Secretory IgA Possesses Intrinsic Modulatory Properties Stimulating Mucosal and Systemic Immune Responses[1]

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Secretory IgA (SIgA) is essential in protecting mucosal surfaces by ensuring immune exclusion. In addition, SIgA binds selectively to M cells in Peyer's patches (PP), resulting in transport across the epithelium and targeting of dendritic cells (DC) in the dome region. The immunological consequences of such an interaction are unknown. In this study, we find that oral delivery of SIgA comprising human secretory component and mouse IgA induces human secretory component-specific Ab and cellular responses in mucosal and peripheral tissues in mice. This takes place in the absence of coaddition of cholera toxin, identifying so far unraveled properties in SIgA. Specific immune responses are accompanied by sustained IL-10 and TGF-β expression in draining mesenteric lymph nodes and spleen. SIgA also triggers migration of DC to the T cell-rich regions of PP, and regulates expression of CD80 and CD86 on DC in PP, mesenteric lymph nodes, and spleen. These results provide evidence that mucosal SIgA reentering the body exerts a function of Ag delivery that contributes to effector and/or regulatory pathways characteristic of the intestinal mucosal compartment. ***The Journal of Immunology,* 2005, 175: 2793–2800.**

Mucosal surfaces comprising the gastrointestinal, respiratory, and urogenital mucosae represent a large port of entry (400 m^2 in humans) (1) for most of the pathogens and thus have to be efficiently protected. This goal is achieved by a combination of constitutive, nonspecific substances (e.g., mucus, lysozyme, lactoferrin, and defensins) (2, 3) and induced specific immune mechanisms involving cellular and Ab responses (4, 5). The chief Ab at mucosal surfaces is secretory IgA (SIgA)[3] (6, 7), a multimeric structure made of polymeric IgA (pIgA) produced by activated B cells (8) in the mucosal epithelium and of secretory component (SC), which is derived from the polymeric Ig receptor that ensures selective transcytosis of pIgA across the epithelial cells of mucous membranes (9). Once in secretions, SIgA binds Ag(s), thus preventing their adhesion to the luminal epithelial surface and facilitating their elimination by peristalsis or mucociliary movements (2), a phenomenon called immune exclusion (10).

SIgA exhibits a remarkable stability in the harsh environment of the gastrointestinal tract (11). Another feature of SIgA is their capacity to adhere selectively to microfold (M) cells irrespective of their Ag-binding specificity (12, 13) in Peyer's patches (PP) (14); these latter are subsequently able to transport SIgA across the epithelium and to bring them in contact with the underlying structure named organized MALT (15). After transport, we found that SIgA Abs are internalized by dendritic cells (DC) in the subepithelial dome (SED) region and associate with CD4$^+$ T cells in the interfollicular region (16). As DC in the SED are anatomically positioned to sample Ag from the intestinal lumen and contribute to the regulation of local immune responses (17, 18), our observation provided a first link between DC targeting and immune activation by SIgA.

The possible immunomodulatory effects of the interaction between DC and SIgA has only been investigated in a small number of in vitro studies using human monocyte-derived DC exhibiting a myeloid phenotype (19, 20). Results obtained from these works gave conflicting results in terms of DC maturation, partly due to differences in preparation and inhomogeneous sources of SIgA. Furthermore, it is highly likely that the properties and functions of these cells differ in their natural tissue environment (21, 22). However, studies examining the role for the luminal to subepithelial transport of SIgA and their relationship with DC in the physiological context are missing.

In this study, we have shown that oral immunization of naive mice with reconstituted SIgA molecules (rSIgA) consisting of mouse pIgA and human SC (hSC) serving as a nonself Ag induced mucosal and systemic responses against hSC in the absence of any mucosal adjuvant. The data support the notion that retrotransported SIgA molecules are able to stimulate a mucosal immune response toward associated nonself Ag via their activating action on DC.

Materials and Methods

Mice

Eight- to 15-wk-old female BALB/c mice (Harlan) were used for this work. All animals housed under conventional conditions in the animal facility of the Centre Hospitalier Universitaire Vaudois were tagged for identification. All experiments involving animals were approved by the State Veterinary Office.

Preparation of hSC and rSIgA molecules

Recombinant hSC molecules were recovered from the supernatant of Chinese hamster ovary cells stably transfected with the pcDNA3:SC expression vector (23) and purified by size exclusion chromatography on a 1.6 × 100-cm Superdex 200 column (Amersham Pharmacia Biotech). Mouse pIgA were obtained from the hybridoma HNK20, purified, and associated to hSC as described (24). Final preparations containing highly purified rSIgA molecules were concentrated using Centricon-100 U up to 2–2.5 mg/ml, 0.22-μm filtered onto membranes, and stored at 4°C until use. As

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[3] Abbreviations used in this paper: SIgA, secretory IgA; CT, cholera toxin; DC, dendritic cell; MLN, mesenteric lymph node; M, microfold; pIgA, polymeric IgA; PP, Peyer's patch; rSIgA, reconstituted SIgA; SC, secretory component; hSC, human SC; SED, subepithelial dome.

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a substitute for immune complexes, we chose to use heterologous rSIgA because of the stability of such molecules in the gastrointestinal tract (11).

Immunizations

Mouse oral immunizations were performed by orogastric intubation with polyethylene tubing under light anesthesia with halothane (Halocarbon Laboratories). The tubing was introduced at a fixed distance of 1.8 cm from the incisors. Immunizations consisted of four administrations of 200–250 μl (depending on the concentration of the administered preparation) at 1-wk intervals, followed by a last booster immunization 5 wk later. Preliminary dose-response experiments were conducted; based on the results of such experiments, groups of 11 mice were immunized with either 100 μg of hSC, 100 μg of hSC plus 5 μg of cholera toxin (CT; Calbiochem) or 500 μg of rSIgA at each administration. All Ag preparations were diluted in PBS.

Blood and saliva recovery

Biological fluids were recovered before immunization and 1 wk after each immunization to examine their Ab content. Serum samples were obtained from whole blood recovered by performing a small incision at the tail of the mice. Saliva was collected from mice injected with 50 μl of a pilocarpin solution (500 μg/ml; Sigma-Aldrich) through small absorbent polyester sticks (Polywicks, 2×25 mm; Polyfiltronics) placed in the mouth of the animal. Sticks were then centrifuged at $10{,}000 \times g$ in a spun column, and saliva eluates were stored at −20°C until use. Previous reports have established that the measure of SIgA in saliva properly reflects the mucosal Ab response at the intestinal level (25, 26).

Measurement of seric and salivary Abs

Abs in biological fluids were measured by ELISA coated with 300 ng of hSC in 50 μl of PBS per well (96 microwell plates, Nunc-immuno Plate Maxisorp surface; Nalge Nunc International). Serial dilutions of biological fluids (1/500 to 1/16,000 for total seric IgG; 1/2 to 1/40 for salivary SIgA; 1/1,000 to 1/80,000 for subclass analysis) were incubated with goat anti-mouse IgG Ab (1/1,000 dilution; Sigma-Aldrich), goat anti-mouse IgA antiserum (1:1,000 dilution, α-chain specific; Sigma-Aldrich), or biotinylated goat anti-mouse IgG1 or IgG2a antisera (1/1,000 dilution; Caltag Laboratories). Detection was performed using HRP or alkaline phosphatase-conjugated secondary reagents, using ortho-1,2-phenylenediamine or 4-nitrophenyl phosphate as substrates. Absorbances were measured at 492 and 405 nm, respectively. The Ab titer for each sample is expressed as the reciprocal of the highest dilution for which the absorbance was 2.5 times greater than the absorbance of control sera at the lowest dilution. Results are given as the means of titer ± SEM. As the titers of salivary IgG were very similar in all experimental groups, this rules out that transudation of Abs affects artifactually the titers of hSC-specific salivary SIgA (data not shown).

Proliferation assays

Mice were sacrificed by cervical dislocation under anesthesia 2 wk after the booster immunization. Proliferation assays were performed with cells isolated from mesenteric lymph nodes (MLN) and spleen of mice as described (27). A total of 5×10^5 cells per well were cultivated in the proliferation medium alone or in the presence of 3 μg/ml purified hSC for 5 days. The diverse immunization groups were then compared on the basis of the stimulation index obtained for each tested mouse obtained by dividing geometric mean of Ag-stimulated cpm by background cpm.

Semiquantitative real-time RT-PCR analyses for cytokine expression

MLN and splenic cells collected as above were cultivated (4×10^6 cells per well) in 24-well culture plate (Corning Costar) for 48 h in proliferation medium, alone or in the presence of 3 μg/ml purified hSC. After harvesting of cells, total RNA was extracted using the guanidinium-thiocyanate/acid phenol method (28). Real-time RT-PCR (50 cycles) to measure expression of IL-4, IFN-γ, and IL-10 was performed as described (29). Data were expressed as $1/(Ct_{cytokine} - Ct_{cyclophilin})$ where Ct represents the cycle number in which the SYBR green fluorescence crossed the threshold value set at 0.2. Production of TGF-β in culture supernatants was assayed in duplicates, after sample acidification, by ELISA using a commercial kit (R&D Systems) following the instructions of the manufacturer.

Tracking of DC migration within PP

Experiments were performed as described (30). Briefly, mice were given intragastrically a suspension of 10^{12} Fluoresbrite Yellow Green microspheres (diameter 0.2 μm; Polysciences) in 500 μl of PBS by intubation with polyethylene tubing under light anesthesia with halothane (Halocarbon Laboratories). The tubing was introduced at a fixed distance of 3.8 cm from the incisors. Forty-eight hours later, mice were given intragastrically (as for microspheres administration) either 50 μg of CT or 500 μg of rSIgA diluted in a final volume of 500 μl of PBS. Based on trial kinetics experiments and data from other experimental systems (31), mice were sacrificed by cervical dislocation 24 (CT) or 38 h (rSIgA) later, respectively. Control mice administered fluorescent microspheres only were sacrificed at diverse time points up to 96 h. Three distal PP per mouse were harvested, embedded in OCT (Miles), frozen in liquid nitrogen-cooled isopentane, and stored at −20°C. Frozen sections (7 μm) were obtained using a Leica Cryostat model CM 1800 apparatus and mounted on SuperFrost Plus microscope slides (Menzel-Gläser). To visualize fluorescent microspheres, sections (60–80 for each experimental setting) were mounted in PBS, examined, and photographed using a Axioplan microscope (Carl Zeiss). For laser scanning confocal microscopy analyses, sections were washed in PBS and blocked for 30 min with a PBS solution containing 2% FCS (PBS-S) and 5% mouse serum. Incubation with biotinylated anti-CD11c (BD Pharmingen) mAb diluted 1/10 was then conducted in PBS-S at room temperature for 2 h. After successive PBS-S washings, sections were incubated with streptavidin-conjugated indocarbocyanine (Cy3) fluorochrome (Jackson ImmunoResearch Laboratories) diluted 1/1000 in PBS-S for 30 min, washed again, and finally mounted in Vectashield (Vector Laboratories). Observations were performed using a TCS NT laser scanning confocal microscope (Leica Microsystems).

Analysis of CD80 and CD86 expression on DC surface

Mice were given once either 10 μg of CT or 500 μg of rSIgA by oral administration as described for the immunization trials. At diverse time points, mice were sacrificed and lymphocytes were isolated from the PP, MLN, and spleen. Freshly isolated cells were washed once with 1 ml of cold PBS solution supplemented with 1% BSA (PBS-BSA) before incubation for 15 min with anti-FcγIIIR/FcγIIR blocking Abs (anti-CD16/CD32; BD Pharmingen) diluted 1/100 in PBS-BSA. Expression of cell surface markers was determined by double staining using either FITC-conjugated anti-CD80 (clone 16-10A1) or anti-CD86 (clone GL1) mAb and PE-conjugated anti-CD11c (clone HL3) mAb (all from BD Pharmingen) diluted 1/100 in PBS-BSA. After coincubation for 20 min on ice (final volume of 100 μl), cells were washed once with cold PBS-BSA solution, resuspended in PBS-BSA, and 0.6 ng/ml propidium iodide was added to exclude dead cells from the analysis by flow cytometry (FACScan; BD Biosciences). Cells recovered from naive mice (time 0) were used to determine the fluorescence background.

Statistical analysis

All statistical analyses were performed using the InStat version 2.01 software from GraphPad Software and the unpaired two-tail Mann-Whitney U test was applied. Significance limit was set at $p \leq 0.05$.

Results

Nonself epitopes comprised within rSIgA complexes are immunogenic via the oral route in the absence of any adjuvant

SIgA was shown to enter PP and be targeted to DC in the SED region (16). This prompted us to investigate whether this property of SIgA is relevant to the capacity to induce subsequent immune responses. To this goal, mice were immunized with rSIgA molecules comprising a SC moiety of human origin serving as a source of nonself epitopes. We chose to use heterologous rSIgA as a substitute for immune complexes because of the stability of such molecules in the gastrointestinal tract (11). Thus, mice received 500 μg of rSIgA per oral administration as described in *Materials and Methods*, or an equimolar amount of hSC (100 μg) combined with 5 μg of CT acting as mucosal adjuvant. Mice in the negative control group were immunized with 100 μg of hSC alone. Mucosal immunization being known to induce mucosal and systemic responses (32), both saliva and blood samples were collected at different time points to examine the Ab production in these fluids. Significant levels of anti-hSC specific SIgA were measured after the third immunization in saliva of animals having received rSIgA

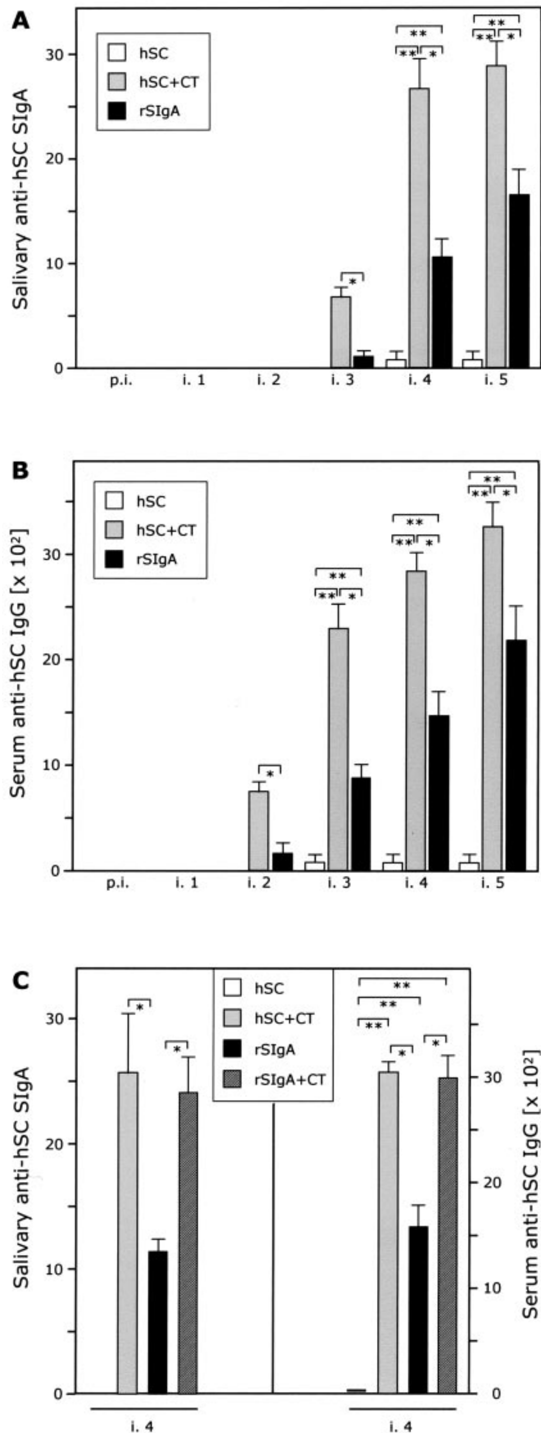

FIGURE 1. Oral immunization with rSIgA induces mucosal and systemic humoral responses in mice. Blood and saliva samples were recovered preimmunization (p.i.) and 1 wk after each immunization with either hSC, hSC plus CT, or rSIgA (i.1 to i.5). End-point titers of salivary anti-hSC-specific SIgA (*A*) and serum anti-hSC-specific IgG (*B*; all subclasses) were measured by ELISA. *C*, Control independent experiment showing levels of hSC-specific SIgA and IgG in mice administered rSIgA plus CT in comparison with immunization using either hSC, hSC plus CT, or rSIgA. Ab levels were measured 1 wk after the fourth immunization, and end-point titers were determined as in *A* and *B*. Bars represent mean values ± SEM obtained from 11 (*A* and *B*) or four (*C*) mice per immunization group. Significant statistical differences are indicated. *, $p < 0.05$; **, $p \leq 0.0005$.

or hSC plus CT (Fig. 1*A*), with subsequent recalls leading to continuous increases in Ab production. The appearance of hSC-specific IgG Ab preceded that of SIgA by 1 wk, with responses again in both groups of mice immunized with rSIgA or hSC plus CT (Fig. 1*B*). When compared with controls given hSC alone, the association of hSC in the form of rSIgA complexes yielded pronounced Ab production that confers to the IgA moiety properties usually encountered when using the mucosal adjuvant CT. Levels of hSC-specific SIgA and IgG Ab in saliva and sera of mice immunized with rSIgA plus CT compare directly with that measured in mice given hSC plus CT (Fig. 1*C*), suggesting a dominant effect of CT over IgA.

We thus sought to determine whether the differences in activating properties of rSIgA as compared with CT reflected themselves at the cellular level. Two weeks after the last booster immunization, mice were sacrificed and T cell proliferation assays were performed with cells recovered from MLN draining the mucosal environment and spleen serving as a marker of the systemic response. At the time of analysis, low stimulation indices were obtained using MLN cells of mice challenged with rSIgA and hSC alone, whereas animals given hSC plus CT exhibited significant increases (Fig. 2*A*). Meanwhile, splenocyte proliferation took place for both groups given rSIgA or hSC plus CT as compared with control animals administered hSC (Fig. 2*B*).

An equivalent Th bias is induced after oral immunization with rSIgA or hSC plus CT

After having quantitatively characterized the immune response triggered by oral administration of various forms of hSC, we got interested in evaluating the possible Th bias associated with this response. To this purpose, we analyzed hSC-specific IgG1 and IgG2a Ab production in sera recovered 1 wk after the final boost. As for total IgG, the highest IgG1 and IgG2a Ab levels were measured in the sera of mice administered hSC plus CT (Fig. 3, *A* and *B*). Similarly, oral delivery of rSIgA led to Ab production of both isotypes (Fig. 3, *A* and *B*), whereas hSC alone yielded 65 times (IgG1) and 60 times (IgG2a) lower titers. IgG1 to IgG2a ratios calculated for each individual mouse revealed equivalent values for the two groups of mice immunized with rSIgA or hSC plus CT

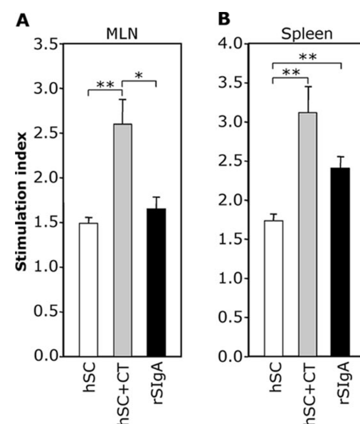

FIGURE 2. Oral immunization with rSIgA induces mucosal (MLN) and systemic (spleen) cellular responses in mice. Mice immunized with either hSC (□), hSC plus CT (▨), or rSIgA (■) were sacrificed 2 wk after the last immunization, and proliferation assays were performed in medium containing 3 μg/ml purified hSC or in the absence of the Ag for 5 days. Bars represent mean values of the calculated stimulation indices ± SEM obtained from 11 mice per immunization group. Significant statistical differences are indicated. *, $p < 0.01$; **, $p < 0.0005$.

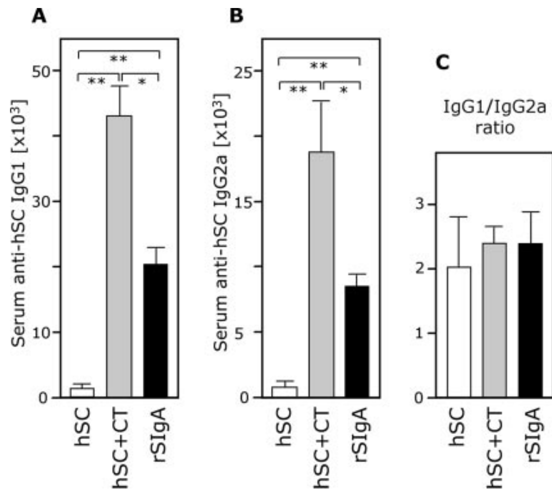

FIGURE 3. An equivalent Th bias is induced after oral immunization with rSIgA or hSC plus CT. Blood samples were collected from mice immunized with either hSC (□), hSC plus CT (▨), or rSIgA (■) 1 wk after the last immunization. Anti-hSC-specific IgG1 (*A*) and IgG2a (*B*) responses were measured by ELISA using serial dilutions. The IgG1 to IgG2a ratio (*C*) was calculated for each individual mouse. Bars represent mean values ± SEM obtained from 11 mice per immunization group. Significant statistical differences are indicated. *, $p < 0.001$; **, $p < 0.0001$.

(Fig. 3*C*). This result indicates that immunization with rSIgA induces a similar Ab ratio bias as does CT.

To gain insight into the possible Th1 or Th2 deviation induced after rSIgA immunization, we analyzed the expression of IL-4, IFN-γ, and IL-10 using real-time RT-PCR, as such an approach was shown to accurately reflect the relative amounts of cytokine measured at the protein level (29), whereas activated TGF-β production was quantitated by ELISA (33). To permit analysis at both the mucosal and systemic levels for individual mouse, cells isolated from MLN and spleen were used. MLN cells isolated from mice immunized either with rSIgA or hSC plus CT significantly up-regulated expression of IL-4 and IFN-γ when compared with the hSC control group (Fig. 4*A*). In MLN, determination of the IFN-γ to IL-4 ratio indicated no measurable immune deviation between rSIgA and hSC plus CT groups (1.19 ± 0.21 vs 1.39 ± 0.23; $p = 0.0845$), suggesting a mixed Th1/Th2 response. Expression of IL-10 remained remarkably stable whatever the immunization procedure (Fig. 4*A*), emphasizing a basal, dominant role of this cytokine in the mucosal context; yet the immunological relevance of low IL-10 expression might be questionable. Interestingly, significantly higher production of TGF-β was observed when using MLN cells from mice that received rSIgA as compared with the two other groups (Fig. 4*C*, *left*). Splenocytes of mice given hSC plus CT up-regulated cytokines toward a Th1 bias (IFN-γ to IL-4 ratio, 1.69 ± 0.33), as seen in the mucosal pulmonary environment (34). In contrast, splenic cells of mice of the rSIgA group maintained a mixed response (Fig. 4*B*; IFN-γ to IL-4 ratio, 1.13 ± 0.14), with maintenance of IFN-γ expression in the rSIgA group as compared with mice given hSC plus CT ($p < 0.0001$). Expression of IL-10 was observed as in MLN, yet it was more pronounced in the case of mice immunized with rSIgA and hSC plus CT. In contrast to MLN, homogenous production of TGF-β by splenocytes recovered from each group was measured (Fig. 4*C*, *right*). Together, this emphasizes the tendency of rSIgA immunization to induce a mixed Th1/Th2, tolerance-biased pattern of cytokines in mucosal tissues.

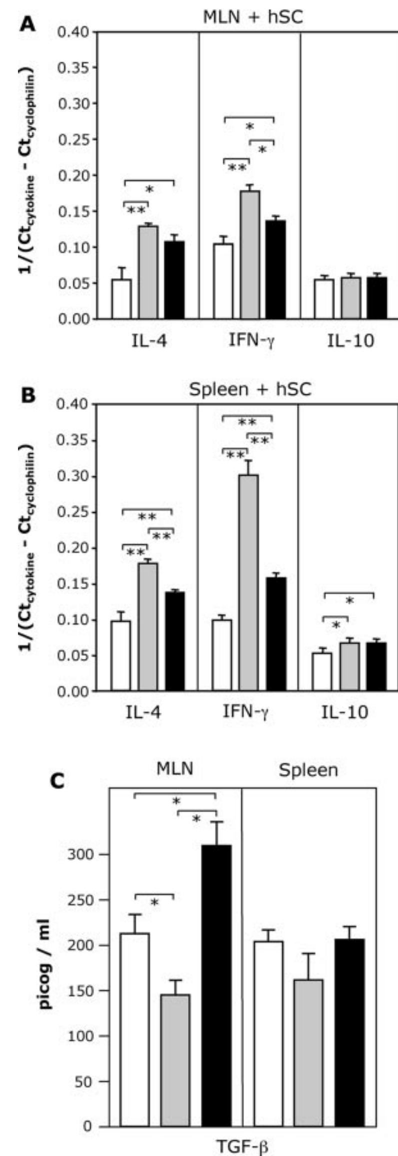

FIGURE 4. Cytokine analysis reveals a more pronounced Th2-biased response in mice administered rSIgA as compared with mice given hSC plus CT. Mice having received orally five times either hSC (□), hSC plus CT (▨), or rSIgA (■) were sacrificed 2 wk after the last immunization. Cells isolated from MLN (*A*), and spleens (*B*) were cultured in the presence of 3 μg/ml purified hSC for 48 h. Expression of IL-4, IFN-γ, and IL-10 was analyzed by real-time RT-PCR (represented as $1/(Ct_{cytokine} - Ct_{cyclophilin})$ ratio). *C*, Production of TGF-β in acidified culture supernatants was determined by ELISA (picograms per milliliter). Bars represent mean values ± SEM obtained from 11 mice per immunization group. Significant statistical differences are indicated. *, $p < 0.05$; **, $p < 0.0005$.

Intragastric administration of rSIgA induces DC migration within PP

The observation that rSIgA bearing a nonself Ag together with the targeting to PP DC trigger modulation of mucosal and systemic immune responses led us to investigate whether rSIgA contributes to induce DC maturation as does CT used as control (35). A feature of DC maturation (36), we examined migration from the SED region in PP to the interfollicular region where Ag presentation to T cells occurs (37). Fluorescent microspheres given intragastrically were accumulating in murine intestinal PP in the absence of external stimulus, yet the strict demonstration of internalization in a particular cell type was not achieved (30). Using laser scanning

confocal microscopy, we first determined by double staining that fluorescent microspheres localize within the cytoplasm of DC present in the SED region (Fig. 5, *A* and *B*). Thus, tracking of moving fluorescent microspheres in the PP reflects migration of DC within the tissue. Analysis of the PP of mice having received fluorescent microspheres 24 h before intragastric rSIgA administration revealed that fluorescent microsphere-loaded DC were present in both the SED region and the interfollicular region 38 h postadministration. (Fig. 5, *C* and *D*). A similar pattern of migration was visualized 24 h after the administration of CT alone to mice previously given fluorescent microspheres (Fig. 5, *E* and *F*). In contrast, no fluorescent microsphere-loaded cells could be detected in the interfollicular region of mice in the absence of stimulus, although fluorescent microsphere-loaded DC were indeed present in the SED region (Fig. 5, *G* and *H*). These results demonstrate that mucosally delivered rSIgA has a direct effect on the capacity of DC in vivo to migrate from sampling tissues to underlying sites of presentation.

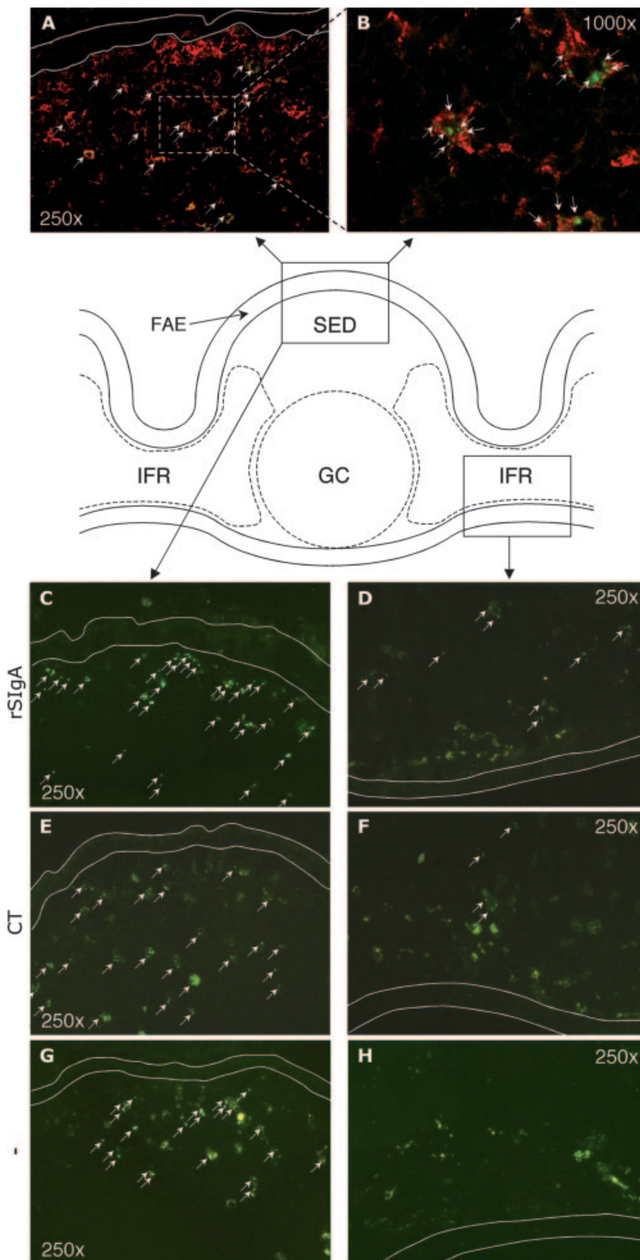

FIGURE 5. Migration of DC from the SED region to the interfollicular region of PP. Mice were given 10^{12} green fluorescent microspheres by intragastric delivery. Animals were kept as such (*A, B, G,* and *H*) or received a single gastric administration of either rSIgA (*C* and *D*) or CT (*E* and *F*) 48 h later. Mice were sacrificed, distal PP were collected, and 7-μm thick sections were prepared. The presence of microspheres within DC labeled with red Cy3-conjugated anti-CD11c mAb was demonstrated using laser scanning confocal microscopy of the SED region (*A* and *B*). The presence of green microspheres in the SED region of PP follicles (*C, E,* and *G*) or in the interfollicular region (*D, F,* and *H*) was visualized by fluorescence microscopy. White arrows indicate the presence of microspheres within DC. FAE, follicular-associated epithelium; IFR, interfollicular region; GC, germinal center. In the scheme, rectangles mark the area of PP under analysis. Magnification scales are indicated on the fluorescence images.

CT and rSIgA differentially affect the expression of costimulatory markers CD80/CD86 on DC along the PP-MLN-spleen pathway

Another main feature of DC maturation is their acquisition of surface-expressed costimulatory molecules including CD80 and CD86 (38). Previous scarce works examining CD80 and CD86 regulation by CT did not allow defining consensus analytical time windows (39, 40). Furthermore, with the exception of one study (41), no direct gastrointestinal mucosal exposure followed by collection of the appropriate cells involved in the immune response to be examined has been achieved to date. Therefore, to get a dynamic view of the in vivo modulation of CD80/CD86 surface expression, we conducted the analysis using time points at 2, 6, 14, 24, or 38 h after a single oral administration of CT or rSIgA. Moreover, to get spatial information, we extended the analysis to diverse lymphoid organs including PP, MLN, and spleen. Flow cytometry analysis performed on groups of four to six mice per time point compared the median fluorescence intensity for CD80/CD86 markers on live DC (Table I). In mice fed CT, significant up-regulation of DC CD80 as compared with time 0 was detected at 14 h in PP and MLN, at 24 h in MLN and spleen, a tissue that maintained high expression at 38 h. The increase in CD80 expression followed different kinetics and pattern in DC of mice administered rSIgA. Only PP and spleen cells displayed a significant up-regulation at 24 and 38 h. Thus, both the prototype mucosal adjuvant CT and rSIgA contribute to selectively modulate CD80 surface expression on DC along the PP-MLN-spleen pathway, although with a different kinetics and intensity. As long as CD86 is concerned, statistically significant differences were observed in PP only. In the case of CT, a raise in surface expression occurred at 14 h, whereas a decrease resulted at 2 and 6 h in mice fed rSIgA.

Discussion

Previous works have shown that SIgA binds selectively to M cells in PP, before being transported across the epithelium underlying DC present in the SED region. However, the possible immunological consequences of SIgA targeting and entering DC were not explored in these studies. The present report investigates the potential mucosal immunogenicity of rSIgA carrying a nonself Ag, and the relationship of this effect on maturation/migration of mucosal DC in vivo. The novelty of our data resides in the demonstration that, besides its already widely described role in the protection of mucosal surfaces, SIgA works as a weak immunopotentiator in the mucosal environment. This resulted in both mucosal and systemic hSC-specific responses identified at the Ab and cellular levels, yet less marked when compared with responses triggered upon mixing of hSC with the prototype mucosal adjuvant CT (Figs. 1 and 2). Analysis of the systemic Ab responses demonstrated that the same Th bias was obtained (Fig. 3), yet

Table I. *Oral administration of CT or rSlgA induces modulation of the costimulatory markers CD80 and CD86 on PP, MLN, and splenic DC*

	Time Postadministration[a]					
	0	2 h	6 h	14 h	24 h	38 h
CD80[b]						
PP						
CT	12.58 ± 2.29[c]	12.37 ± 2.30	12.13 ± 1.28	**17.08 ± 3.27***[d]	12.61 ± 1.19	14.75 ± 3.92
rSlgA	11.69 ± 1.59	10.51 ± 1.40	10.48 ± 2.39	10.97 ± 1.92	**13.63 ± 1.27***	**15.22 ± 2.11****
MLN						
CT	14.57 ± 3.12	12.00 ± 2.10	**10.74 ± 1.52***	**19.38 ± 3.75***	**20.77 ± 6.85***	16.13 ± 3.24
rSlgA	13.70 ± 3.59	10.42 ± 1.49	11.45 ± 3.50	14.26 ± 1.42	13.48 ± 2.25	13.85 ± 3.93
Spleen						
CT	25.92 ± 4.94	26.55 ± 5.33	27.60 ± 5.86	30.04 ± 5.46	**39.78 ± 12.56***	**40.02 ± 4.65****
rSlgA	25.75 ± 6.23	24.50 ± 4.67	18.13 ± 7.97	31.01 ± 4.53	**34.43 ± 3.60***	**34.84 ± 9.80***
CD86[b]						
PP						
CT	11.30 ± 1.20	12.25 ± 1.43	10.75 ± 0.31	**14.80 ± 1.67****	11.18 ± 20.6	11.86 ± 2.66
rSlgA	10.65 ± 1.37	**8.23 ± 0.73****	**8.76 ± 0.71***	10.41 ± 1.53	10.81 ± 2.24	10.80 ± 2.44
MLN						
CT	19.01 ± 2.61	17.89 ± 2.19	16.15 ± 1.30	21.11 ± 4.39	21.95 ± 7.55	17.06 ± 2.58
rSlgA	17.53 ± 3.07	14.34 ± 0.74	15.31 ± 1.39	16.95 ± 2.13	20.64 ± 8.20	16.67 ± 3.96
Spleen						
CT	13.79 ± 2.65	15.06 ± 1.63	15.39 ± 2.33	15.80 ± 2.29	17.13 ± 6.05	13.39 ± 1.34
rSlgA	14.48 ± 1.69	17.14 ± 3.88	15.51 ± 2.19	17.18 ± 3.90	15.66 ± 2.25	14.75 ± 5.46

[a] Mice were administered 10 μg of CT or 500 μg of rSlgA, and cells from PP, MLN, and spleen were isolated 2, 6, 14, 24, or 38 h later. Naive mice were used as control (time 0).
[b] Cells were double stained with PE-labeled anti-CD11c mAb and FITC-labeled mAb to CD80 or CD86 and analyzed based on gating of CD11c$^+$ DC.
[c] Values represent the mean of the median fluoresence intensities of CD80 or CD86 expression ± SEM obtained from four to nine mice per time point.
[d] Significant statistical differences between various time points and time 0 are highlighted in bold characters.
*, $p < 0.05$.
**, $p < 0.01$.

refined definition using cytokine expression patterns by cells isolated from MLN and spleen (Fig. 4) revealed that immunization with rSIgA rather induced a mixed Th1/Th2 type of response.

Of note, in both cellular proliferation and cytokine assays, the intensity of responses and differences between groups were more marked in the spleen than in MLN. This feature is most likely due to the fact that at the time T cell proliferation and cytokine expression were measured, the frequency of effector/memory cells was greater in the spleen than in draining MLN (42). Although cells in the present study were recovered from mice 2 wk after the final boost, our results confirm both different tissue-specific turnover and kinetics of persistence (43, 44) also in the context of immunization with rSIgA. Overall, although intrinsically weaker than those determined using splenocytes, the responses observed with cells isolated from MLN indicate Ag-specific activation of the mucosal immune system after rSIgA immunization.

Our findings that similar levels of IL-4 were produced in the MLN of mice given rSIgA and hSC plus CT, whereas less IFN-γ is produced in response to immunization with rSIgA might provide a first clue as to how inflammation is limited without abolishing immune responses. Homeostasis of inflammation can occur through IL-4 serving as a differentiation factor for TGF-β producing cells (45, 46). In this respect, the highest production of TGF-β, a cytokine related to intestinal regulatory T cells (47), was measured with cells recovered from the MLN of mice given rSIgA (Fig. 4*C*). Moreover, splenic cytokine analyses revealed a small but significant up-regulation of IL-10 expression in mice immunized with hSC plus CT or rSIgA (Fig. 4*B*). Such an IL-10 expression may be also correlated to the generation of regulatory T cells as it has been observed after immunization with CT (48). This suggests that rSIgA and hSC plus CT oral immunization did not override local production of noninflammatory cytokines, yet the expression of others involved in IgA switch (IL-4) and immune reaction in lymphoid organs (IFN-γ) promoting the induction of effector T cells was increased. Consistent with this, CT in vivo down-regulates rather than induce inflammatory stimuli produced by DC, including IL-6, IL-18, IL-12, and TNF-α, as compared with more proinflammatory stimuli including LPS and CD40L for example (48, 49). From another point of view, TGF-β and IL-10 act in concert to promote IgA isotype switch (50), and could additionally be important for the induction and maintenance of mucosal SIgA production.

Moreover, our results demonstrate that a single mucosal administration of rSIgA induced 1) migration of the DC from the SED region to the interfollicular region (Fig. 5) and 2) increased expression of costimulatory molecules CD80 and CD86 on these cells along the PP-MLN-spleen pathway (Table I). Comparison with CT used as a control adjuvant indicates that SIgA exhibits distinct intrinsic adjuvant-like properties, which marks this class of Ab with so far unraveled immunoregulatory functions. Of note, the modulation of CD80/CD86 surface expression on DC induced by one single administration of rSIgA was different time-wise and in terms of abundance from that observed after a single administration of CT (41, 48, 51). Although rather modest, the up-regulation of CD80/CD86 expression by DC isolated from their physiological context agrees with data obtained from in vitro-derived DC directly exposed to CT (49, 52) and intestinal DC (53).

CD80 and CD86 can be actively involved in the choice of induction of immune tolerance vs immune activation (54). A recent perception of this phenomenon is based on the concept that CD80 is probably the more potent costimulatory molecule in terms of T cell activation (55, 56) but that the outcome of this activation would be directed by CD86 (57), the up-regulation of which would induce an effector/proinflammatory instead of a tolerogenic/anti-inflammatory type of immune response. In support of this, the CD80 up-regulation observed after both rSIgA and hSC plus CT

administration (Table I) thus could account for the induction of Ab and cellular responses (Figs. 1 and 3). Differences in the intensities of the responses between immunization groups might be explained by the rapid down-regulation of CD86 seen after rSIgA administration and the more delayed up-regulation observed after hSC plus CT administration (Table I). This stresses that in the SIgA context, DC are prone to induce a more regulated/anti-inflammatory, yet still effector immune response, than that mediated by exposure to CT.

The present work provides evidence that SIgA can act as a weak mucosal immunopotentiator inducing effector immune responses in a noninflammatory context favorable to preserve local homeostasis of the gastrointestinal tract. Given that SIgA is transported across M cells in PP (16) and triggers mucosal and systemic immune responses (this work), it is fully conceivable that retrotransport of SIgA-Ag complexes could significantly contribute to immune regulation by providing a mechanism for luminal Ag to gain access to professional APC such as DC in the SED region. As suggested by our data, the integrity of the intestinal epithelial barrier thus would be preserved through multiple mechanisms including classical Ag neutralization mediated by SIgA, and possibly IgG in secretion (58), trapping by lamina propria DC (59), and mixed Th1/Th2 mucosal and systemic responses (IL-4, IFN-γ) accompanied by the maintenance of regulatory T cells expressing IL-10 and TGF-β (60, 61). In terms of humoral immunity at mucosal surfaces, SIgA Ab thus appear to combine properties of a neutralizing agent (immune exclusion) and of an immunomodulator, revealing a novel facet of their complex functioning.

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Disclosures

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