-selectin, whereas IgA ASCs induced by oral and i.r. immunization expressed α4β7 in the absence of L-selectin (45, 46). Our results suggest instead that i.n. immunization does not generate α4β7-expressing lymphocytes, because Ag-
specific IgA ASCs collected from draining CLNs, blood, and spleen of mice immunized by the i.n. route were unable to bind to MAdCAM-1. Aside from species differences between mice and humans, the discordance between the previously published results and our results can also be explained by the different methodology used to identify $\alpha_4\beta_7$-expressing IgA ASCs: In fact, in the earlier reports, $\alpha_4\beta_7^+$ cells were separated from $\alpha_4\beta_7^-$ cells using magnetic beads coated with an $\alpha_4\beta_7$-specific Ab, whereas in the current study we performed a functional adhesion assay on MAdCAM-1–coated wells. However, when we analyzed Ag-specific IgA ASCs by flow cytometry, we found that $\sim 50\%$ of CD138 $^+$ sIgA $^+$ VLP $^+$ lymphocytes induced by i.n. immunization were positively stained for $\alpha_4\beta_7$, although with a much lower intensity than lymphocytes of mice immunized by the i.r. route. Therefore, it is possible that many $\alpha_4\beta_7^-$ Ag-specific IgA ASCs in the blood of intranasally immunized volunteers were selected as $\alpha_4\beta_7^+$ in the magnetic separation, even though their levels of integrin expression would have probably been too low to allow MAdCAM-1 adhesion and intestinal tropism. In addition, we show that, unlike human IgA ASCs (45–47), the majority of CD138 $^+$ sIgA $^+$ VLP $^+$ cells in the blood of immunized mice are L-selectin $^-$. This finding is consistent with the idea that murine PCs do not recirculate into peripheral LNs, as they are also unresponsive to the chemokines CXCL13, CCL19, and CCL21 (18, 48).

According to the current paradigm, T and B cell homing to the small intestine requires the expression of both $\alpha_4\beta_7$ and CCR9, as if these receptors were the “molecular password” that authorizes lymphocyte access into this “restricted” organ. In fact, it is known that mice that are genetically deficient for the integrin $\beta_7$, or express a mutated $\beta_7$ chain that keeps the $\alpha_4\beta_7$ heterodimer in a persistently adhesive conformation, display fewer intestinal lymphocytes than do control mice (31, 49). In addition, fewer IgA ASCs and intraepithelial T cells have also been observed in the small intestine of Ccr9 $^{-/-}$ or Ccl25 $^{-/-}$ mice (16, 17, 19, 21). However, some of the
data presented in this article do not seem to fit with the above model. First, we showed that CCR9 was not required for small intestinal tropism of IgA ASCs, because immunization by the i.r. route induced Ag-specific IgA ASCs that did not respond to CCL25 but were nevertheless able to migrate into the small intestinal LP. The chemokine CCL28 did not seem to compensate for the lack of CCL25 responsiveness in our model (50), because Ag-specific IgA ASCs were not decreased in the small intestine and colon of Ccr10−/− mice immunized by the i.r. route, in comparison with control Ccr10−/− mice (nor in the colon of Ccr10−/− Itgb7−/− mice; data not shown). Second, we report that even the integrin αb7 was not absolutely necessary for IgA ASC migration into the gut. In fact, whereas the number of Ag-specific IgA ASCs was ~80% lower in the small intestine and ~50% lower in the cecum of Itgb7-deficient mice immunized with VLPs by the i.r. route, the number of total IgA ASCs was only ~50% lower in the small intestine, and not significantly lower in the cecum, showing that lymphocyte extravasation into the gut is still possible even in the absence of αb7. More strikingly, when mice were immunized by the i.r. route, the numbers of Ag-specific IgA ASCs in the small intestinal LP of Itgb7−/− mice were even higher than those of control mice. These findings can be easily explained by envisaging leukocyte migration into the different organs as a highly competitive process, in which cells that lack a proper homing receptor combination are not intrinsically excluded from entering into a tissue, but rather present a competitive disadvantage against those that do express the right molecules. Therefore, IgA ASCs induced by i.n. immunization are normally excluded from the gut because they express low αb7 levels and cannot efficiently compete with αb7-high IgA ASCs that are constantly generated in the GALT in response to intestinal Ags. On the contrary, in Itgb7-deficient mice GALT-derived lymphocytes are deprived of αb7 and no longer benefit from “privileged” access into the gut. In consequence, even IgA ASCs primed in other mucosal inductive sites, such as those elicited by i.n. immunization in LNs draining the respiratory tract, can now efficiently occupy the PC niches of the intestinal LP. In addition, we can assume that the decreased number of Ag-specific IgA ASCs found in the gut of Itgb7−/− mice immunized by the i.r. route derives not only from “denied” access to the intestinal mucosa but also from a reduced advantage to overcome the competition with IgA ASCs normally destined to other organs. Therefore, in the absence of organ-specific homing receptors, the compartmentalization of the mucosal immune system ceases, not only because of impaired lymphocyte entry into a given organ (which may be moderate, as in the case of IgA ASCs in the gut of Itgb7−/− mice), but also, and perhaps more importantly, because lymphocytes elicited against a tissue-specific pathogen now promiscuously migrate into several unrelated organs. In support of this hypothesis, it has recently been reported that whereas in normal mice the CDR3 sequences of small intestinal IgA ASCs are distinct from those of colonic IgA ASCs, the repertoires displayed by the two IgA ASC populations become instead, largely overlapping in Ccr9-deficient mice (51). In conclusion, we have shown that i.r. immunization induces IgA ASCs that localize not only in the colon, but also in the small intestine, and display a peculiar gut-homing receptor profile in which the integrin αb7 is expressed in the absence of the CCL25 receptor CCR9. Besides extending our knowledge of lymphocyte homing, the results reported above may also be important in the rational development of vaccines for the prevention of mucosal infections and, in particular, those transmitted by enteric pathogens.

Acknowledgments
We thank Angie C. Williams and Yoji Shimizu (University of Minnesota Medical School, Minneapolis, MN) for help in setting up the adhesion assay and Raymond Kwan (Stanford University School of Medicine, Stanford, CA) for help with the chemotaxis assay.

Disclosures
The authors have no financial conflicts of interest.

References
INTESTINAL HOMING OF IgA AB-SECRETING CELLS


Supplemental Material

**Supplemental Table I.** Total IgA ASCs ($\times 10^6$ cells) in the organs of BALB/c mice immunized by the i.n. or i.r. route.

<table>
<thead>
<tr>
<th></th>
<th>i.n.</th>
<th>i.r.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>68,708 ± 3,645</td>
<td>65,124 ± 6,049</td>
</tr>
<tr>
<td>Peyer’s patches</td>
<td>925 ± 225</td>
<td>1,031 ± 84</td>
</tr>
<tr>
<td>Cecum</td>
<td>8,839 ± 1,873</td>
<td>10,844 ± 379</td>
</tr>
<tr>
<td>Colon (ascending)</td>
<td>45,335 ± 3,278</td>
<td>37,847 ± 2,642</td>
</tr>
<tr>
<td>Lung</td>
<td>11,451 ± 750 **</td>
<td>73 ± 3</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>311 ± 77</td>
<td>378 ± 20</td>
</tr>
</tbody>
</table>

Mice were immunized with VLPs and CT by either the i.n. or the i.r. route, as reported in Fig. 1, and total IgA ASCs were measured by ELISPOT, in the indicated organs, three weeks after the last immunization. Data are mean ± SEM of 6 mice per group, whose organs were pooled two by two for analysis. For colons 12 mice per group were used (**$p < 0.01$).
Supplemental Table II. Total IgA ASCs \((\times 10^{-6} \text{ cells})\) in the organs of \(Itgb7^{+/-}\) and \(Itgb7^{-/-}\) mice immunized by the i.r. route.

<table>
<thead>
<tr>
<th>Organ</th>
<th>(Itgb7^{+/-})</th>
<th>(Itgb7^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>34,432 ± 4,947 (\text{ (35,205 ± 2,764)})</td>
<td>16,718 ± 3,994 (\ast) (\text{ (13,385 ± 2,109 (\ast\ast))})</td>
</tr>
<tr>
<td>Cecum</td>
<td>10,500 ± 2,506 (\text{ (10,890 ± 1,084)})</td>
<td>8,177 ± 940 (\text{ (7,116 ± 985)})</td>
</tr>
<tr>
<td>Colon (ascending)</td>
<td>19,754 ± 1,906 (\text{ (12,272 ± 2,335)})</td>
<td>8,433 ± 2,667 (\ast\ast) (\text{ (9,064 ± 4,097)})</td>
</tr>
<tr>
<td>Lung</td>
<td>240 ± 70 (\text{ })</td>
<td>254 ± 55 (\text{ })</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>650 ± 69 (\text{ })</td>
<td>743 ± 112 (\text{ })</td>
</tr>
</tbody>
</table>

\(Itgb7^{+/-}\) and \(Itgb7^{-/-}\) mice were immunized with VLPs and CT by the i.r. route, as reported in Fig. 6, and total IgA ASCs were measured by ELISPOT, in the indicated organs, three weeks after the last immunization. Data in parenthesis are the numbers of total IgA ASCs measured in the gut of 2 and 4 (small intestine), 3 and 3 (cecum) or 6 and 6 (colon) non-immunized \(Itgb7^{+/-}\) and \(Itgb7^{-/-}\) naive mice, respectively. Data are mean ± SEM (\(\ast p < 0.05; \ast\ast p < 0.01\)).
**Supplemental Table III.** Total IgA ASCs (×10^{-6} cells) in the organs of *Ccr10^{+/−}* and *Ccr10^{-/-}* mice immunized by the i.r. route.

<table>
<thead>
<tr>
<th>Organs</th>
<th><em>Ccr10^{+/−}</em></th>
<th><em>Ccr10^{-/-}</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>27,338 ± 4,124</td>
<td>38,070 ± 7,081</td>
</tr>
<tr>
<td>Colon (ascending)</td>
<td>14,112 ± 1,109</td>
<td>14,276 ± 1,868</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>633 ± 122</td>
<td>1,142 ± 177 *</td>
</tr>
<tr>
<td></td>
<td>(250 ± 38)</td>
<td>(422 ± 48 *)</td>
</tr>
</tbody>
</table>

*Ccr10^{+/−}* and *Ccr10^{-/-}* mice were immunized with VLPs and CT by the i.r. route, as reported in Fig. 7, and total IgA ASCs were measured by ELISPOT, in the indicated organs, three weeks after the last immunization. Data in parenthesis are the numbers of total IgA ASCs measured in the bone marrow of 12 *Ccr10^{+/−}* and 11 *Ccr10^{-/-}* non-immunized naive mice. Data are mean ± SEM (*p < 0.05).*
Supplemental Figure 1

A

- CD138 (Syndecan-1) APC-A
- sIgA FITC-A
- FSC-A
- SSC-A
- Lymphocytes
- CD45R/B220 PerCP-Cy5.5-A
- sIgD, CD90.2 eFluor450-A
- sIgD- CD90-
- B220-
- VLP PE-Cy7-A
- CT i.n.
- VLP/CT i.n.
- CT i.r.
- VLP/CT i.r.

B

- VLP/CT i.n.
- Isotype control PE-A
- Q1 Q2 0.2%
- Q1 Q2 0.3%
- VLP/CT i.r.
- Isotype control PE-A
- Q3 Q4 48.5%
- Q3 Q4 40.2%
- VLP/CT i.n.
- L-selectin (CD62L) PE-A
- Q1 Q2 2.1%
- Q1 Q2 1.6%
- VLP/CT i.r.
- L-selectin (CD62L) PE-A
SUPPLEMENTAL FIGURE 1. Integrin $\alpha_4\beta_7$ and L-selectin expression on antigen-specific CD138$^+$ sIgA$^+$ lymphocytes in the blood of mice immunized by the i.n. or the i.r. route. (A and B) Mice were immunized every other week, by either the i.n. or the i.r. route, three times with 5 $\mu$g of VLPs together with 3 $\mu$g of CT and a fourth time with 20 $\mu$g of VLPs together with 3 $\mu$g of CT. Two additional groups of control mice were mock-immunized four times, by either the i.n. or the i.r. route, with 3 $\mu$g of CT only. Five days after the last immunization, blood was collected. Lymphocytes were isolated by density gradient purification and antigen-specific IgA ASCs were analyzed by FACS for $\alpha_4\beta_7$ or L-selectin expression. (A) Gating strategy used to identify VLP-specific IgA ASCs. After excluding CD90$^+$ and sIgD$^+$ cells with a dump gate, B220$^-$ CD138$^+$ sIgA$^+$ lymphocytes stained with fluorescent antigen were detectable in the blood of mice immunized with VLPs together with CT, but not in the blood of mice mock-immunized with CT only (numbers are the percentage of VLP$^+$ cells within the gated CD138$^+$ sIgA$^+$ lymphocytes; blue dots inside the lymphocyte population, in the FSC vs. SSC plot, represent CD138$^+$ sIgA$^+$ cells). (B) Representative flow cytometry plots, showing VLP binding and $\alpha_4\beta_7$ or L-selectin expression on B220$^-$ CD138$^+$ sIgA$^+$ lymphocytes in the blood of mice immunized with VLPs together with CT (isotype control staining is reported for comparison; numbers are the percentage of positive cells within the VLP$^+$ populations).