

Targeting of Secretory IgA to Peyer's Patch Dendritic and T Cells after Transport by Intestinal M Cells¹

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In addition to being instrumental to the protection of mucosal epithelia, secretory IgA (SIgA) adheres to and is transported by intestinal Peyer's patch (PP) M cells. The possible functional reason for this transport is unknown. We have thus examined in mice the outcome of SIgA delivered from the intestinal lumen to the cells present in the underlying organized mucosa-associated lymphoreticular tissue. We show selective association of SIgA with dendritic cells and CD4⁺ T and B lymphocytes recovered from PP in vitro. In vivo, exogenously delivered SIgA is able to enter into multiple PP lining the intestine. In PP, SIgA associates with and is internalized by dendritic cells in the subepithelial dome region, whereas the interaction with CD4⁺ T cells is limited to surface binding. Interaction between cells and SIgA is mediated by the IgA moiety and occurs for polymeric and monomeric molecular forms. Thus, although immune exclusion represents the main function of SIgA, transport of the Ab by M cells might promote Ag sampling under neutralizing conditions essential to the homeostasis of mucosal surfaces. *The Journal of Immunology*, 2004, 172: 3026–3033.

Immunity at the mucosal surfaces, which are the port of entry for many pathogens, is essential in preventing infection. To protect these very thin and sensitive surfaces, an elaborated set of mechanisms prevents attachment, colonization, and possible damage by pathogenic agents. Physicochemical and enzymatic mechanisms include mucus, glycocalyx, lactoferrin, peroxidase, defensins, and peristalsis at the intestinal level. Moreover, epithelial cells, the organized mucosa-associated lymphoreticular tissue-immunoreactive cells distributed within the mucosal tissue, and Ab act in concert to promote induction of an immunological barrier comprising innate, inflammatory, and adaptive components (1).

In the gastrointestinal (GI)³ tract, Peyer's patches (PP) have been studied in detail as a major inductive site for mucosal secretory IgA (SIgA) responses. Luminal Ag gain access to the PP via transfer across specialized epithelial cells known as M cells (microfold cells), which are scattered among the columnar epithelial cells of the follicle-associated epithelium (2–4). SIgA serves as the first line of defense against microbes by agglutinating potential invaders and facilitating their clearance by peristaltic and mucociliary movements (5). SIgA, but not IgM or IgG, interacts with rabbit, mouse, and human M cell apical membranes (6–9). The purpose of the IgA-M cell interaction is undefined to date. Selec-

tive binding of SIgA to the apical surface of M cells requires domains C α 1 and C α 2 of IgA (9), but not associated secretory component (SC) as for mucus anchoring (10). Although it has been proposed that M cells may mediate the transepithelial transport of Ab from the intestinal lumen to the organized mucosa-associated lymphoreticular tissue (7, 11), no formal demonstration has been reported to date.

Possible interaction between SIgA and target cells in PP has not been investigated in in vivo conditions. In vitro, the interaction between IgA/SIgA and monocyte-derived dendritic cells (DCs) exhibiting a myeloid phenotype yielded contrasted results in DC maturation, most likely due to differences in preparation and inhomogeneous sources of SIgA (12, 13). Because it is highly likely that the properties and functions of these cells in their natural tissular environment will differ (14, 15), the binding of SIgA under more physiological conditions remains to be substantiated. In contrast to soluble Ag that are destroyed by digestion before they can be taken up, free SIgA exhibits intrinsic stability when exposed to proteases found in the GI tract (16–18). In support of this, antigenic epitopes carried by SIgA are immunogenic when given intragastrically (19, 20). DC that form a dense network in the subepithelial dome (SED) region and more occasionally found in association within the M cell pocket (21) might interact with transcytosed SIgA-containing immune complexes. SIgA-Ag complexes could alternatively be sampled by B lymphocytes also residing in the M cell pocket (22, 23).

As a first step to resolve the immunological significance of M cell binding and transport of SIgA, we explored the fate of SIgA within the lymphoid follicle in murine PP. In vitro, we observed selective binding to PP DC and CD4⁺ T and B cells, but not to monocyte/macrophages and CD8⁺ T cells. Administration of SIgA into a ligated intestinal loop comprising a PP resulted in tissue sections showing colocalization with DC and CD4⁺ T cells in the SED region and interfollicular region (IFR), respectively. In contrast, no costaining with B cells was observed under identical experimental conditions. Internalization of SIgA by DC in the SED region was demonstrated by laser scanning confocal microscopy. Targeting of SIgA to PP DC suggests that SIgA-containing immune complexes not only are cleared by peristalsis, but might also

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³ Abbreviations used in this paper: GI, gastrointestinal; Cy3, indocarbocyanin; DC, dendritic cell; GALT, gut-associated lymphoreticular tissue; GFP, green fluorescent protein; IFR, interfollicular region; IgAm, monomeric IgA; IgAp/d, polymeric/dimeric IgA; PP, Peyer's patch; SAV, streptavidin; SC, secretory component; SED, subepithelial dome; SIgA, secretory IgA.

enter the body to boost an ongoing immune response in the absence of concomitant inflammation.

Materials and Methods

Abs and reagents

For flow cytometry, PE- or FITC-labeled anti-CD3 (17A2, rat IgG2b), anti-CD19 (1D3, rat IgG2a), anti-CD4 (L3T4, rat IgG2a), anti-CD11b/MAC-1 (M1/70, rat IgG2b), anti-CD11c (HL3, hamster IgG), and anti-CD8a/Ly-2 (53-6.7, rat IgG2a) were purchased from BD Biosciences. The FDC M2 hybridoma (anti-follicular DC rat IgG) was a gift from M. Kosco-Vilbois (Serono Pharmaceutical Research Institute, Geneva, Switzerland). After production, purified IgG were biotinylated with EZ-Link biotin-LC-hydrazide, according to the manufacturer's instructions (Pierce, Rockford, IL). For immunofluorescence, mAb against CD4 (L3T4, rat IgG2a) and biotinylated mAb against CD11c (N418, hamster IgG) were used (BD Biosciences, Mountain View, CA). Biotinylated mAb against B220 (RA3-6B2, rat IgG2a) was obtained from Caltag Laboratories (Burlingame, CA). Streptavidin (SAV) coupled to FITC was obtained from DAKO Cytomation (Carpenteria, CA). SAV and anti-rat Ig coupled to indocarbocyanin (Cy3) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). SC:green fluorescent protein (GFP) was produced in CHO cells stably transfected with the expression vector pcDNA3 comprising the coding sequence for GFP (Invitrogen, Carlsbad, CA) fused to the 3' end of the sequence coding for human SC (24). Emission of GFP fluorescence in the FITC channel permits tracking by flow cytometry.

Protein concentration

IgA harvested from hybridoma MB2 (7) was concentrated using an ultrafiltration system (Labscale TFF system; Millipore, Bedford, MA) equipped with a 100-kDa molecular mass cut-off membrane (Millipore). Ab-containing medium was reduced to 10 ml and separated by size exclusion chromatography (see below). Supernatants containing SC:GFP were concentrated as described above, using a 50-kDa molecular mass cut-off membrane (Labscale TFF system; Millipore). The volume was brought to 10 ml and further reduced to a final volume of 1–2 ml using a Centricon-50 filtration unit (Millipore).

Purification of the various IgA molecular forms and SC

To separate monomeric, dimeric, and polymeric IgA from mouse hybridomas, concentrated Ab were chromatographed onto two 1-m columns (100 × 2.6 cm) filled with Sephacryl S-300 beads (Amersham Pharmacia Biotech, Arlington Heights, IL) coupled to a fast protein liquid chromatography system with continuous monitoring at 278 nm (25). The identity of the separated IgA forms was ensured by SDS-PAGE under nonreduced denaturing conditions (1% SDS), followed by immunoblotting with goat anti-mouse IgA α -chain (Sigma-Aldrich, St. Louis, MO) or rabbit anti-human SC (24) and appropriate HRP-conjugated secondary Ab. Recombinant SC (10) and SC:GFP proteins were purified from concentrated supernatants by Con A-agarose chromatography (Vector Laboratories, Burlingame, CA) (26) and were stored at 4°C in PBS.

Chemical modification of IgAp/d and SC

Biotinylation. Coupling of biotin to purified IgAp/d or SC on sialic acid residues was performed as follows. PBS containing 1 mg of Ab was changed to 0.1 M sodium acetate (pH 5.5) using several cycles of ultrafiltration on a Centricon-100 filtration unit (Millipore). The protein was then incubated with 2 mM sodium periodate on ice in the dark for 30 min. Oxidation was stopped by the addition of a 1/10 (v/v) dilution of 150 mM glycerol, which was subsequently removed by several washes with 0.1 M sodium acetate (pH 5.5) using a Centricon-100 filtration unit (Millipore). Biotin-LC-hydrazide (Pierce) in DMSO was added to a 5-mM final concentration and was incubated with IgAp/d at room temperature for 2 h. The buffer was finally changed to PBS using a Centricon-100 filtration unit.

Conjugation of Cy3 to IgAp/d, IgAm, and SC. Protein samples were concentrated to 1 mg/ml in PBS using a Centricon-100 filtration unit (Millipore). Conjugation of Cy3 was conducted using FluoroLink mAb Cy3 (Amersham Pharmacia Biotech), strictly following the procedure provided by the manufacturer.

In vitro association of IgAp/d with SC

Various forms of recombinant SIgA were obtained by combining in PBS the various molecules of IgAp/d with SC:GFP (green SIgA), IgAp/d:biot with SC (SIgA:biot), or IgAp/d:cy3 with SC (red SIgA) for 1 h at room temperature in a 1:1 stoichiometric ratio (18).

Recovery of mouse PP cells

Mice (4–6 wk old) were purchased from Harlan Breeders (Indianapolis, IN). Freshly collected PP in plain MEM α medium (Invitrogen) were digested for 15 min at room temperature on a horizontal shaker with 0.5 mg/ml prewarmed (37°C) collagenase (Sigma-Aldrich) solution. After enzymatic treatment, PP were passed over a 70- μ m pore size nylon mesh (Falcon; BD Biosciences), and the dissociated tissue was gently forced through using a syringe pestle. The cell harvest was then passed through a 40- μ m pore size nylon mesh (Falcon; BD Biosciences) and kept on ice in RPMI 1640 medium (Sigma-Aldrich) complemented with 10% FCS (Invitrogen) before use.

Determination of the PP composition by flow cytometry

Cells (5×10^5) from the PP cell suspension were incubated with anti-CD11b (1/250), anti-CD11c (1/250), anti-CD19 (1/200), anti-CD3 (1/200), anti-CD4 (1/200), and anti-CD8a (1/250) mAb labeled with either FITC or PE or with biotinylated anti-FDC mAb, followed by SAV:FITC detection. Abs and cells were incubated on ice in PBS-1% BSA for 1 h, then washed three times with PBS-0.1% BSA. Dead cells were excluded from the analysis by selection in the presence of 1 μ g/ml propidium iodide solution (Sigma-Aldrich). Flow cytometric analysis was performed on a FACScan flow cytometer (BD Biosciences), and data were processed using either the LYSIS II or CellQuest software (BD Biosciences).

Association of SC and SIgA with cells isolated from PP

Protocol I (whole cell labeling). Cells (5×10^5) recovered from PP were incubated with 1.2 μ g of SC:GFP or 3.6 μ g of green SIgA in a final volume of 30 μ l of PBS. Cells were washed twice with PBS-0.1% BSA before analysis by flow cytometry as described above. For the experiment shown in Fig. 1E, up to 18 μ g of green SIgA was mixed with PP cells.

Protocol II (cell type-specific labeling). After incubation with cell type-specific PE-labeled Ab, 5×10^5 cells from PP were incubated for 25 min at room temperature with SC:GFP (1.2 μ g) or green SIgA (3.6 μ g) in a final volume of 30 μ l of PBS. Each incubation was followed by two washing steps with PBS-0.1% BSA. In inhibition assays, a 5-fold molar excess of IgAp/d or SIgA was added to the cells 25 min before incubation with biotinylated molecules. The percentage of PP cells showing association with SIgA and SC was determined by flow cytometry. Propidium iodide treatment excluded dead cells from the analysis.

In vitro internalization assay

Cells (5×10^5 /ml) recovered from PP were incubated with 480 ng of SIgA:biot for 25 min at room temperature. The cells were then washed with PBS-0.1% BSA to remove nonspecifically bound SIgA:biot and were incubated for 1 h on ice with SAV:FITC (1/500). After two washes with PBS-0.1% BSA, the cells were incubated at 37°C for 25 min to allow internalization of the surface SIgA:biot molecules associated with SAV:FITC. Half the cells for each condition were submitted to tryptic digestion for 10 min at room temperature using 500 μ l of prewarmed (37°C) tryptic solution (Invitrogen) with gentle mixing every other minute. These digestion conditions allowed recovery of the same relative percentages of DC, B, and T cells in the PP as established in the absence of tryptic exposure. Trypsin activity was then neutralized with 1 ml of RPMI 1640 medium containing 10% FCS. After two washes in PBS-1% BSA, the degree of association with DC (CD11c⁺), T cells (CD4⁺), and B cells (CD19⁺) was determined by flow cytometry. Internalization of SIgA:biot-SAV:FITC was determined by comparison of the percentage of SIgA:biot-SAV:FITC-labeled cells incubated at 37°C and subjected to tryptic digestion with that of SIgA:biot-SAV:FITC-labeled cells incubated at 37°C in the absence of enzyme (see Fig. 5). Control of the efficiency of the tryptic digestion was obtained by comparing the percentage of SIgA:biot-SAV:FITC-labeled cells incubated at 4°C and exposed to trypsin with that of SIgA:biot-SAV:FITC-labeled cells incubated at 4°C without tryptic treatment.

Ligated loops of intestine

Mice starved overnight were injected i.p. with 100 μ l of anesthetics (10 mg/ml Ketazol-100 (Gräub, Bern, Switzerland) and 0.2% Rompun (Bayer, Leverkusen, Germany) in PBS)/10 g of body weight. The abdomen was incised, and the peritoneal lining was opened to expose the intestines. The ileum was ligated loosely to prevent ischemia and tissue damage at ~0.5 cm of each side of one PP, using surgical thread. Fifteen micrograms of green SIgA or red SIgA in 100–120 μ l of PBS was injected into the lumen of the ligated loop using a 0.5 ml of U-100 insulin syringe (gauge 29G1/2; BD Biosciences). The intestine was then reintroduced into the abdomen, and the peritoneal cavity and skin around the initial incision were sewn back. After awakening, mice were kept for up to 4 h before sacrifice. The

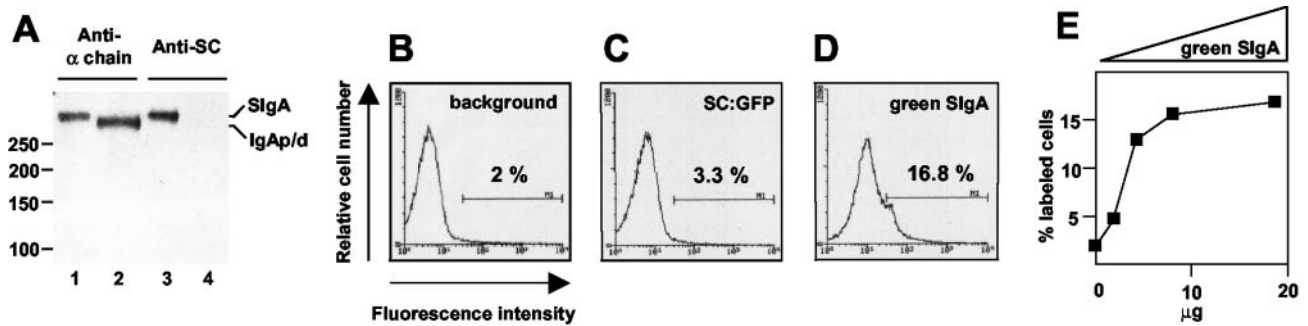


FIGURE 1. Selective interaction *in vitro* between SIgA and cells recovered from mouse PP. *A*, Covalent association between IgAp/d and SC:GFP was assayed by immunodetection, as indicated on the top of the lanes. *Lanes 1* and *3*, Green SIgA; *lanes 2* and *4*, IgAp/d. Relative m.w. are marked alongside the picture. *B*, *C*, and *D*, *In vitro* association of green SIgA and SC:GFP with cells recovered from mouse PP. Cells and GFP-labeled proteins were incubated for 25 min at room temperature before flow cytometric analysis. *B*, Cell autofluorescence. *C*, Signal detected after incubation with SC:GFP alone. *D*, Fluorescence obtained after incubation with green SIgA. The panels shown are representative of four independent experiments. *E*, Binding saturation of SIgA mixed with PP cells. Increasing amounts of green SIgA were incubated with a constant amount of cells recovered from PP, and the association was measured by flow cytometry.

ligated intestinal loop was extensively washed on ice with cold RPMI 1640 medium complemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate (all from Invitrogen) to remove excess of fluorescent SIgA from the lumen. The PP was then prepared for immunofluorescence imaging.

Preparation of tissue sections and immunolabeling

The tissue containing one PP was prepared as frozen sections. For histology, tissue was fixed in 4% paraformaldehyde-PBS (Sigma-Aldrich) for 2 h, washed, and quenched for 30 min in 50 mM NH₄Cl in PBS. It was then embedded in OCT (Sakura Finetek, Torrance, CA) and snap-frozen in isopentane (Sigma-Aldrich) cooled in liquid nitrogen. Frozen sections (7 μm thin) were produced and incubated for 10 min at room temperature. Sections were then washed in PBS and blocked for 30 min in PBS containing 5% normal mouse serum and 2% FCS. All incubations with specific cell type biotinylated mAb (anti-CD11c (1/10), anti-B220 (1/50), anti-CD4 (1/100)) were conducted in PBS containing 2% FCS at room temperature. After successive 2% FCS-PBS and PBS washings, sections were incubated with SAV-Cy3 (1/1000) for 30 min, washed again, and finally mounted in Citifluor (Citifluor, Kent, U.K.).

Fluorescence microscopy

The sections were examined under a fluorescence microscope (Axioskop, Zeiss, Oberkochen, Germany) equipped with a UV source and coupled to a color Coolview camera (Photonic Science, East Sussex, U.K.). Yellow spots resulting from colocalization of green and red signals were identified using the color range function in Photoshop (Adobe Systems, Mountain View, CA).

Laser scanning confocal microscopy

A laser scanning confocal microscope (TCS NT; Leica, Deerfield, IL) was used to examine the cellular localization of fluorescent SIgA previously detected in tissue sections. Excitation was obtained with an argon-krypton laser, with lines set at 488 nm for FITC excitation and 568 nm for TRITC excitation; the emitted light was filtered through appropriate filters (BF 530/30 for FITC, LP 590 for TRITC). Images of 512 × 512 pixels were taken with a ×100 objective (NA 1.4). The optical resolution of the objective used is given at 139 nm. For each field, a digitized series of optical sections, with 390-nm spacing between sections (z-axis), was collected on the host computer; cross-talk between the different channels was checked by appropriate inactivation of the specific laser line. The images were processed using Imaris software (Bitplane, Zurich, Switzerland) on an Octane 2 computer (Silicon Graphics, Mountain View, CA).

Statistics

Student's *t* test was used to interpret the significance of differences between experimental groups (presented as the mean ± SD).

Results

SIgA associates *in vitro* to cells recovered from mouse PP

Using flow cytometry, we first investigated whether SIgA exhibit selective association with cells recovered from mouse PP. Our

isolation procedure defines the following PP composition: 56.5 ± 2% CD19⁺ B cells, 30.3 ± 1.7% CD3⁺ T cells (23.3 ± 4.6% CD4⁺ vs 5.3 ± 1.5% CD8⁺), 9.0 ± 2% CD11b⁺ monocyte/macrophages, 2.1 ± 0.3% CD11c⁺ DC, and 0.8 ± 0.1% FDC-M2⁺ follicular cells (numbers in agreement with previous analyses of PP content) (27–29). To track binding of SIgA to PP cells, we thus prepared a recombinant protein consisting of GFP fused to the C terminus of SC (SC:GFP), and reconstituted fluorescent SIgA upon combination with IgAp/d (Fig. 1A). Covalent IgAp/d-SC:GFP complexes were thus obtained, which reflects the proper assembly of SIgA, and they are referred to as green SIgA in the rest of the paper. The background fluorescence intensity was examined using PP cells incubated with PBS (Fig. 1B). The interaction with SC:GFP alone yielded a similar low signal intensity (Fig. 1C). In contrast, nearly 17% of the cells incubated with green SIgA were found in association with PP cells (Fig. 1D). Titration with increasing amounts of green SIgA did not allow improvement of the percentage of interacting Ab, arguing for selective and saturable binding to a particular population of cells recovered from PP (Fig. 1E). No similar binding properties were observed when using cells recovered from mouse spleen, further arguing for tissue-specific interaction of SIgA (K. Kadaoui, unpublished observations).

Selective association of SIgA and SC to cell types recovered from mouse PP

Saturable binding suggests that not all cell types and subtypes present in PP are prone to interact with SIgA. To examine this issue, we extended our analysis by mixing green SIgA with PP cells that were, in turn, lit up with specific PE-labeled Abs. The number of double-labeled cells was divided by the absolute number of cells belonging to a particular cell type, and the ratio was expressed as a percentage. Consistent with the association data in Fig. 1, SC:GFP alone showed limited binding to DC, CD4⁺ T cells and B lymphocytes compared with SIgA (Fig. 2, lanes 1, 5, and 9 vs lanes 2, 6, and 10). In contrast, CD8⁺ T cells and macrophages did not exhibit significant differences in association that might reflect either nonspecific interaction or nonselective absorptive capacities (Fig. 2, lanes 13–16). When a 5-fold molar excess of IgAp/d was added to the cells before incubation with fluorescent SIgA, a marked and significant reduction in binding capacity was observed for DC ($p < 0.003$) and CD4⁺ T ($p < 0.001$) cells (Fig. 2, lanes 3 and 7), whereas the effect was close to the limit of significance ($p < 0.02$) for B cells (Fig. 2, lane 11). Interestingly, inhibition by SIgA did not favor any additional drop in association with DC, CD4⁺ T cells, and B lymphocytes, indicating that SC is

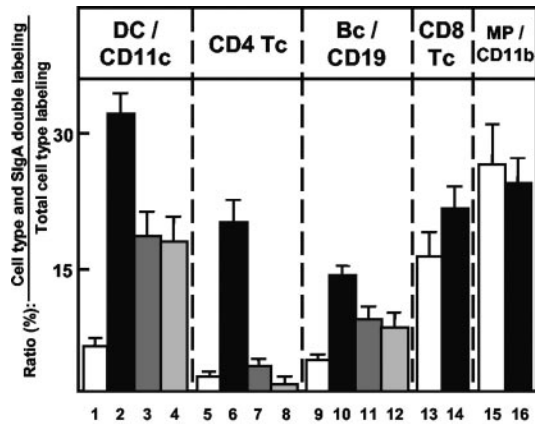


FIGURE 2. Association of SIgA, respectively SC, to cell populations present in mouse PP. The data shown represent the ratio of a particular cell type found in association with fluorescent proteins divided by the total amount of this cell type, expressed as a percentage. Tc, T cells; Bc, B lymphocytes; MP, macrophages. □, SC:GFP binding; ■, green SIgA binding; ▒, inhibition of green SIgA binding by IgAp/d; ▨, inhibition of green SIgA binding by SIgA. Only cell types exhibiting significant differential binding between the SC and SIgA ligands were tested in inhibition assays. Results are the mean ± SD from five or six independent experiments.

probably not involved in binding (Fig. 2, lanes 4, 8, and 12), in agreement with the weak interaction it is capable of when incubated alone. We show later in the paper that the incomplete inhibition of SIgA binding to DC is due to internalization (see Fig. 5). These experiments demonstrate that DC and CD4⁺ T and B cells exhibiting differential binding between SIgA and SC are most likely the cell types in PP that together contribute to the selective binding observed in Fig. 1. Globally, this results in one-third of DC, one-fifth of CD4⁺ T cells, and 15% of B cells in PP found in association with SIgA.

In vivo targeting of SIgA to mouse PP cells

To verify the relevance of the data obtained *in vitro*, we were interested in tracking the outcome of SIgA in the physiological context. To guarantee limited dilution, we chose to administer the green SIgA in a ligated intestinal loop comprising a PP. Because ligation is leaky (see *Materials and Methods*), the fate of the Ab downstream of the site of injection was also investigated in the next three PP (Fig. 3A). After a 4-h incubation in the mouse, individual PP cells were recovered, and the presence of associated green fluorescence was assessed by flow cytometry. More than 5% of cells in the PP at the site of SIgA injection were found to be positive (Fig. 3B, lane 1). Downstream PP 2 and 3 comprised 3 and 2.5% of fluorescent cells, respectively (Fig. 3B, lanes 2 and 3), whereas the percentage of green cells in the more distant PP was just above the autofluorescence measured in the control mice (Fig.

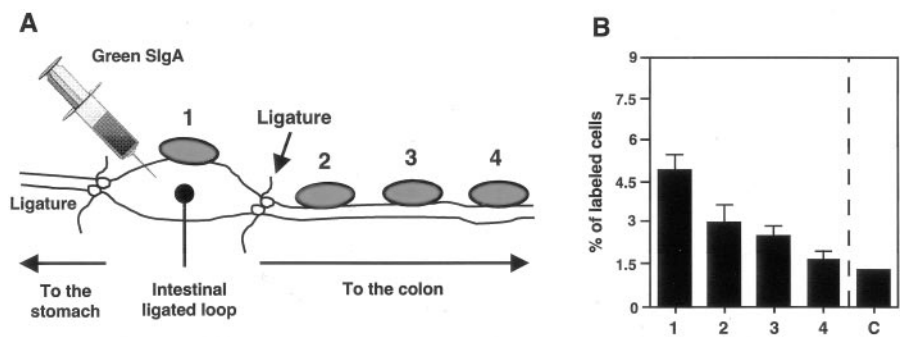
3B, lanes 4 and C). These results indicate that SIgA uptake by PP indeed occurs *in vivo* and appears not to depend simply on possible overpressure at the site of injection represented by the ligated intestinal loop. Transfer of SIgA from the lumen to PP that occurred *in vivo* prompted us to pursue the identification of the cell types targeted.

Fate of SIgA administered in a ligated intestinal loop containing a PP

Together with *in vitro* binding data, the uptake *in vivo* of SIgA by PP strongly suggests that DC, CD4⁺ T and B lymphocytes might be privileged targets for the Ab once internalized in the PP lymphoid follicle. After injection of green SIgA into a ligated loop, we generated PP sections that we examined by immunofluorescence using mAb specific for DC (Fig. 4A, B, and C) and CD4⁺ T cells (Fig. 4D, E, and F). Consistent with previous observations (30, 31), DC were found in the SED region mainly. Green SIgA molecules were detected in the same area of the section, which colocalized with most of DC (stained in red), as shown by the appearance of yellow spots (white arrowheads). Colocalization with CD4⁺ T cells was observed in the bottom part of the SED region, yet to a lesser extent, and at the bottom of the IFR (Fig. 4D, E, and F). Conversely, no convincing colocalization of signals for B cells and green SIgA could be obtained on the numerous sections analyzed (J. Rey, unpublished observations), indicating that *in vitro* data could not be confirmed by *in vivo* analysis in this particular case. Sections prepared from control mice did not yield any signal when examined under identical microscope settings. To exclude that the green fluorescence might result from GFP clipped off green SIgA, we performed a series of identical experiments using direct labeling of IgAp/d with Cy3 (red), followed by reconstitution with SC. Fig. 4G shows that red SIgA colocalizes with DC stained in green in the SED region, as pinpointed by arrowheads; some SIgA can also be observed in close association with the surface of the PP, consistent with previous observations (9, 32, 33). Abundant B cells detected in green in the upper part of the germinal center and more scarcely in the SED region do not colocalize with red SIgA (Fig. 4H). Similar to the picture in Fig. 4G, some SIgA are observed at the surface of the PP, and B cells are detected in what looks like the pocket of an M cell (Fig. 4H, white arrowheads).

To gain further insight into the molecular partner in SIgA involved in the colocalization with DC *in vivo*, we administered IgAp/d and IgAm labeled with Cy3 into ligated intestinal loops and tracked binding to DC in the SED region by immunofluorescence. Both molecular forms of the Ab were found in association with DC, as exemplified by the detection of yellow colocalization spots (Fig. 4, I and J). In contrast, SC: Cy3 remained distributed around labeled DC (Fig. 4K). Inasmuch as IgAm does not contain

FIGURE 3. Targeting *in vivo* of exogenously delivered SIgA to mouse PP. A, Green SIgA was injected into a ligated intestinal loop comprising a PP, marked 1 in the scheme. Ligation was performed to allow diffusion of green SIgA along the intestine. B, Four hours postinjection, PP (numbered 1–4 along the intestinal tract) were taken out independently, and association of the green Ab with PP cells was assessed by flow cytometry for each individual patch. Lane C corresponds to the background fluorescence intensity of PP cells isolated from a control mouse.



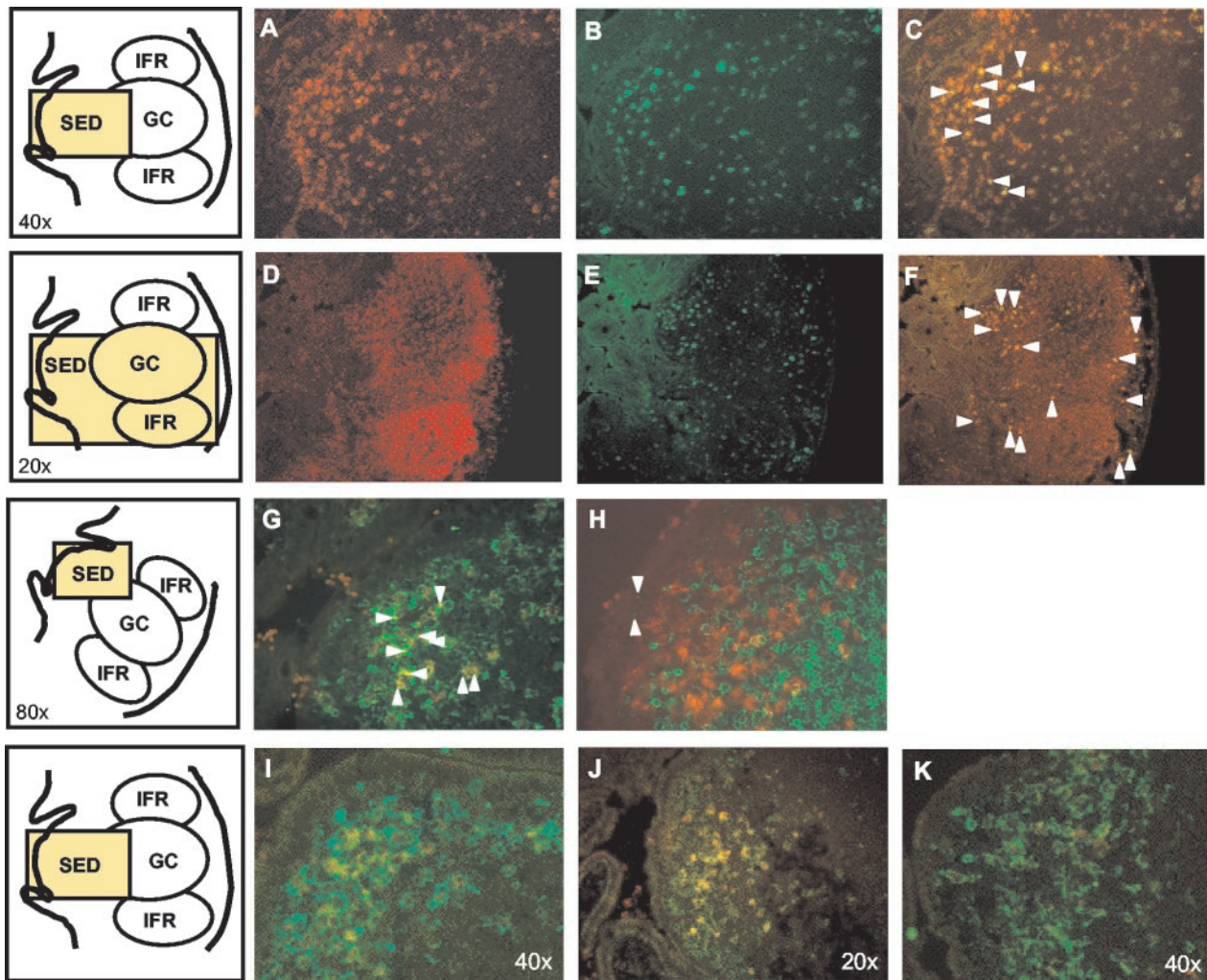


FIGURE 4. Outcome of SIgA *in vivo* after administration in a ligated intestinal loop comprising a PP. *A*, Visualization by Cy3-mediated immunofluorescence of DC in the SED region. Green SIgA molecules (*B*) colocalize with DC (arrowheads), yielding yellow staining (*C*). Cy3-labeled CD4⁺ T lymphocytes (*D*) associate with green SIgA (*E*), as shown by the detection of yellow signals in the SED region and IFR (*F*, arrowheads). *G*, Confirmation of colocalization is obtained using red SIgA and FITC-stained DC. *H*, Fluorescence associated with B cells (green) and red SIgA displays no overlap. Colocalization of red IgAp/d (*I*) and red IgAm (*J*) with DC (green) occurs, as reflected by the appearance of yellow spots, in contrast to SC: Cy3 (*K*). GC, germinal center. Fluorescence microscopy of sections comprising a PP. Pale yellow rectangles in drawings on the left indicate the area of PP under analysis. Magnification scales are marked on the drawings on the left (*A–H*) or directly on the fluorescence image (*I–K*).

J chain and fails to bind SC, it is likely that specific interactions occur between the Fc portion of IgA and a receptor to be identified.

DC from mouse PP internalize SIgA in vitro

We then hypothesized that DC, known to be potent APC (34), can internalize exogenously delivered SIgA. To test this assumption, we cocultured PP cells with biotinylated SIgA that we decorated using FITC-conjugated SAV. According to the sketch in Fig. 5, cells capable of internalizing SIgA will yield preserved fluorescence after tryptic digestion *in vitro*, whereas cells capable of surface binding only will lose fluorescence after exposure to trypsin. Similar to the experiments in Fig. 2, the percentages reported in Fig. 5*B* correspond to the signal of double-labeled cells divided by the signal of all cells of a particular cell type. The two left columns indicate that most DC remain SIgA positive after treatment with trypsin (■), suggesting that internalization took place during the incubation time (50 min overall) preceding the addition of the enzyme. In contrast, CD4⁺ T cells as well as B lymphocytes lost SIgA labeling under identical tryptic conditions, arguing for their

incapacity to protect the Ab by internalization or surface shielding (Fig. 5*B*).

DC from mouse PP internalize SIgA in vivo

Although *in vitro* and *in vivo* data showed remarkable similarity in the experimental settings assayed to date, we wanted to confirm *in vivo* the possible internalization of SIgA by DC. Green SIgA was administered into a ligated intestinal loop, sections were obtained, and labeling with specific PE-mAb for DC, CD4⁺ T and B lymphocytes was conducted. Laser scanning confocal microscopy analysis reveals that DC in the SED region have internalized SIgA (green arrowheads) that also colocalize (yellow arrowheads) at the cell surface (Fig. 6*A*). The absence of complete double labeling (red arrowheads) correlates with the data and images in Figs. 2 and 4. No internalization can be detected in the case of CD4⁺ T cells examined in the IFR, yet surface colocalization is confirmed (Fig. 6*B*, yellow arrowheads). The few green spots on the picture correspond to SIgA located in the neighborhood of T cells, as established by the observation of

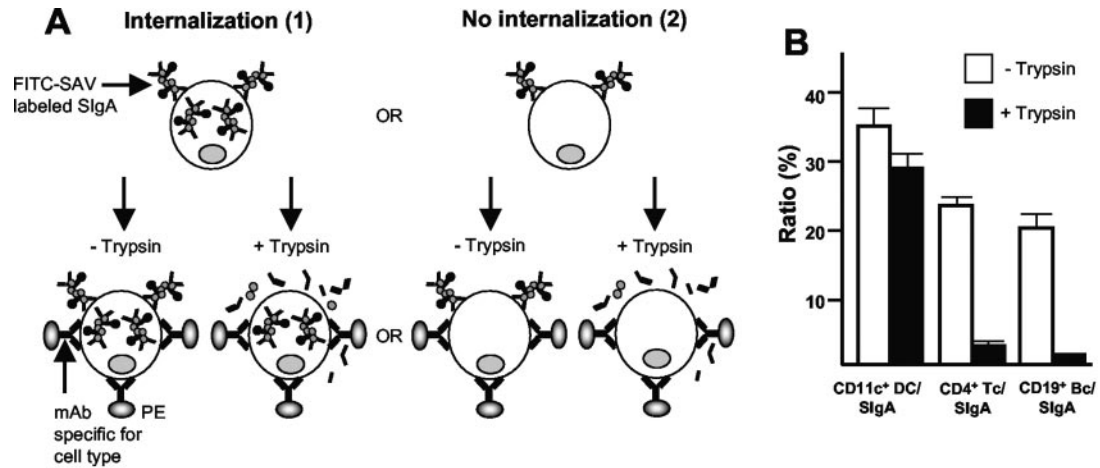


FIGURE 5. In vitro assay of SIgA binding and internalization by cells isolated from PP. *A*, The sketch represents the outcome of SIgA:biot-SAV:FITC as a function of their possible internalization (pathway 1) or surface binding (pathway 2). *B*, Double labeling with cell type-specific mAb and fluorescent SIgA in the absence of trypsin reflects surface binding and/or internalization (□). Upon exposure to trypsin, preserved double labeling resulting from internalization is found for DC only (■). Bars represent the mean ± SD of cell labeling obtained in three independent experiments.

successive plans. B lymphocytes observed in the germinal center do not bind or internalize SIgA (Fig. 6C). We conclude that PP DC are equipped to bind and internalize SIgA capable of entering the epithelium through M cell.

Discussion

Previous works demonstrated that SIgA binds specifically to M cells (7), but not to neighboring epithelial cells (9). M cells are specialized structures in the PP follicle-associated epithelium capable of taking up bacteria, viruses, and macromolecules for later presentation in the GALT (35, 36). Transport selectivity highlights the unique importance of M cells as being the primary site determining which type of Ag can be presented subsequently to the immune system. The present work describes the fate of SIgA after M cell binding and migration in the M cell pocket. We first show in vitro that SIgA Abs interact selectively with defined populations of PP cells, namely DC, T, and B lymphocytes, supporting the idea that SIgA might not stop their journey in the M cell pocket. Second, we confirm and extend in vitro data under near-physiological conditions in vivo, leading to the demonstration that SIgA is in-

deed found in association with DC and CD4⁺ T cells in the SED region and IFR of PP; SIgA is internalized by DC in the SED region. Third, the selective binding observed both in vitro and in vivo involves the IgA moiety of SIgA, not SC. Given the noninflammatory properties usually assigned to IgA, we speculate that mucosal entry of SIgA-containing immune complex might partly explain how this class of Abs balances homeostasis at mucosal surfaces.

DC in the SED region are anatomically positioned to sample Ag from external surfaces (37) and contribute to the regulation of intestinal responses depending on the presence or the absence of inflammatory signals (38, 39). Three phenotypically distinct subsets of DC in PP have been characterized (30, 40, 41). CD11c⁺CD11b⁺ myeloid DC are found in the SED region of PP, CD11c⁺CD8⁺ lymphoid DC localize in the T cell-rich IFR, whereas CD11c⁺ DC that lack either CD8α or CD11b are present in both the SED region and IFR. DC in the SED region show an immature phenotype, in that they phagocytose particulate and endocytose soluble Ag, synthesize MHC class II and invariant chains, and poorly stimulate resting T cells (42). Upon IL-6

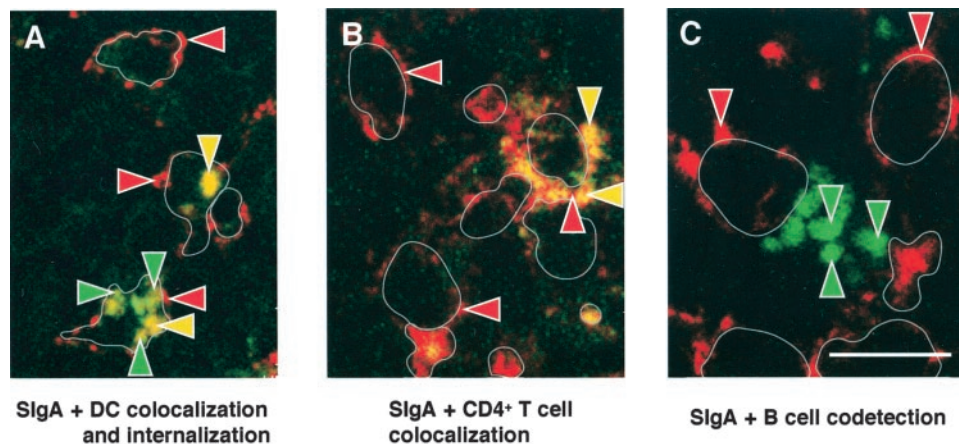


FIGURE 6. PP DC are capable of internalizing exogenously delivered SIgA. Analysis by laser scanning confocal fluorescent microscopy of the entry of green SIgA into PP DC in the SED region. White lines indicate cell circumference, which varies, as all cells are not shown in the same plan. *A*, Red arrowheads mark CD11c surface labeling; green arrowheads indicate internalization of SIgA; yellow arrowheads show surface colocalization. *B*, Green SIgA associates with CD4⁺ T cells (red arrowheads) in the IFR, displaying colocalization on the cell surface (yellow arrowheads). *C*, B lymphocytes (red arrowheads) in the germinal center do not bind or internalize SIgA (green arrowheads). White bar = 10 μm.

secretion, CD11c⁺CD11b⁺ myeloid DC contribute to IgA production (43); whether SIgA internalization by this subset of DC favors IL-6 secretion requires investigation. Furthermore, internalization of SIgA molecules by DC in the SED region of PP argues for possible Ag transport to the IFR (21) and mesenteric lymph nodes (44) under neutralized conditions. If we assume that the initiation of a mucosal immune response depends on both the recognition of foreign Ag and the modulation of APC inductive capacity by inflammatory or danger signals (45–47), then the Ag complexed with SIgA might favor further activation of the immune response under reduced or absent inflammatory conditions.

The interaction of SIgA with CD4⁺ T cells might appear more surprising. A scrutiny of the literature of the eighties that preceded the cloning of human CD89 (FcαRI) reveals that numerous papers reported on IgA binding receptors on murine T cells present in PP (48–54). The functional significance of (S)IgA-binding T cells remains to be determined. An intriguing possibility would be to regulate the expression of PP T cell cytokines (55) involved in the development of mucosal B cells into IgA-producing plasmocytes. Given that SIgA favors surface expression of IgA binding receptors on T cells in IgA inductive and effector tissues (56), this would contribute to potentiate isotype-specific mucosal immune response (57). Whether this represents the molecular rationale to explain the noninflammatory properties of SIgA is currently being examined in our laboratory. Moreover, the correlation found between in vivo and in vitro data will serve to reappraise the controversial nature of the receptor (58, 59). In conclusion, binding of SIgA to T cells in PP might represent another possible mechanism of local immunomodulation leading to a cytokine profile dominated by IL-4, IL-10, and TGF-β in mucosa (60, 61).

During primary infection, uptake by M cells can result in a microbial load greater than can be controlled and contained by the cellular immune defenses concentrated within mucosal lymphoid follicles. In the frame of secondary (recall) responses, association with pre-existing neutralizing SIgA capable of transporting them to underlying APC will leave infectious microorganisms detectable by the epithelial sampling machinery, but in a form that should limit the risk of overwhelming the local immune protection system. The modulating effect of SIgA could be exerted by directing the Ag to mucosal cells that display IgA receptors and by masking bacterial and viral epitopes in the local environment where the infection occurs. The strong correlation in SIgA binding observed using either PP DC ex vivo or within the tissue suggests that the biochemical characterization of mechanisms involving Ag processing, activation of surface markers, and capacity to trigger T cells with polarized cytokine expression is conceivable. We anticipate that such approaches should permit comparison in the near future of the differential modulatory effects on DC of SIgA-containing immune complexes or Ag alone.

Although our data argue for M cell as the privileged means of SIgA entry, we cannot strictly exclude alternative mechanisms. Similarly to intraepithelial DC capable to trap luminal Ag (62) and in view of their strategic location, it can be postulated that DC in the SED region protruding their dendrites across the dome epithelium may capture Ag, including SIgA as well. Consistent with the absence of IgA binding data to neighboring enterocytes outside the PP (9), no binding to DC in the lamina propria lining the epithelium could be detected. Thus, in contrast to *Listeria monocytogenes* injected into a ligated loop of the rat ileum with or without PP that can translocate within 15 min to deep organs with similar efficiencies (63), SIgA requires a PP to permit reuptake. Another advantage of using the M cell pathway is that the Ab or immune complex may be carried through by pinocytosis and released into the pocket (31), but only rarely goes into phagolysosomes (36).

Consequently, the integrity of antigenic molecules taken up from the lumen is preserved and, in the case of intrinsically stable SIgA, prompts selective targeting to underlying APC.

In summary, we propose that in addition to their contribution to immune exclusion, SIgA entering the mucosa through PP may act as an important immunomodulator to preserve the integrity of the intestinal barrier. Together with Th2 and Th3 cell polarization, it is thus reasonable to speculate that these features complement each other to govern mucosal homeostasis in the GI tract. Future studies should also explore whether IgA deficiency might contribute to the defect in immune regulation encountered in intestinal inflammatory diseases.

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