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Systemic Flagellin Immunization Stimulates Mucosal CD103+ Dendritic Cells and Drives Foxp3+ Regulatory T Cell and IgA Responses in the Mesenteric Lymph Node

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Mucosal immunity is poorly activated after systemic immunization with protein Ags. Nevertheless, induction of mucosal immunity in such a manner would be an attractive and simple way to overcome the intrinsic difficulties in delivering Ag to such sites. Flagellin from Salmonella enterica serovar Typhimurium (FliC) can impact markedly on host immunity, in part via its recognition by TLR5. In this study, we show that systemic immunization with soluble FliC (sFliC) drives distinct immune responses concurrently in the mesenteric lymph nodes (MLN) and the spleen after i.p. and s.c. immunization. In the MLN, but not the spleen, sFliC drives a TLR5-dependent recruitment of CD103+ dendritic cells (DCs), which correlates with a diminution in CD103+ DC numbers in the lamina propria. In the MLN, CD103+ DCs carry Ag and are the major primers of endogenous and transgenic T cell priming. A key consequence of these interactions with CD103+ DCs in the MLN is an increase in local regulatory T cell differentiation. In parallel, systemic sFliC immunization results in a pronounced switching of FliC-specific B cells to IgA in the MLN but not elsewhere. Loss of TLR5 has more impact on MLN than splenic Ab responses, reflected in an ablation of IgA, but not IgG, serum Ab titers. Therefore, systemic sFliC immunization targets CD103+ DCs and drives distinct mucosal T and B cell responses. This offers a potential “Trojan horse” approach to modulate mucosal immunity by systemically immunizing with sFliC. The Journal of Immunology, 2012, 189: 5745–5754.

Dendritic cells (DCs) are a bridge between the innate and the adaptive immune responses, which are required for the priming of adaptive T cell responses in the T zone of secondary lymphoid tissues (SLT) (1, 2). Extensive phenotypic and functional characterization has led to the establishment of several DC subsets in lymphoid and nonlymphoid tissues (3), which reveals that there is specialization of DC subsets influenced by their location. Thus, the phenotype and nature of the DC that initially encounters Ag is likely to influence markedly the outcome of the adaptive response.

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Abbreviations used in this article: ASC, Ag-secreting cell; DC, dendritic cell; GC, germinal center; LN, lymph node; LP, lamina propria; MFI, mean fluorescence intensity; MLN, mesenteric lymph node; sFliC, soluble FliC; SLT, secondary lymphoid tissue; Treg, regulatory T cell; WT, wild-type.

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at this site because intestinal DCs are refractory to TLR4 stimulation (17, 18). This is highlighted by the role for TLR5-mediated stimulation of CD103 resulting in rapid IL-23 production and resistance to bacterial colonization (19). Flagellin proteins such as FltC have marked immunomodulatory functions and can promote responses against themselves in the absence of exogenous adjuvant (20). Recently, we showed that the Ag-specific response to soluble FltC (sFltC) in the spleen after systemic administration was predominantly Th2 as determined by the selective induction of IL-4 and GATA-3 and not IFN-γ or T-bet. In contrast, when FltC was administered as a surface-localized Ag on Salmonella Typhimurium, the Ag-specific response was predominantly Th1 (21, 22). This dichotomy of T cell responses to the same Ag given in two different contexts in the spleen reflected the Th1 polarizing influence of monocyte-derived DCs found in the T zone after Salmonella Typhimurium infection (23). Similarly, Th1 responses are seen to OVA when expressed by Salmonella Typhimurium and Th2 responses when the protein is alum-precipitated (24). Therefore within the same anatomical site, the response to the same Ag can differ depending upon the context in which it is encountered.

In this study, we show that systemic immunization with sFltC induces the recruitment of CD103+ DCs from the LP to the MLN in a TLR5-dependent manner. This recruitment of CD103+ DCs was independent of i.p. or s.c. immunization, and CD103+ DCs were responsible for the priming of CD4 T cells in the MLN. The consequence of priming was 2-fold. First, there was a selective induction of IgA FltC-specific plasma cell responses. Therefore, systemic administration of sFltC can prime systemic and mucosal responses concurrently, with responses in each site having both shared and individual immune signatures. Thus, targeting TLR5 through systemic routes may be an attractive route for modulating immunity in the mucosa.

Materials and Methods

Mice and Abs

Specific pathogen–free 6- to 8-wk C57BL/6 mice were purchased from Harlan Sprague-Dawley, SM1 transgenic (25), TCRβ−/−, TLR5−/−, and BoyJ mice were maintained in-house. All animal procedures were carried out in strict accordance with local ethical and U.K. Home Office approval. Abs are listed in Supplemental Table I.

Flagellin preparation and immunizations

Soluble, recombinant sFltC was generated as described (22) as a his-tagged recombinant protein after nickel affinity chromatography and immunoprecipitation with an FltC-specific monoclonal. LPS contamination was typically <1 endotoxin unit/300 μg protein. Mice were immunized i.p. or s.c. in the footpad with 20 μg recombinant sFltC. Where stated, 15 μg TLR-grade LPS (Alexis Biochemicals) or 20 μg R848 (InvivoGen) was used. Where stated, 35-d sFltC-primed mice were boosted with 20 μg sFltC, and the responses were evaluated 4 d postboost.

Cell preparation and FACS

Single-cell suspensions from spleens and lymph nodes (LN) were generated by mechanical disruption and collagenase IV (Worthington Biochemical) digestion (400 U/ml; 25 min; 37°C). When required, DCs were negatively enriched using MACS beads and LS columns according to the manufacturer’s instructions (CD19, CD5, and DX5 beads, purity ≥85%; Miltenyi Biotec). Cells were then processed for multicolor FACS analysis after anti-CD16/32 blocking. Primary mAbs or isotype controls were added for 20 min at 4°C. Intracellular Foxp3 staining was performed according to the manufacturer’s instructions (eBioscience) after extracellular staining and cell fixation/permeabilization for 30 min at 4°C. Intracellular staining was performed using anti-Foxp3 Ab diluted in permeabilization buffer. Cells were analyzed using a FACSCalibur (BD Biosciences) and CyAn ADP (Beckman Coulter) flow cytometers and FlowJo software version 9.3.2.

Immunohistochemistry and confocal microscopy

Immunohistochemistry was performed as described previously (26) with tissues frozen in liquid nitrogen after being embedded in Tissue-TEK OCT compound (Bayer Healthcare) prior to freezing. Small intestines were flushed with cold PBS and arranged in a concentric circle before freezing in the absence of OCT compound. After cutting, this ensured that cross sections of the whole length of the intestine could be examined. For all samples, cryostat sections (6 μm) were dried at room temperature for 1 h and subsequently fixed in cold acetone for 20 min before air drying and storage at −20°C.

After thawing, cryosections were incubated with primary unlabelled Abs for 45 min at room temperature before addition of either HRP-conjugated or biotin-conjugated secondary Abs. Fc-binding cells were identified as described (22) using soluble biotinylated FltC. Subsequently, streptavidin ABCComplex alkaline phosphatase (Dako) was added and signal was detected using diaminobenzidine for HRP activity and naphthol AS-MX phosphate with Fast Blue salt and levamisole for alkaline phosphatase activity. Images were acquired with a Leica microscope (DM5000) using ×10 and ×20 objectives.

Confocal microscopy was performed on frozen sections as previously described (27). Staining was performed in PBS containing 10% FCS, 0.1% sodium azide, and sections were mounted in 2.5% 1,4-diazabicyclo(2,2,2) octane (pH 8.6) in 90% glycerol in PBS. After incubation with primary Abs (1 h, room temperature), secondary Abs were added (30 min; room temperature). In some instances, Hoechst 33342 (Molecular Probes) was used (10 μg/ml for 10 min, room temperature) to label nuclei and identify tissue architecture. Confocal images were acquired using a Zeiss LSM510 laser scanning confocal microscope with a Zeiss Axiovert 100M. Signals obtained from lasers were scanned separately and stored in four non-overlapping channels as pixel digital arrays of 2048 × 2048 (×10 objective) or 1024 × 1024 (×63 objective).

Generation of SM1 chimeras and assessment of T cell priming

SM1 chimeras were generated as described (23) by transfer of 107 CFSE-labeled splenocytes i.v. into C57BL/6, TLR5−/− or BoyJ mice (as stated) 24 h before immunization. After immunization, spleens were collected and cells processed for multicolor FACS analysis as described earlier.

In vitro coculture for T cell priming and Foxp3 induction

TCRβ−/− mice were immunized with sFltC (20 μg, i.p.) for 24 h. Spleen and MLN were collected, and single-cell suspensions were prepared as described earlier. DCs were pre-enriched using MACS beads (anti-CD19 and DX5) before staining with CD11c and CD103 to FACS sort CD103+ CD11c+ and CD103− CD11c− from the MLN and CD11c+ from the spleen (≥97% purity). SM1 T cells were MACS enriched (CD5+ selection) and CFSE labeled. DCs and T cells were cocultured for 4 d (1:30 ratio DC/T) before analysis by FACS.

ELISPOT analysis

Plates (MultiScreen; Millipore) were coated overnight at 4°C with 5 μg/ml sFltC and blocked with 1% BSA for 1 h at 37°C before plating cell suspensions. Single-cell suspensions were adjusted, and 5 × 105 cells were added per well in triplicate. Cells were cultured for 6 h at 37°C. Plates were washed three times with PBS–0.05% Tween and incubated overnight at 4°C with optimal dilutions of detection Abs (alkaline phosphate–conjugated anti-IgG and anti-IgA; Southern Biotech). Plates were washed three times with PBS–TWEEN before developing the reaction with Sigma Fast BCIP/NBT (Sigma Aldrich). Spots were counted using the AID ELISPOT Reader System and AID software version 3.5 (Autoimmune Diagnostika). Counts were expressed as SFUs/5 × 105 cells.

sFltC-specific ELISA

ELISA plates were coated with 5 μg/ml sFltC (2 h at 4°C) and blocked with 1% BSA overnight at 4°C. Serum was diluted 1:100 in PBS–0.05% Tween, was added and further diluted stepwise; plates were incubated for 1 h at 37°C. Bound Abs were detected using alkaline phosphate–conjugated goat anti-mouse IgG and IgA Abs (Southern Biotech). Reaction was developed with Sigma Fast p-nitrophenylphosphate (Sigma Aldrich). Relative reciprocal titers were calculated by measuring the dilution at which the serum response reached a defined ODmax.

Statistics

Statistics were calculated using the non-parametric Mann–Whitney sum of ranks test using the Analyze-It program (Analyze-It), with p values ≤0.05 accepted as significant.
Results

Intraperitoneal immunization with sFliC induces the accumulation of DCs in the MLN

We have recently shown that Th1 responses to Salmonella Typhimurium are mediated by monocyte-derived DCs that accumulate in the SLT draining the site of infection (23). Control experiments showed that sFliC had minimal effects on total numbers of DCs in the spleen. During these studies, we also examined the DC response after systemic sFliC immunization in other SLT. Notably, 24 h after i.p. immunization with 20 μg sFliC, a significant increase in DC frequency and numbers (Fig. 1) was observed in the MLN compared with nonimmunized control mice. In contrast, DC numbers after sFliC immunization were comparable to those of control mice in the spleen and the inguinal and brachial LN. As expected (28, 29), sFliC was able to induce CD86 up-regulation on DCs from all SLT examined, showing that although numbers did not increase, these DCs still responded to sFliC. This DC activation in SLT was TLR5 mediated, as it was not observed in TLR5−/− mice (Fig. 1). These experiments show that the systemic immunization with sFliC induces an increase of DCs in the MLN but not in the spleen or inguinal or brachial LN.

Systemic immunization with sFliC selectively recruits CD103+ DCs into the MLN in a TLR5-dependent manner

CD103+ DCs are known to migrate from the LP to the MLN and also to be high expressers of TLR5 (11), and therefore we focused on changes in this population in SLT after sFliC immunization. CD103+ DCs were defined on the basis of their forward and side scatter, their lack of CD3 expression, and their positive staining for CD11c and high expression of MHC class II (Fig. 2A). After i.p. immunization with 20 μg sFliC, CD103+ DC proportions and numbers were evaluated in the MLN, spleen, inguinal LN, and brachial LN. There was a clear increase in the frequency and total numbers of the CD103+ DC population in the MLN, but not in other sites after sFliC immunization (Fig. 2A). Because sFliC is a ligand for TLR5, we addressed whether this accumulation of CD103+ DCs was TLR5 dependent. Wild-type (WT) and TLR5−/− mice were immunized with sFliC for 24 h. In the absence of TLR5, no increase in CD103+ DCs was detected in the MLN (Fig. 2B).

To test if this accumulation of CD103+ DCs in the MLN was a transitory event, a time course was performed evaluating CD103+ DC numbers after sFliC immunization. CD103+ DC numbers in the MLN peaked at 24 h, and by 48 h the numbers sharply decreased close to the control; at 72 h postimmunization, DC numbers had returned to nonimmunized levels (Fig. 2C).

Finally, CD103+ DCs can migrate from the LP (11). Therefore, we examined whether the CD103+ DC increase observed in the MLN after sFliC immunization might correlate with a reduction of their frequency in the LP. To test this, we performed confocal microscopy on intestine and MLN sections from nonimmunized and 24-h sFliC-immunized WT mice. At 24 h, an increase in MLN CD103+ DCs was apparent, supporting the increase observed by flow cytometry, and these cells were primarily localized to the T zone. We then examined how this correlated with changes in the density of CD103+ DCs (CD11c+CD103+) in the LP. To do this, intestine sections were analyzed after sFliC immunization and the frequency of CD103+ DCs per 200 μm of villus length evaluated. This showed that there was a significant decrease in CD103+ DCs in the villi of sFliC-immunized mice (Fig. 2D, lower panel). This is consistent with the concept that these cells migrated from the LP to the MLN.

These experiments raised the possibility that the recruitment of CD103+ DCs reflected a general response to systemic exposure to TLR stimulation. To examine this, we assessed DC responses in the MLN after i.p. immunization with 20 μg Salmonella Typhimurium LPS or 20 μg R848 [a TLR ligand known to stimulate DC migration in the intestine (30)] or 20 μg sFliC. Although LPS was able to induce CD86 expression on CD11c+ DCs in the spleen and MLN, indicating the LPS reached these sites, it did not result in an increase in the proportion or total number of DCs (gated as shown in Fig. 1A) (Fig. 3A). In contrast, immunization with R848 resulted in both DC activation and the selective recruitment of CD103+ DCs to the MLN to levels comparable to those observed after sFliC immunization (Fig. 3A, 3B). These results showed that systemic sFliC or R848 immunization both result in an accumulation of CD103+ DC numbers in the MLN and is in stark contrast, whereas after LPS immunization there was an absence of CD103+ DCs.

FIGURE 1. sFliC i.p. immunization induces DCs recruitment to the MLN. (A) Gating strategy to define DCs is shown first. Representative dot plots of spleen, MLN, and inguinal and brachial LN cell suspensions from nonimmunized (N.I.) or sFliC-immunized (i.p.) mice 24 h after immunization. Frequency of MHC-II+CD11c+ cells is stated next to the gate. Histograms to the right show CD86 expression in WT and TLR5−/− mice within the previously defined gate; isotype control (black line filled), nonimmunized (gray line), and sFliC-immunized (gray line filled) samples are shown. MFI is stated. (B) Absolute number of CD11c+ cells per organ. Experiments were independently repeated three times with four mice per group. **p ≤ 0.01.
DC accumulation in the MLN. This indicates that not all TLR ligands have the capacity to induce increased CD103+ DC accumulation.

**Optimal T cell priming in the MLN after systemic sFliC requires CD103+ DCs and TLR5**

To address how the recruitment of CD103+ DCs to the MLN could impact T cell priming in vivo, we took advantage of the SM1 transgenic mouse, where CD4 T cells are specific for a peptide in Salmonella Typhimurium FliC (25). SM1 splenocytes were CFSE labeled and adoptively transferred into WT recipients 24 h before immunizing with 20 μg sFliC either i.p. or s.c. in the foot. T cell activation in the spleen, MLN, popliteal and brachial LN was assessed by CD69 expression 18 h after immunization by flow cytometry (Fig. 4A). After i.p. immunization, nearly all SM1 T cells upregulated CD69 in all sites assessed. Nevertheless, careful assessment of mean fluorescence intensity (MFI) levels of CD69 expression revealed differences. As expected, after i.p. immunization CD69 expression was highest in the spleen and brachial LN, whereas after s.c. immunization CD69 expression was highest in the popliteal LN. Despite these differences in systemic sites, expression of CD69 was similar in the MLN irrespective of the route of immunization indicating that this efficient priming was not a consequence of immunizing through a single route. In addition, sFliC immunization induced CD4 T cell priming in the spleen and brachial and inguinal LN without inducing a determinable difference in total DC numbers in these sites (Figs. 1, 4A). Because sFliC induced T cell activation in the MLN to similar levels after s.c. and i.p. immunization, we assessed whether immunization through both routes resulted in a similar accumulation of CD103+ DCs in the MLN. Indeed this was the case; the recruitment of CD103+ DCs in the MLN was independent of the immunization route. This increase in CD103+ DCs in the MLN was selective, as there was no increase in CD103+ DCs in the popliteal LN after s.c. immunization, even though this lymph node drains the site where sFliC was administered during s.c. immunization (Fig. 4B). Similar results were also seen in endogenous T cells (data not shown).

CD103+ DC recruitment to the MLN after sFliC is TLR5 dependent (Fig. 2B), and T cell priming could occur without changes in DC numbers (Figs. 2A, 4A). Therefore, we hypothesized that if CD103+ DCs were involved in CD4 T cell priming in the spleen and brachial and inguinal LN without inducing a determinable difference in total DC numbers in these sites (Figs. 1, 4A). Because sFliC induced T cell activation in the MLN to similar levels after s.c. and i.p. immunization, we assessed whether immunization through both routes resulted in a similar accumulation of CD103+ DCs in the MLN. Indeed this was the case; the recruitment of CD103+ DCs in the MLN was independent of the immunization route. This increase in CD103+ DCs in the MLN was selective, as there was no increase in CD103+ DCs in the popliteal LN after s.c. immunization, even though this lymph node drains the site where sFliC was administered during s.c. immunization (Fig. 4B). Similar results were also seen in endogenous T cells (data not shown).
CD69 expression and CD62L downregulation in the spleen and MLN (Fig. 4C). In both WT and TLR5<sup>−/−</sup> chimeras, levels of T cell activation were similar in the spleen, indicating that in this site priming occurs without the contribution of CD103<sup>+</sup> DCs. In contrast, in the MLN, T cell activation in SM1 cells in TLR5<sup>−/−</sup> chimeras was impaired relative to the WT chimeras, with lower numbers of activated T cells observed. Furthermore, assessment of proliferation, by examining CFSE dilution 48 h after immunization, showed that whereas T cells had divided similarly in the spleen and popliteal and brachial LN in both sets of chimeras, there was a clear lag in the division of SM1 T cells in the MLN of TLR5 chimeras. This suggests that in the absence of TLR5, there is selectively impaired T cell priming in the MLN but not in the spleen.

The defective CD103<sup>+</sup> DC recruitment in TLR5<sup>−/−</sup> mice and priming in the MLN in TLR5<sup>−/−</sup> chimeras suggested that CD103<sup>+</sup> DCs made significant contributions to T cell priming in this site. To address the contribution of CD103<sup>+</sup> DCs to T cell priming, WT mice were i.p. immunized with 20 µg sFltC, and 24 h later CD103<sup>+</sup> and CD103<sup>−</sup> DCs were cell sorted from the MLN and popliteal LN in both sets of chimeras, and SM1 T cells were CFSE labeled and cultured in vitro in the presence of sFltC. These experiments showed that CD103<sup>+</sup> DCs were able to induce T cell proliferation more effectively than CD103<sup>−</sup> DCs (Fig. 4D). This shows that CD103<sup>+</sup> DCs capture sFltC in vivo, and in the MLN are the major APC subset that present sFltC and prime CD4 T cells.

**CD103<sup>+</sup> DCs can prime T cells for Foxp3 expression in the MLN after sFltC immunization in vivo**

Because CD103<sup>+</sup> DCs can prime for the induction of Foxp3 in CD4 T cells (6, 10, 31), we wanted to address if sFltC immunization would induce Foxp3<sup>+</sup> Tregs. We first studied this ex vivo by sorting CD103<sup>+</sup> and CD103<sup>−</sup> MLN DCs after sFltC immunization. These sorted DCs were then cocultured with naïve SM1 CD4 T cells for 4 d (1:30 ratio DC/T). The CD103<sup>+</sup> DCs were able to prime T cells when in the presence of sFltC, and T cell division and Foxp3 induction was evaluated. As shown in Fig. 5A, both subsets of DCs were able to prime T cells when
CD103+ DCs induced to sFliC prime Ag-specific Foxp3+ Tregs in the MLN. (A) CD103+ and CD103- DCs were cell sorted from 24-h sFliC-immunized mice. Foxp3 induction in primed T cells was evaluated by coculturing the sorted DC populations with SM1 CFSE-labeled CD4+ cells (1:30 ratio) in the presence of 5 μg/ml sFliC. Representative dot plots show T cell division, and Foxp3 expression was evaluated after 4-d culture. Experiments were independently repeated three times with four mice per group. (B) Graphs show the absolute number of transferred cells and the frequency of Foxp3+ T cells in the spleen and MLN of nonimmunized (N.I.) and sFliC-immunized mice. Experiments were independently repeated three times with four mice per group. (C) Composite confocal representative images of spleen and MLN sections showing CD3 (blue) and Foxp3 (red) and CD45.2 (green) (scale bar, 25 μm). Representative dot plots show the frequency of transferred T cells in the spleen and MLN in nonimmunized (N.I.) and sFliC-immunized mice. Experiments were independently repeated three times with four mice per group. (D) WT mice were nonimmunized or sFliC-immunized. At day 5 postimmunization, Tregs were gated as CD25+Foxp3+ TGFβ+ and assessed in the spleen and MLN. Histogram shows TGF-β expression (gray line) and isotype control (black line filled). Graphs show absolute number of Foxp3+ T cells. (E) Foxp3 induction in the spleen and MLN in WT (open bars) and TLR5−/− (dashed bars) mice after 5 d of sFliC was provided in culture, but only CD103+ DCs could induce Foxp3 expression in SM1 T cells to a significant level. To assess if this reflected the response in vivo, CD45.2-expressing SM1 T cells were transferred into CD45.1-expressing BoyJ mice, and 24 h after transfer, mice were immunized with sFliC. After 3 d, the T cell response of the transferred cells was assessed in the spleen and MLNs using FACS (Fig. 5B) and confocal microscopy (Fig. 5C). This showed that SM1 T cells had expanded in both sites after sFliC immunization, but the frequency of Foxp3 expression in the transferred T cells was significantly greater in the MLN. Furthermore, confocal microscopy identified clusters of SM1 T cells expressing Foxp3 localized within the T zone (Fig. 5C).

Next we assessed whether these results reflected the use of transgenic T cells by examining the response in endogenous WT CD4 T cells. WT mice were immunized with sFliC for 5 d, and Foxp3 induction was evaluated by FACS. A significant increase in the frequency of Foxp3+ CD4 T cells was observed after sFliC immunization in the MLN, but not in the spleen (Fig. 5D). These Foxp3+ CD4 T cells expressed CD25 and had surface-bound TGF-β, indicating that these cells are Tregs. Finally, to confirm that Foxp3 induction in endogenous cells after sFliC immunization was associated with CD103+ DC recruitment to the MLN, WT or TLR5−/− mice were immunized with sFliC, and Foxp3 expression in CD4 T cells in the spleen and MLN was assessed 5 d later. This showed that in TLR5−/− mice, where CD103+ DC numbers in the MLN do not increase after sFliC immunization (Fig. 2B), there was no induction of Foxp3+ T cells (Fig. 5E), whereas numbers of Foxp3+ T cells in the spleen were not affected by sFliC immunization or TLR5 expression. Collectively, these results show that CD103+ DCs can prime for Treg development in Ag-specific CD4+ T cells in the MLN.

sFliC immunization induces the selective induction of specific IgA responses in the MLN

We have shown the response in the MLN after systemic immunization is dependent upon the TLR5-mediated migration of CD103+ DCs from the LP to the MLN. In the absence of CD103+ DC recruitment, T cell activation in the MLN after sFliC immunization is negligible. Therefore, we examined whether the recruitment of CD103+ DCs was associated with a selective IgA Ab response in the MLN, a role proposed for such DCs (13). To do this, we first examined B cell responses in situ using immunohistochemistry at 4 d after sFliC boost. We chose 4 d because we have previously shown there is a pronounced sFliC-specific IgG extrafollicular plasmablast and germinal center (GC) response in the spleen at this time (22). This showed that although GCs were present in both groups, only MLN from sFliC-immunized mice contained FliC-binding cells in extrafollicular foci and GC. Furthermore, supporting the concept that these were Ag-specific GCs, FliC-specific plasmablasts could be observed in the proximal follicle, consistent with these being recent GC emigrants (27) (Fig. 6A and data not shown). To assess the Ab response in greater depth, we assessed by ELISPOT assay the Ig isotype of FliC-specific Ab-secreting cells in the spleen and MLN of mice primed once for 4 d or primed for 35 d and boosted for 4 d with sFliC i.p. (Fig. 6B). This showed that the relative frequency of FliC-specific IgG Ab-secreting cells in the spleen and MLN of mice primed once for 4 d or primed for 35 d and boosted for 4 d with sFliC i.p. (Fig. 6B). This showed that the relative frequency of FliC-specific IgG Ab-secreting cells (ASCs) was similar in the spleen and MLN in primed or primed-boosted mice. Nevertheless, in the MLN a marked IgA response was observed in boosted mice that was largely absent from the spleen. This IgA response was
also observed in the MLN of 4-d primed mice albeit to a lower level, indicating this switching is local and rapid at this site.

To examine if the IgA response reflected the TLR5-dependent recruitment of CD103+ DCs, we assessed the Ig response in the spleen and MLN of primed and boosted WT and TLR5 mice as before. This showed that FliC-specific IgG ASCs in the spleen remained detectable in TLR5−/− mice, although at a lower frequency than in WT mice. In contrast, in the MLN both IgG and IgA responses were nearly completely abrogated. This indicates that the induction of FliC-specific IgG and IgA responses in the MLN are highly dependent upon TLR5 (Fig. 6C). To examine if these observations had only a minor, local influence, we examined the anti-FliC serum Ab responses. These showed that IgG and IgA responses were readily detectable in WT mice, but there were diminished and absent IgG and IgA responses respectively in TLR5−/− mice (Fig. 6D). This supports the concept that the CD103+ DC population is responsible for promoting IgA switching in the MLN after sFliC immunization.

Finally, we examined IgA and IgG switching in the MLN and popliteal LN after i.p. or s.c. immunization in the foot. Independent of the route of Ag administration, FliC-specific IgA responses were detectable in the MLN at frequencies substantially higher than in the spleen or popliteal LN, mirroring the recruitment of CD103+ DCs after i.p. and s.c. immunization. As expected, after i.p. immunization, IgG ASCs were readily detected in the spleen and MLN, but were largely absent in the popliteal LN. However, after s.c. immunization in the popliteal LN, IgG ASCs were readily detectable, but IgA responses were absent (Fig. 6E). Therefore, our results suggest that sFliC induces selective IgA switching in the MLN that is dependent upon the recruitment of CD103+ DCs to the MLN in a TLR5-dependent manner.

Discussion

In this study, we show that immunization with sFliC has the capacity to drive selectively the recruitment of CD103+ DCs to the MLN, prime for Treg induction, and promote IgA switching. Flagellin is an important immunodominant and immunomodulatory protein with adjuvant properties, with a clear, although not exclusive (32), dependence on TLR5 for these activities (33). Previously, we showed that the immune response to flagellin within the same SLT can show significant plasticity (21). We have developed these findings further to show that the response to sFliC
can differ in different SLT responding to the same immunization. This discriminatory capacity in the MLN largely depends upon the interaction between sFltC and TLR5, as loss of TLR5 resulted in ablated T cell priming in the MLN but not the spleen. This is compatible with recent work showing that in TLR5-deficient mice, there was delayed priming of FltC-specific T cells in the MLN and Peyer’s patches after *Salmonella* Typhimurium infection (34). In a separate study, loss of TLR5 also resulted in defective priming after flagellin, which could not be compensated by coimmunizing with additional TLR ligands (35). As expected, immunization with R848 could drive CD103+ DC recruitment. This molecule induces DC migration to the MLN through TLR7/8 ligation and not TLR5. The mechanism of R848 action is known to be through an indirect activity on plasmacytoid DCs. These cells then secrete high levels of type I IFN, which in turn acts on CD103+ DCs resulting in their activation and migration to the MLN (30, 36). We hypothesize that the ability of sFltC to drive CD103 DC migration to the MLN is through the direct ligation of TLR5 on LP CD103+ DCs. This reflects the highest expression of TLR5 found on LP DCs (18) and possibly means that they have a greater efficiency at taking up this Ag than other DC subsets with lower TLR5 expression. Our findings that CD103+ DCs do not migrate in the absence of TLR5 and that they are sufficient to prime T cells indicates that they carry Ag and are therefore efficient at capturing circulating flagellin. If flagellin’s interaction with CD103+ DCs is direct, then it would indicate that flagellin acts through a mechanism distinct to that of R848. The inability of systemically administered LPS to have a similar effect on CD103+ DC recruitment has been reported previously (17) and probably reflects the low level of TLR4 mRNA expression associated with this cell type. This supports the concept that there is a differential sensitivity of DCs in the LP to TLR ligands. A hyporesponsiveness to LPS may reflect its ubiquitous presence in the digestive tract and its translocation across the intestinal epithelium to the lymph nodes (37). Where the translocation of LPS occurs from the intestine, local LPS concentrations are obviously likely to be greater than those elsewhere, and so there may be significant advantages in not continuously triggering immune responses at this site. In contrast, only a limited spectrum of organisms are flagellated, and only a proportion of such have flagella with the appropriate motifs that trigger TLR5 [β and γ proteobacteria (38)]. Although many TLR ligands share a common signaling pathway, and MyD88 plays an important role in TLR5 biology (28), not all activities in which TLR5 is involved may require MyD88. One such function may be a role for TLR5 as a scavenger for flagellin, enabling the cell more efficiently to present processed flagellin peptides via MHC class II to CD4 T cells (35). This aspect is particularly relevant to flagellin, which is of a small number of proteinaceous TLR ligands and thus able to be presented in such a manner. Nevertheless, whether CD103+ DC activation via TLR5 requires in vivo encounter with flagellin or the migration of DCs to the MLN (41). The potential of CD103+ DCs to drive a tolerogenic response has been assessed in vivo using an Ag targeting strategy (47). This revealed a marked influence on the induction of tolerance by the addition of adjuvant. In contrast, the antigenicity intrinsic to flagellin is sufficient to drive a CD103+ DC population that can induce priming of both tolerogenic and effector T cell phenotypes, with the greater capacity to induce the former in the mucosa.

IgA is the classical Ab isotype associated with mucosal immunity, and its induction through systemic administration of protein Ag is atypical. In this study, we show that the generation of the IgA response is overwhelmingly in the MLN but not other LNs and was therefore not reflective of a systemic induction of IgA. Loss of TLR5 had a more profound effect on IgA than IgG responses. Sustained IgG titers after flagellin in TLR5−/− mice has been described, where NLRC4 can provide some compensation for its loss (32, 42). The near absolute loss of IgA to sFltC in TLR5−/− mice suggests that TLR5 may have a nonredundant role in the generation of IgA in this response.

Although the processes through which IgA is induced is not entirely clear, its induction has been observed after in vitro culture of flagellin-pulsed CD103+ DCs with naïve B cells. Nevertheless, under such circumstances, the induction of IgA is T-independent, and it is not clear if the IgA is flagellin-specific or if flagellin acted to stimulate the DCs (13). In our studies, isotype switching to flagellin is highly dependent upon the presence of T cells, at least in part because peritoneal B1 cells appear to not respond to this protein after i.p. immunization (22, 48). Therefore, the presence of CD103+ DCs may contribute to T-dependent and T-independent switching to IgA. One soluble factor associated with IgA induc-
tion is TGF-β (49, 50). We were unable to identify TGF-β production by freshly isolated CD103+ DCs ex vivo (data not shown), whereas surface-bound TGF-β was readily detectable on Tregs after sFliC (Fig. 5D), indicating T cells may be one potential source of this cytokine. On the basis of this and other studies (51, 52), it is reasonable to suggest that the parallel development of Ag-specific IgA and Treg responses is related to the recruitment of CD103+ DCs to the MLN. Previous work has identified links between Tregs, Treg function, Treg differentiation, and IgA switching (53). One issue that will need to be addressed in greater detail is the relationship between Treg and T follicular helper cells in regulating switching to IgA. Although Treg responses in GCs have been shown to aid the specificity of the response and reduce the risk of autoimmunity (54), it is also likely that they can contribute to the magnitude of the switched response to IgA (50–53).

The induction of Foxp3 priming after sFliC is in contrast to the strong proinflammatory cytokine responses observed to flagellin in multiple models (55). Nevertheless, systemically administered FljB, another Salmonella Typhimurium flagellin, has been administered to humans without notable inflammation-associated symptoms such as fever (56), although it is unclear what impact this has on IgA or mucosal responses. Furthermore, we and others have shown that soluble flagellin can induce Th2 responses, and these may help counter some of the inflammatory effects induced by flagellin on the innate immune system (21, 22, 28, 57). Further evidence that flagellin may not produce overt local inflammation is the relationship between Treg and T follicular helper cells during the induction of tolerance to Ags directly or to Ags conjugated to itself.

Disclosures

The authors have no financial conflicts of interest.

References


14. Bergqvist, P., A. Stensson, N. Y. Lycke, and M. Bemark. 2010. T cell-mediated intestine is that during inflammatory conditions, CD103+ DCs are impaired at driving tolerogenic responses (58). This would imply that if sFliC induced pronounced inflammation, then it was not sufficient to prevent CD103+ DCs from sFliC immunized mice from priming Tregs. Therefore it is tempting to suggest that systemic administration of flagellin may have the potential to drive tolerizing (e.g., through Treg induction) or active (e.g., through specific IgA responses) mucosal responses. Systemically administered flagellin and derived polypeptides have been shown to have marked and diverse effects on the host such as conferring protection from radiation systemically and in the intestine and preventing colonization of selective E. coli strains in cattle (59–61). Indeed, this function of flagellin may be mediated through IgA Ab induced mucosally. Thus, the effects of flagellin on the host, inflammatory or otherwise, may not necessarily be negative. Indeed, it may be possible that flagellin through TLR5 could help balance inflammation at the intestinal mucosa through the induction of tolerance to Ags directly or to Ags conjugated to itself. Therefore, systemically administered flagellin may be used as a carrier directly to target the induction of mucosal Ig responses and avoid the challenges of delivering Ag via the oral route or the difficulties involved in delivering intact Ag to the appropriate mucosal site. Because flagellin has been given to humans (62, 63), its mechanisms and sites of action may enable its translation for use against other conditions, such as inflammatory bowel diseases, where novel treatments are urgently required.

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