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Gut-resident CX3CR1^{hi} macrophages induce tertiary lymphoid structures and IgA response in situ

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Abstract

Intestinal mononuclear phagocytes (MPs) are composed of heterogeneous dendritic cell (DC) and macrophage subsets necessary for the initiation of immune response and control of inflammation. Although MPs in the normal intestine have been extensively studied, the heterogeneity and function of inflammatory MPs remain poorly defined. We performed phenotypical, transcriptional, and functional analyses of inflammatory MPs in infectious *Salmonella* colitis and identified CX3CR1⁺ MPs as the most prevalent inflammatory cell type. CX3CR1⁺ MPs were further divided into three distinct populations, namely, *Nos2*⁺CX3CR1^{lo}, *Ccr7*⁺CX3CR1^{int} (lymph migratory), and *Cxcl13*⁺CX3CR1^{hi} (mucosa resident), all of which were transcriptionally aligned with macrophages and derived from monocytes. In follow-up experiments in vivo, intestinal CX3CR1⁺ macrophages were superior to conventional DC1 (cDC1) and cDC2 in inducing *Salmonella*-specific mucosal IgA. We next examined spatial organization of the immune response induced by CX3CR1⁺ macrophage subsets and identified mucosa-resident *Cxcl13*⁺CX3CR1^{hi} macrophages as the antigen-presenting cells responsible for recruitment and activation of CD4⁺ T and B cells to the sites of *Salmonella* invasion, followed by tertiary lymphoid structure formation and the local pathogen-specific IgA response. Using mice we developed with a floxed *Ccr7* allele, we showed that this local IgA response developed independently of migration of the *Ccr7*⁺CX3CR1^{int} population to the mesenteric lymph nodes and contributed to the total mucosal IgA response to infection. The differential activity of intestinal macrophage subsets in promoting mucosal IgA responses should be considered in the development of vaccines to prevent *Salmonella* infection and in the design of anti-inflammatory therapies aimed at modulating macrophage function in inflammatory bowel disease.

INTRODUCTION

Intestinal mononuclear phagocytes (MPs), represented by dendritic cells (DCs) and macrophages (Mφs), form a heterogeneous cellular network within the mucosa of both mice and humans (1). Most of the conventional DC (cDC) compartment in the normal intestine is phenotypically marked by the expression of the αE subunit of integrin CD103, developmental origin from the bone marrow progenitor pre-DC, and dependency on the growth factor FLT3L (2). Similar to cDCs in secondary lymphoid organs, intestinal cDCs are further divided on the basis of their dependence on transcription factors into basic leucine zipper transcriptional factor ATF-like 3 (BATF3)–dependent CD11b[−] cDC1 and interferon regulatory factor 4–dependent CD11b⁺ cDC2 subsets (3–5). The remaining, CX3CR1⁺ MPs were identified in *Cx3cr1*^{GFP/WT} mice (6) as green fluorescent protein (GFP)⁺ cells with the CD11c⁺ and major histocompatibility complex class II (MHCII)⁺

antigen-presenting phenotype reminiscent of cDCs (7). Subsequent developmental, functional, and transcriptional studies have classified CX3CR1⁺ MPs as Mφs due to their unique properties that distinguished them from cDCs including development from monocytes, dependency on colony stimulatory factor 1 (CSF1), and poor ability to migrate to the mesenteric lymph nodes (MLNs) (2, 8–12).

Accumulating evidence supports the idea of unique contributions of distinct intestinal MP subsets to the initiation and control of immune responses (3, 10, 11). Although intestinal cDCs have been thought to act as key players in the induction of adaptive immunity (3), recent functional studies in vivo have demonstrated the importance of CX3CR1⁺ MPs in the induction of T helper 17 (T_H17) and immunoglobulin G (IgG) responses against bacterial and fungal commensals (13, 14). In addition, studies using a systemically administered model ovalbumin (OVA) antigen suggested involvement of CX3CR1⁺ MPs in the induction of IgA and CD8 responses (15). In contrast, other studies described the immunoregulatory role of CX3CR1⁺ MPs, mainly due to their expression of interleukin-10 (IL-10) and IL-10 receptor (16–20). Thus, CX3CR1⁺ MP-restricted depletion of IL-10 receptor or genes associated with its pathway was sufficient to drive spontaneous inflammatory bowel disease (IBD) (17, 21, 22) and required CX3CR1⁺ MP-derived IL-23 (23). Similarly, gut commensal bacterial sensing through myeloid differentiation primary response protein 88 (MyD88) in Mφs drove spontaneous IBD when unopposed by IL-10 (24). Detailed analysis of CX3CR1⁺ MPs in the normal and inflamed intestine revealed their heterogeneity and identified mucosa-resident CX3CR1^{hi} (named Mφs) and lymph-migratory CX3CR1^{int} (named monocyte-derived DCs) populations (25), although other studies have classified the CX3CR1^{int} population as bona fide DCs (26, 27). The recent functional studies, however, did not account for the topography of the immune response induced by the resident versus migratory CX3CR1⁺ MP populations. Furthermore, how the labor is divided between heterogeneous inflammatory MP subsets in response to enteric infection is not entirely clear.

To revisit heterogeneity of inflammatory MPs and determine their functions in response to enteric infection, we performed extensive phenotypical, transcriptional, and functional in vivo and ex vivo analyses of intestinal inflammatory MPs in a model of *Salmonella* colitis. We found that CX3CR1⁺ MPs, and not cDCs, are a required antigen-presenting cell (APC) responsible for the mucosal *Salmonella*-specific IgA response. We established heterogeneity of CX3CR1⁺ MPs and identified mucosa-resident CX3CR1^{hi} Mφs as the APC responsible for recruitment and activation of CD4⁺ T and B cells to the sites of *Salmonella* invasion, followed by the development of tertiary lymphoid structures (TLSs) and the local pathogen-specific IgA response. Our findings revealed that under conditions of infectious colitis, mucosa-resident CX3CR1^{hi} Mφs are immunogenic because they drive adaptive immune responses locally in parallel with the pathogen-specific IgA response induced by the lymph-migratory CX3CR1^{int} population in the MLNs. In summary, we demonstrate the importance of inflammatory mucosa-resident CX3CR1^{hi} Mφs in TLS formation and function.

RESULTS

Inflammatory MPs are dominated by CX3CR1^{lo}, CX3CR1^{int}, and CX3CR1^{hi} Mφ subsets

To study the immune response of heterogeneous inflammatory MPs to enteric infection, we used a model of infectious colitis induced by oral infection with wild-type (WT) *Salmonella* strain SL1344 in mice on a *Salmonella*-resistant background. In this model mimicking human salmonellosis (28, 29), mice are pretreated with a single dose of streptomycin that allows for preferential entry of *Salmonella* into the cecal and colonic mucosa instead of Peyer's patches (fig. S1), followed by systemic pathogen dissemination. Because composition of the MP system becomes more complicated in the inflamed intestine through the recruitment of myeloid cell populations from the blood (3), we characterized the heterogeneity of intestinal inflammatory MPs in *Cx3cr1*^{WT/GFP} mice by analyzing the cell surface phenotype of large bowel (LB) MPs using flow cytometry (fig. S2A). As compared with steady-state, inflamed colon contained the CD103⁺CD11b⁻ population, likely representing cDC1 (4), and the CD11b⁺ population, which we further separated into three subsets, CX3CR1^{lo}, CX3CR1^{int}, and CX3CR1^{hi}, based on their GFP level. The CD103⁺CD11b⁺ CX3CR1^{lo} population thought to represent most of cDC2 (4) was not detectable in the inflamed intestine (Fig. 1A).

Unbiased t-distributed stochastic neighbor embedding (t-SNE) analysis of cells labeled with an expanded antibody panel that included additional cDC/Mφ-specific markers, XCR1 to mark cDC1 (30), CD101 to mark cDC2 (5), and CD64 and CD16/32 to mark Mφs (11, 31), showed that CD103⁺CD11b⁻XCR1⁺ (X-C motif chemokine receptor 1) (cDC1) and CX3CR1⁺ populations were positioned in separate clusters in both the normal and inflamed LBs. The phenotype of the cDC1 population remained unchanged before and after infection, whereas the phenotype of CX3CR1⁺ populations was markedly altered by inflammation, with the CX3CR1^{lo}, CX3CR1^{int}, and CX3CR1^{hi} populations becoming phenotypically distinct (Fig. 1, B and C). In contrast, the CD103⁺CD11b⁺CD101⁺ cells (cDC2) appeared as a discrete subset only at steady state (Fig. 1, B and C, and fig. S2B). Comparative expression analysis of selected cell surface markers showed that CX3CR1⁺ populations expressed lower levels of cDC-specific markers CD103, XCR1, and CD101 and were positive for Mφ-specific IgG Fc receptors I, II, and III recognized by CD64 and CD16/32 monoclonal antibody (mAb), consistent with their classification as Mφs (Fig. 1D). Quantitatively, inflammation increased the number and radically shifted the proportions of MP subsets with CX3CR1^{lo} and CX3CR1^{int} cells being the most abundant (Fig. 1E and fig. S1E).

The phenotypic identity of inflammatory MP subsets was further confirmed through transcriptional profiling of sorted cells (fig. S2C) by RNA sequencing (RNA-seq) using the rationale established by the ImmGen Consortium (10, 11). The expression levels of differentially expressed genes were obtained by comparing MP subsets before and after infection with two control cDC and Mφ populations: cDC2 from normal small bowel (SB) and normal peritoneal Mφs. As expected, clustering analysis of MP subsets placed SB cDC2 and peritoneal Mφs in two most distant clusters. Similar to the flow cytometry data, normal and inflammatory cDC1 clustered with SB cDC2, and inflammatory CX3CR1⁺ MP subsets clustered with peritoneal Mφs. In contrast, normal CX3CR1⁺ MPs fell into a separate cluster

together with normal cDC2, suggesting that the LB cDC2 is contaminated with CX₃CR^{lo} population. The distances between individual subsets (height) that reflect their differences in gene expression were smaller among inflammatory CX₃CR⁺ MP subsets, suggesting that infection decreases their variability by committing cells to a specific functional activity. We next compared the expression of the established cDC and M ϕ signature genes defined by the ImmGen Consortium (10, 11) among MP subsets and controls (SB cDC2 and peritoneal M ϕ s). Consistently, normal and inflammatory cDC1 populations expressed higher levels of cDC-specific genes (e.g., genes encoding cDC-specific growth factor receptor *Flt3* and *Kit*), whereas CX₃CR⁺ MPs expressed higher levels of M ϕ -specific genes, particularly during infection (e.g., genes encoding receptor for apoptotic cells *Mertk* and IgG Fc receptors *Fcgr1* and *Fcgr3*). The only exception was the normal cDC2 subset that expressed markers of both cDCs and M ϕ s, likely reflecting its heterogeneity (Fig. 1G and table S1).

One of the key characteristics that discriminate intestinal M ϕ s from cDCs is their monocyte-derived origin (2, 8, 12). We therefore depleted monocytes known to express CCR2 (32) from the blood of control and infected mice using CCR2 mAb (12, 33) and found that monocyte depletion markedly reduced the number of CX₃CR^{lo}, CX₃CR^{int}, and CX₃CR^{hi} populations (Fig. 1H), further corroborating their M ϕ identity. When compared with cDCs, the CX₃CR^{lo}, CX₃CR^{int}, and CX₃CR^{hi} populations had significantly higher proportions of CCR2⁺ cells and at the levels similar to blood monocytes as quantified in transgenic *Ccr2*^{WT/RFP} *Cx3cr1*^{WT/GFP} mice that express red fluorescent protein (RFP) under control of *Ccr2* promoter (34) in addition to GFP (Fig. 1I and fig. S2D). A population of CCR2-dependent CD103⁻CD11b⁺ DCs thought to represent a subpopulation of cDC2 has been described in the normal SB (27). We and others have shown that the signaling receptor NOTCH2 (neurogenic locus notch homolog protein 2) controls cDC2 development (35, 36). To ensure that CX₃CR⁺ MP subsets do not represent a subpopulation of cDC2, we analyzed inflammatory MP populations in *Itgax*^{Cre} *Notch2*^{fllox (fl)/fl} (*Itgax:Notch2*^{-/-}) mice and *Itgax:Notch2*^{-/-} *Cx3cr1*^{WT/GFP} mice but found no reduction of total M ϕ or CX₃CR⁺ subset number (fig. S2, E and F). Collectively, we defined the diversity of intestinal MP subsets in the *Salmonella*-infected LB and demonstrated that the increase in MPs in the inflamed bowel was mainly driven by the expansion of heterogeneous monocyte-derived CX₃CR^{lo}, CX₃CR^{int}, and CX₃CR^{hi} M ϕ populations.

CX₃CR⁺ M ϕ subsets are required for the development of the mucosal *Salmonella*-specific IgA response

The analysis of RNA-seq data revealed significant differences between cDC1 and M ϕ s in expression levels of gene pathways known to be associated with MP functions such as microbial sensing, phagosome and lysosome function, bactericidal responses, production of inflammatory and anti-inflammatory cytokines, and induction and regulation of adaptive immunity. Although both inflammatory cDC1 and M ϕ s expressed gene signatures associated with antigen presentation, transcriptional profiles of inflammatory M ϕ subsets were characterized by the highest expression of genes required for B cell and IgA responses (Fig. 2A and table S3). Thus, transcriptional analysis of inflammatory MPs predicted the importance of CX₃CR⁺ M ϕ subsets in the *Salmonella*-specific IgA response.

To test our predictions, we first compared the ability of Mφs and cDC1 to respond to *Salmonella* infection in vivo using mice that lacked either mucosal Mφs [treated with CSF1R (CSF1 receptor) mAb] (37, 38) or cDC1 (*Batf3*^{-/-} mice) (39). Treatment with CSF1R mAb before infection significantly reduced the numbers of CX3CR1^{lo} and CX3CR1^{int} Mφs and nearly completely depleted CX3CR1^{hi} Mφs, whereas the numbers of cDC1 were unaffected (fig. S3A). The same Mφ depletion protocol has no significant impact on the composition of MPs in the MLNs (37). The survival, body weight, and *Salmonella* colonization (fig. S3B) of Mφ- and cDC1-deficient mice and their corresponding controls were followed after infection. Both Mφ- and cDC1-deficient mice succumbed to infection, albeit the latter with slower kinetics (Fig. 2B). Both models exhibited a reduced loss in body weight compared with corresponding controls during the early phase of infection (Fig. 2C), suggesting a delay in the inflammatory response. Although colonization of the cecum by *Salmonella* was normal in Mφ-depleted mice, *Salmonella* spread to the MLNs and spleen was accelerated; thus, *Salmonella* infection is poorly controlled in the absence of Mφs. In contrast, cDC1 mice had fewer *Salmonella* colony-forming units (CFU) in the cecum and MLNs at the earlier time points (Fig. 2D), suggesting the importance of cDC1 in the entry of *Salmonella* into the tissue.

We next assessed the antibody response to infection. Most *Salmonella*-infected Mφ- and cDC1-depleted mice succumbed to infection before the peak of the antibody response at 4 to 5 weeks after infection. To overcome this obstacle, we considered infection with attenuated *Salmonella* strains; however, they have been shown to have an altered infection route (40, 41). We therefore used a clinically relevant approach by rescuing mice with oral antibiotics after they developed inflammation. Measurement of fecal lipocalin-2 (42) revealed that mucosal inflammation was reduced in Mφ-deficient mice throughout infection in contrast to cDC1-deficient mice (Fig. 2E). We also found that Mφ-deficient mice failed to develop a *Salmonella*-specific mucosal IgA response but had an insignificantly reduced systemic IgA response and a normal systemic IgG response (Fig. 2F). Because plasma cells producing luminal IgA home to the gut mucosa after being generated in the gut-associated lymphoid tissue (GALT) (43), we monitored the kinetics of the gut-associated *Salmonella*-specific IgA cellular response. *Salmonella*-specific IgA-producing cells were detected in the mucosa before the MLNs (week 1), and their levels plateaued at 3 weeks after infection (fig. S3C). Numbers of *Salmonella*-specific IgA-producing cells were markedly reduced in Mφ-deficient mice throughout infection (Fig. 2G). cDC1-deficient mice had delayed *Salmonella*-specific mucosal IgA responses at the earlier time points, likely due to delayed *Salmonella* colonization of the tissue and delayed inflammation, but the mucosal IgA response at later time points was normal. Although the *Salmonella*-specific systemic IgA response was also normal in infected cDC1-deficient mice, the systemic IgG response was significantly reduced (Fig. 2F), which likely accounts for their poor survival. In line with the early delay in the mucosal IgA response, cDC1-deficient mice had moderately reduced numbers of mucosal *Salmonella*-specific IgA-producing cells only at the early time point (Fig. 2G).

Although cDC2 are a miniscule population among LB inflammatory MPs, we also tested cDC2-deficient *Itgax*^{Cre}*Notch2*^{fl/fl} (*Itgax:Notch2*^{-/-}) mice. cDC2-deficient mice had normal survival rate and showed no major defects in *Salmonella*-specific mucosal IgA and systemic IgG responses, but had significant reduction of the systemic IgA response (fig. S3, D to H).

Because *Batf3* and *Notch2* control cDC1 and cDC2 development systemically (35, 36, 39), cDC1 and cDC2 from the mucosa and MLNs could be responsible for the induction of systemic *Salmonella*-specific antibody responses. Together, our data point toward a preferential role of M ϕ s in promoting intestinal inflammation and show that gut CX3CR1⁺ M ϕ subsets are necessary for the induction of the pathogen-specific mucosal IgA response.

CX3CR1⁺ M ϕ subsets are required for the development of mucosal TLSs

Comparison of the three M ϕ subsets among each other showed differences in the expression of key genes involved in innate and adaptive immune responses, particularly upon inflammation. For instance, gene signatures related to reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, such as *Ncf1*, *Ncf2*, *Ncf4*, and *Nos2*, were enriched in CX3CR1^{lo} M ϕ s, suggesting that the main role of this population is the early innate control of infection (Fig. 3A and table S4). CX3CR1^{int} M ϕ s were characterized by the higher expression of genes related to migration to the MLNs such *Ccr7* and *Cxcr4* (Fig. 3A and table S4). In contrast, CX3CR1^{hi} M ϕ s appeared as tissue-resident cells with the highest expression of anti-inflammatory cytokines, most notably *Il10* and *Tgfb1*, and simultaneous expression of inflammatory genes, e.g., *Inf1b1*, *Tnf*, and IL12 members *Il12b* and *Ebi3* (Fig. 3A and table S4). CX3CR1^{hi} M ϕ s showed the strongest signature for genes involved in the induction of B cell and IgA responses, including *Tgfb1*, *Cxcl13*, and *Icosl* (Fig. 3A and table S4), suggesting that they induce immune responses locally within the mucosa.

Ectopic solitary lymphoid tissue is a form of mucosal GALT. Isolated lymphoid follicles develop postnatally upon colonization by the microbiota mainly in the SB around cryptopatches; additional TLSs may develop during inflammation and are consistently found around inflammatory lesions caused by IBD (44). We observed that the mucosa of *Salmonella*-infected colons contained a large number of B220⁺ B cell aggregates of various sizes and locations rarely found in the normal colon (Fig. 3, B and C). Consistent with features of TLSs, these B cell follicles contained MAdCAM⁺ (mucosal vascular addressin cell adhesion molecule 1) (45) high endothelial venules (Fig. 3D).

TLSs serve as a site of the IgA response to a pathobiont segmented filamentous bacterium (46), and studies of TLSs in other organs point to the importance of antigen presentation in their formation (47). CX3CR1⁺ M ϕ s and CD4⁺ T cells, as well as GL7⁺B220⁺ germinal center (GC) B cells and clusters of IgA⁺B220⁺ cells, were also present within TLSs (Fig. 3D). Within the mucosa, TLSs formed around or adjacent to the sites of *Salmonella* penetration of the bowel wall (Fig. 3E).

GC B cells purified from the infected colon by fluorescence-activated cell sorting (FACS) (fig. S5A) expressed genes essential for IgA class switch recombination (48, 49), such as *Aicda*, IgA germline (aGT), and mature rearranged IgA (I μ -C α) transcripts (PST) (fig. S5B). Consistently, flow cytometry analysis of cells isolated from macroscopic TLSs, cecal and colonic patches, and the rest of the mucosa confirmed that TLSs were enriched with B cells, GC B cells, and CD4⁺ T cells (Fig. 3F and fig. S5C), yet TLSs did not have discrete GCs (Fig. 3D) similar to the extrafollicular B cell response to systemic *Salmonella* in the spleen (50). To determine whether B cells within TLSs acquired specificity to *Salmonella*, cells isolated from TLSs, mucosa, LB patches, and MLNs were cultured in the presence of CD40

mAb, IL-21, and B cell-activating factor (BAFF) to promote differentiation of GC B cells into antibody-producing cells (51, 52). Upon such stimulation, cells from TLSs provided the most significant expansion of *Salmonella*-specific IgA-producing cells (Fig. 3, G and H). These results demonstrate that colonic TLSs that develop in response to *Salmonella* infection serve as the ectopic mucosal sites of the pathogen-specific IgA response.

Because CX3CR1⁺ cells were detected within TLSs (Fig. 3D), we asked whether CX3CR1⁺ Mφ subsets were required for the development of TLSs. We measured the number and size of TLSs in Mφ-deficient mice before and after infection and found that both parameters were significantly reduced after infection (Fig. 3, I to K). In contrast, cDC1 depletion had no impact on TLS development (Fig. 3, L and M). Collectively, CX3CR1⁺ Mφs drive the development of TLSs that serve as ectopic sites of the *Salmonella*-specific IgA response.

Antigen presentation by CX3CR1⁺ Mφ subsets is required for the development of the mucosal *Salmonella*-specific IgA response and TLSs

Next, we asked whether intestinal CX3CR1⁺ Mφ subsets act as APCs. The IgA response to luminal antigens can be T cell independent or require antigen presentation by intestinal APCs to T cells (53). We therefore asked whether induction of *Salmonella*-specific mucosal IgA and TLS development depend on antigen presentation by Mφs to CD4⁺ T cells through MHCII. We used *Lyz2^{Cre/WT}H2-Ab1^{fl/fl}* (*Lyz2:MHCII^{-/-}*) mice in which MHCII expression and antigen presentation are predominantly lost by Mφs [(37) and fig. S6, A to C]. *Lyz2:MHCII^{-/-}* mice succumbed to infection with delayed kinetics but showed no differences in the early body weight loss, fecal lipocalin-2 levels at early time points, and *Salmonella* colonization (Fig. 4, A to D), suggesting that the initial inflammatory response and innate control of *Salmonella* remained preserved.

We then followed the *Salmonella*-specific antibody response. The *Salmonella*-specific mucosal IgA response was nearly absent in *Lyz2:MHCII^{-/-}* mice (Fig. 4E) similar to Mφ-deficient mice and despite gradually increasing mucosal inflammation (Fig. 4D). In contrast to Mφ-deficient mice, systemic IgA and IgG production was significantly reduced in *Lyz2:MHCII^{-/-}* mice (Fig. 4E). This discrepancy in systemic antibody responses between Mφ-deficient and *Lyz2:MHCII^{-/-}* mice could be explained by the fact that Mφs in both the intestine and MLNs are affected in *Lyz2:MHCII^{-/-}* mice in contrast to Mφ-deficient mice, in which Mφ depletion is restricted to mucosal Mφs (37), although partial loss of MHCII in cDCs and B cells could also contribute to the systemic defect in antibody responses (fig. S6A). Consistent with the low *Salmonella*-specific IgA response, the number of *Salmonella*-specific IgA-producing cells in the mucosa of *Lyz2:MHCII^{-/-}* mice was strongly reduced throughout infection (Fig. 4F).

Lyz2:MHCII^{-/-} mice also developed fewer TLSs, which were significantly smaller than in WT mice (Fig. 3G–I). Consistent with these findings, mucosal T follicular helper (Tfh) cells and GC B cell: were also significantly reduced in *Lyz2:MHCII^{-/-}* mice as compared with controls, in contrast to the similar total B cell numbers, indicating that B cell recruitment into the mucosa is intact in *Lyz2:MHCII^{-/-}* mice (Fig. 4, J and K, and fig. S6D). CD4⁺ T cell-depleted mice had a similar phenotype (fig. S6, E to K). Together, these data suggest that the mucosal *Salmonella*-specific IgA response and TLS development require

presentation of *Salmonella* antigens to CD4⁺ T cells by CX3CR1⁺ Mφs, although the experiments do not establish the site of antigen presentation, i.e., mucosa versus MLNs.

Migration of CX3CR1^{int} Mφs to the MLNs is not required for TLS formation and function

Migration of antigen-loaded APCs from the mucosa to the MLNs is thought to be essential for T cell activation and induction of adaptive immune responses against luminal antigens (54, 55). Transcriptional analysis of intestinal inflammatory MP subsets suggests that they have different trafficking patterns in the infected mucosa, particularly in regard to their migration to the MLNs (Fig. 3A). CCR7 is a key chemokine receptor that directs trafficking of immunocytes from the peripheral tissues to the draining lymph nodes (56, 57). We found that CX3CR1^{int} Mφs were the only inflammatory Mφ population that expressed *Ccr7* (Figs. 3A and 5A) and were present in the mesenteric prenodal lymph of infected mice together with CD103⁺ cDC1 and cDC2 (Fig. 5B). CX3CR1^{int} Mφs were still present in the prenodal lymph of Mφ-deficient mice but reduced (Fig. 5C) in contrast to the nearly complete depletion of CX3CR1^{hi} Mφs from the mucosa (fig. S3A), suggesting that the *Salmonella*-specific IgA defect in Mφ deficient mice was mainly driven by the lack of mucosa-resident Mφs

To test whether the TLSs and mucosal *Salmonella*-specific IgA response would develop in the absence of Mφ migration to the MLNs, we developed *Ccr7*^{fl/fl} mice (fig. S7, A to C) and crossed them to *Lyz2*^{Cre/Cre} mice to obtain *Lyz2*^{Cre/WT} *Ccr7*^{fl/fl} (*Lyz2:Ccr7*^{-/-}) mice in which *Ccr7* expression was predominantly abrogated in Mφs (fig. S7D). *Lyz2:Ccr7*^{-/-} mice had no marked changes in intestinal MP subsets in the infected LB (fig. S7E), survived infection, and showed no difference in early body weight loss or *Salmonella* colonization compared with infected control mice (Fig. 5, D to F), indicating that the initial inflammatory response and innate immune control of *Salmonella* were unaffected. However, mucosal inflammation in *Lyz2:Ccr7*^{-/-} mice was stronger than in control mice (Fig. 5G). Furthermore, infected *Lyz2:Ccr7*^{-/-} mice almost completely lost CX3CR1^{int} Mφs in their lymph (Fig. 5, H and I), in contrast to Mφ-deficient mice. *Lyz2:Ccr7*^{-/-} mice had normal TLS number upon infection, although these TLSs were larger compared with control mice (Fig. 5, J and K). Because TLS formation was preserved in *Lyz2:Ccr7*^{-/-} mice, we predicted that their *Salmonella*-specific mucosal IgA response would be only partially reduced. The *Lyz2:Ccr7*^{-/-} mice produced intermediate levels of *Salmonella*-specific mucosal IgA compared with control mice and mice infected without streptomycin pretreatment (negative control) to restrict *Salmonella* invasion and immune responses to Peyer's and LB patches (58). In contrast, systemic IgA and IgG production were normal (Fig. 5L), and the number of *Salmonella*-specific IgA-producing cells in the mucosa of *Lyz2:Ccr7*^{-/-} mice was not significantly affected at a later time point of the infection (Fig. 5M). Combined, these data support the idea that the *Salmonella*-specific IgA response is able to develop independently of CX3CR1^{int} Mφ migration to the MLNs and does so through antigen presentation by mucosa-resident Mφs within intestinal TLSs. The stronger mucosal inflammatory response in *Lyz2:Ccr7*^{-/-} mice suggests a role for CCR7 in controlling mucosal inflammation.

Mucosa-resident CX3CR1^{hi} Mφs induce TLS-associated T cell and B cell IgA responses

To confirm that the *Salmonella*-specific CD4⁺ T cell response takes place in mucosal TLSs, we first established a model to visualize the *Salmonella*-specific CD4⁺ T cell response in vivo using adoptive transfer of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled congenic CD45.1⁺ OT-II T cells after infection with *Salmonella* expressing OVA (*SL-OVA*; fig. S8A). We found that OT-II T cell proliferation was reduced in both LB and MLNs of *Lyz2:MHCII*^{-/-} mice (fig. S8, B and C). Although these data confirm the importance of Mφs in the activation of *Salmonella*-specific CD4⁺ T cells, the changes in the LB could be secondary to the changes in the MLNs, because T cells activated in the MLNs home into the mucosa (59). To address this issue, we used immunodeficient *Ccr7*^{-/-} mice, in which lymph nodes are impaired, cell egress from the mucosal GALT is blocked (56), and antibody responses to *Salmonella* are poor, despite the intact composition of intestinal MPs and development of TLSs (fig. S9, A to E). We found that OT-II T cells proliferated in the LB of *Ccr7*^{-/-} mice with frequencies similar to WT mice; however, the OT-II proliferation was abrogated when Mφs were depleted in *Ccr7*^{-/-} mice (Fig. 6, A and B). In addition, imaging of LBs from infected WT and *Ccr7*^{-/-} mice after OT-II cell transfer revealed that mucosal TLSs contain clusters of CD45.1⁺ donor cells (Fig. 6C), most of which are OT-II cells (fig. S8A), confirming that TLSs are ectopic sites where Mφs present *Salmonella* antigen to T cells. Although we were unable to determine the exact phenotype of OT-II cells within TLSs due to technical limitations, the data shown in Fig. 4K suggest their Tfh identity.

GC B cell responses in the secondary lymphoid organs require expression of CXCL13 by stromal cells, followed by recruitment of CXCR5⁺ T and B cells (60, 61). The CXCL13/CXCR5 axis has also been shown to be required for the development of TLSs in the inflamed intestine (62, 63). To identify a mucosa-resident Mφ population that drives the formation of TLSs in response to infection, we screened each Mφ population for the expression of genes involved in the GC and IgA B cell responses including *Cxcl13* (64) and identified CX3CR1^{hi} Mφs as the only population capable of both of these functions (Figs. 3A and 6D). Although all Mφs expressed higher levels of *Tnfrsf13a* [encoding B cell growth factor APRIL (a proliferation inducing ligand); fig. S9F], CX3CR1^{hi} Mφs were the only population that expressed *Tnfrsf13b* (encoding B cell growth factor BAFF) and *Cxcl13* before infection and further up-regulated their expression after infection along with expression of *Il10* and *Tgfb1* (Fig. 6D). Consistently, CX3CR1^{hi} Mφs were enriched in TLSs (Fig. 6E). We also identified a B220⁺ subset of CX3CR1^{hi} Mφs, which was represented by doublets of Mφs and B220⁺ cells (fig. S9G), so it was likely derived from TLSs. B220⁺CX3CR1^{hi} Mφs expressed higher levels of *Cxcl13* while maintaining *Il10* expression in contrast to B220⁻CX3CR1^{hi} Mφs or pure B cells (Fig. 6F). Immunofluorescence microscopy of mucosal TLSs co-localized CXCL13 with the *Cx3cr1*-GFP⁺ population (Fig. 6G). An additional cell type showed immunoreactivity for CXCL13 (Fig. 6G) and was likely a subset of CD45⁻ stromal cells, which were expressing intermediate levels of *Cxcl13* (Fig. 6F). Consistently, recruitment of CXCR5⁺ B cells by sorted CX3CR1^{hi} Mφs in a transwell assay was reduced by addition of CXCL13-blocking antibody (Fig. 6H). Thus, mucosa-resident CX3CR1^{hi} Mφs are the most likely Mφ subset responsible for TLS induction.

We then tested our hypothesis *ex vivo*. When inflammatory intestinal MP subsets were cocultured with CFSE-labeled OT-II T cells in the presence of *SL-OVA*, CX3CR1^{hi} Mφs were superior to other Mφ populations in inducing OT-II T cell proliferation (Fig. 6, I and J) and at similar levels as cDC1 (fig. S9, H and I). Similarly, CX3CR1^{hi} Mφs isolated from inflamed LB were superior to other MPs in inducing GC B cell phenotype in naïve B cells (Fig. 6, K and L) and driving the switch of the B cell antibody response from IgG to IgA (Fig. 6M).

Last, we developed an *in vivo* mouse model that selectively lacked MHCII expression in mucosal CX3CR1^{hi} Mφs by modifying a previously published protocol based on *Cx3cr1*^{CreER} knock-in/knockout mice that express a Cre-ERT2 (tamoxifen-inducible estrogen receptor) fusion protein and enhanced yellow fluorescent protein as detailed in Materials and Methods. In previous fate mapping experiments, tamoxifen treatment of *Cx3cr1*^{CreER} mice led to labeling of almost 100% of blood monocytes, 60% of splenic cDC1, and 40% of splenic cDC2 (65). We crossed *Cx3cr1*^{CreER} mice with *H2-Ab1*^{fl/fl} mice to generate *Cx3cr1*^{CreER}*H2-Ab1*^{fl/fl} (Cre:fl/fl) mice and titrated down the tamoxifen dose to achieve preferential targeting of CX3CR1^{hi} Mφs (see Materials and Methods for details and fig. S9J). As a result, Cre:fl/fl mice had a nearly complete loss of MHCII in CX3CR1^{hi} Mφs, whereas MHCII expression in cDC1, CX3CR1^{lo/int} Mφs, and B cells was mostly preserved (Fig. 6N and fig. S9K). When we quantified TLS formation and early mucosal *Salmonella*-specific IgA response in Cre:WT/fl and Cre:fl/fl littermates, we found that Cre:fl/fl mice developed fewer TLSs, which were significantly smaller than in control mice, and showed delayed accumulation of *Salmonella*-specific IgA-producing cells in the LB mucosa (Fig. 3, O to Q). We did not follow these mice long term due to the transient nature of MHCII depletion. Together, our data demonstrate that mucosa-resident CX3CR1^{hi} Mφs drive the local IgA response to infection through CXCL13-dependent recruitment of T and B cells into the sites of *Salmonella* invasion, *in situ* induction of *Salmonella*-specific CD4⁺ T cell and GC B cell responses, and IgA class switching by B cells via TLS formation.

DISCUSSION

Our study uncovered the heterogeneity of inflammatory MPs in infectious *Salmonella* colitis and established their identities as either cDCs or Mφs. CX3CR1⁺ MPs that were the majority of inflammatory MPs were further divided into three functionally distinct populations, namely, *Nos2*⁺CX3CR1^{lo} (bactericidal gene expression signature), *Ccr7*⁺CX3CR1^{int} (lymph migratory), and *Cxcl13*⁺CX3CR1^{hi} (tissue resident), all of which were transcriptionally associated with Mφs, derived from monocytes, and developed independently of NOTCH2, a signaling receptor known to control cDC2 development. We demonstrated that intestinal CX3CR1⁺ Mφ subsets were superior to cDC1 and cDC2 in inducing the *Salmonella*-specific T cell-dependent IgA response and determined the topography of the immune response induced by heterogeneous Mφs. We showed that the mucosa-resident CX3CR1^{hi} Mφ subset was the APC responsible for recruitment and activation of CD4⁺ T and B cells to the sites of *Salmonella* invasion, followed by TLS formation and the local pathogen-specific IgA response, i.e., independently of migration of CX3CR1^{int} Mφs to the MLNs (Fig. 7). Collectively, the study closed a knowledge gap in our understanding of the mechanisms used by the enteric MP system to contain enteroinvasive infection.

The CX3CR1^{int} population has been previously referred to as monocyte-derived DCs because of the ability to traffic to the MLNs (25). In another study, a phenotypically similar population found in the intestinal lymph was referred to as bona fide DCs because it lacked F4/80 expression and expanded in response to systemic FLT3L treatment (26). In the light of our findings, we suggest that these classifications should be revisited.

The developmental hierarchy of CX3CR1⁺ Mφ subsets is yet unknown. Different depletion kinetics of CX3CR1^{lo}, CX3CR1^{int}, and CX3CR1^{hi} Mφs after monocyte depletion together with the expression levels of particular genes suggests their sequential differentiation in the following order: monocytes → CX3CR1^{lo} Mφs → CX3CR1^{int} Mφs and monocytes → CX3CR1^{lo} Mφs → CX3CR1^{hi} Mφs (Fig. 7). If correct, this hypothesis raises a question of what are the molecular switches that regulate Mφ migration versus tissue residency and whether they can be manipulated by *Salmonella*. Alternatively, findings from a recent study on monocyte-derived MPs in the spleen raise a possibility that CX3CR1^{lo}, CX3CR1^{int}, and CX3CR1^{hi} populations begin segregating in the bone marrow by differentiating from distinct monocyte progenitors (66). Intestinal inducible nitric oxide synthase (iNOS)⁺ DCs have been proposed to induce IgA class switching (67), although this study was based on the analysis of *Nos2*^{-/-} mice and MP identity was not assessed in detail. In our hands, *Nos2*⁺CX3CR1^{lo} Mφs are less effective in driving T and B cell responses, suggesting the importance of iNOS in the differentiation of more functional CX3CR1^{int} and CX3CR1^{hi} Mφs.

Tissue Mφs are able to acquire additional subspecialization based on their immediate microenvironment. Thus, a recent study of Mφ heterogeneity in several organs including the lung, dermis, heart, and adipose tissue demonstrated that most of parenchymal Mφs were associated with either vasculature or peripheral nerves (68). A phenotypically and transcriptionally distinct population of intestinal Mφs resides in the outer smooth muscle layer of the intestines (muscularis externa). Within the muscle, Mφs are positioned along nerve fibers and adjacent to neuronal cell bodies of the enteric nervous system (ENS), being most abundant in the myenteric and the deep muscular plexuses (69). The analogous Mφ population is associated with the neural network in the mucosa (70), but the proportional contribution of these cells to the total pool of mucosal Mφs has not been assessed. In this study, we did not separate mucosa from the rest of intestinal layers as we have performed in our previous studies (2, 69, 71), assuming that the vast majority of inflammatory MPs will reside in the infected mucosa, which is supported by our data. For example, *Bmp2* highly and selectively expressed by ENS-associated muscularis Mφs (69) is poorly expressed by intestinal inflammatory Mφs that we have characterized (RNA-seq dataset). Further analysis, particularly using single-cell RNA-seq technology of inflammatory MPs, should be able to determine organ-specific niches of gut inflammatory MPs.

TLSs that contain CXCL13⁺ MPs are consistently found around inflammatory lesions caused by IBD (44, 72). Although TLSs were shown to exacerbate colitis in dextran sulfate sodium-treated RORγ (retinoic acid receptor-related orphan receptor γ)-deficient mice (73), the contribution of TLSs to intestinal immunity in humans and pathogenesis of IBD has not been established. To rationalize the physiological relevance of having local mucosal immune responses, one can speculate that having a compartmentalized antibody response at

the site of infection would restrict systemic spread of enteroinvasive pathogens such as *Salmonella*. Alternatively, it has been suggested that intestinal TLSs form to mediate local immunity when lymph drainage to regional lymph nodes becomes impaired (74), and this concept has been recently proven in the mouse lung (75). IBD is thought to result from the dysregulated immune response to luminal commensal microbes (76). With evidence of TLS development in IBD, it is plausible that TLSs in IBD serve as mucosal sites of pathological commensal-specific immune responses (46), but whether they are effective sites of IgA responses remains to be determined. Thus, recent single-cell analysis of intestinal mucosa from patients with Crohn's disease identified a pathogenic cellular module that consisted of IgG-producing plasma cells, inflammatory MPs, and activated T cells and stroma cells (77), suggesting that dysregulation of TLS function with preferential IgG response may contribute to the pathophysiology of IBD (78). Moreover, identifying antigens that drive TLS development in IBD might provide important cues to establishing its etiology. Similarly, recent findings showing that the presence of tumor-associated TLSs in melanoma and sarcoma positively correlates with response to immunotherapy highlight the involvement of TLSs in tumor pathogenesis, although their role is protective (79–81).

Earlier studies defined CX3CR1⁺ Mφs as tolerogenic due to their IL-10 production, poor ability to migrate to lymph nodes, and capacity to restrict the mucosal inflammatory response through activation of homeostatic regulatory mechanisms (16–18, 20, 82). It was suggested that mucosa-resident CX3CR1⁺ Mφs at steady state induce tolerance to enteric antigens in a stepwise fashion after the activation step initiated by DCs in the MLNs (82). Our study, however, demonstrates that under conditions of infectious colitis, the CX3CR1^{hi} population is immunogenic: CX3CR1^{hi} Mφs that also express *II10* activate CD4 T and B cells locally in parallel to immune events in the MLNs. The ability of CX3CR1^{hi} Mφs to produce IL-10 is likely aimed to prevent excessive tissue damage during the immune response in situ, which is unnecessary if the immune response is localized to the lymph nodes, although a recent study linked IL-10 signaling pathway in Mφ with enhanced antimicrobial resistance to *Salmonella* by limiting Mφ production of eicosanoid prostaglandin E2 (83). Our finding that TLS-associated B220⁺CX3CR1^{hi} Mφs maintain *II10* expression at the same level as their extrafollicular counterparts is in line with these findings, yet only a single-cell analysis will be able to determine how this control is organized in situ.

Previously, CX3CR1⁺ Mφs in the SB were found to take up soluble dietary antigens and transfer them to cDC2 through a gap junction–dependent membrane exchange. This antigen transfer was shown to be important for driving T regulatory (T_{reg}) cell differentiation and induction of oral tolerance to food antigens (84). Whether CX3CR1⁺ Mφs are able to transfer antigens of microbial origin and induce the immune response to microbiota and infection indirectly through cDC2 has not been tested. Because cDC2 are scarce in the inflamed LB, it seems unlikely that this pathway would substantially contribute to the *Salmonella*-specific IgA response induced by the CX3CR1⁺ Mφ subsets in our model.

Given the potential dual pro- and anti-inflammatory nature of mucosa-resident CX3CR1^{hi} Mφs, TLSs may function as crossroads of mucosal T cell immunity by directing T cells toward effector or regulatory phenotypes. This idea is in line with the plasticity of intestinal

T helper cells. T_H17 cells have been shown to develop toward a T_{fh} cell program in the environment of Peyer's patches, a process shown to promote T cell-dependent IgA responses (85). In additional reports, T_{reg} cells were also shown to convert into T_{fh} cells to promote intestinal IgA responses (86, 87), whereas other studies have argued that T_{reg} cells that express markers of T_{fh} cells are essential for control rather than promotion of the GC reaction (88, 89).

Several biologic anti-inflammatory therapies for IBD that are either Food and Drug Administration–approved or in phase 2 and 3 clinical trials target M ϕ function by neutralizing tumor necrosis factor- α (TNF- α), IL-12, IL-23, and IL-1R or by blocking their signaling pathways (90, 91), although these therapies also lead to immuno-suppression and the increased potential for opportunistic infections (92). Our murine study finds that at least some of the molecules neutralized by these therapeutic agents are differently expressed among the three M ϕ subsets (Fig. 3 and fig. S4). Despite the evolving knowledge of M ϕ heterogeneity in IBD-affected intestine (77, 93), the impact of these anti-inflammatory therapies on intestinal M ϕ diversity and subset-specific function is unknown. By advancing the understanding of how the mucosal immune response driven by heterogeneous M ϕ s is organized anatomically, our findings provide the foundation for future studies to develop more sophisticated approaches for IBD therapies and oral vaccine development to prevent *Salmonella* infection.

MATERIALS AND METHODS

Study design

We performed phenotypical, transcriptional, and functional analysis of inflammatory CX3CR1⁺ MPs in infectious colitis induced by *Salmonella enterica* using in vivo and ex vivo experiments. Mice were infected with the WT *Salmonella* strain SL1344 after pretreatment of mice with streptomycin to promote *Salmonella* colonization of the LB mucosa, followed by inflammation. Infection without streptomycin or with attenuated *Salmonella* strains alters the route of colonization and limits the immune response to the organized GALT without mucosal inflammation (29, 58). Because mice on C57BL/6 (B6) background are sensitive to WT *Salmonella* infection, we used mouse strains backcrossed to the *Salmonella*-resistant 129S1 background. To test the role of different MP subsets in mucosal immune responses in vivo, we used an antibody-mediated depletion of M ϕ s, a M ϕ -specific Cre/loxP transgenic mouse approach to modulate M ϕ function, and cDC1- and cDC2-deficient knockout and transgenic mice. Because of different antibody responses in males and females (94), we used females for all experiments.

Experimental animals

B6, 129S1, *Lyz2*^{Cre/Cre}, *H2-Ab1*^{fl/fl}, *Batf3*^{-/-}, OT-II, B6.CD45.1, *Cx3cr1*^{GFP/GFP}, *Ccr7*^{-/-}, *Notch2*^{fl/fl}, *Itgax*^{Cre}, *Ccr2*^{RFP/RFP}, and *Cx3cr1*^{CreER} (*Litt*) mouse strains were obtained from the Jackson Laboratory (Bar Harbor, ME). *Ccr7*^{fl/fl} mice were developed by the Aifantis Lab. The 11.02-kb region used to construct the targeting vector was designed such that the short homology arm (SA) extends about 1.9-kb 5' to exon 3. The long homology arm (LA) ends 3' to exon 3 and is 6.10 kb long. The loxP/FRT-flanked Neo cassette is inserted on the

5' side of exon 3, and the single loxP site is inserted 3' of exon 3. The target region is 2.98 kb and includes exon 3. The bacterial artificial chromosome was subcloned into a ~2.4-kb backbone vector (pSP72, Promega) containing an ampicillin selection cassette for retransformation of the construct before electroporation. A pGK-gb2 loxP/FRT Neo cassette was inserted into the gene as described in the project schematic (fig. S7, A to C). The pGK cassette was subsequently deleted with FLP after the chimera stage. B6, *Lyz2*^{Cre/Cre}, *H2-Ab1*^{fl/fl}, *Batf3*^{-/-}, *Ccr7*^{-/-}, *Ccr7*^{fl/fl}, *Notch2*^{fl/fl}, *Itgax*^{Cre}, and *Cx3cr1*^{CreER} mice were backcrossed for at least three generations to *Salmonella*-resistant 129S1 background and genotyped to confirm the presence of the *Nramp1* (*Slc11a1*) allele responsible for resistance to *Salmonella* infection (95). *Lyz2*^{Cre/WT} *H2-Ab1*^{fl/fl}, *Lyz2*^{Cre/WT} *Ccr7*^{fl/fl}, *Cx3cr1*^{CreER/WT} *H2-Ab1*^{fl/fl}, and *Itgax*^{Cre} *Notch2*^{fl/fl} mice were created using the *Salmonella*-resistant backcrossed strains. For Mφ phenotypical and transcriptional analysis and in vitro culture experiments, *Cx3cr1*^{GFP/GFP} and *Ccr2*^{RFP/RFP} *Cx3cr1*^{GFP/GFP} mice were bred with 129S1 mice to obtain *Cx3cr1*^{GFP/WT} and *Ccr2*^{RFP/WT} *Cx3cr1*^{GFP/WT} F1 generations. To analyze Mφ populations, *Salmonella*-resistant *Batf3*^{-/-} *Cx3cr1*^{GFP/GFP}, *Ccr7*^{-/-} *Cx3cr1*^{GFP/GFP}, *Ccr7*^{fl/fl} *Cx3cr1*^{GFP/GFP}, and *Notch2*^{fl/fl} *Cx3cr1*^{GFP/GFP} mice were created and bred with *Batf3*^{-/-}, *Ccr7*^{-/-}, *Lyz2*^{Cre/WT} *Ccr7*^{fl/fl}, and *Itgax*^{Cre} *Notch2*^{fl/fl}, respectively, and littermates of F1 generations were used for experiments. Because there is a difference in antibody responses in male and female mice (94), we used females for all experiments. All animals were housed under specific pathogen-free conditions. To minimize differences in genetic background and microbiota between control and experimental groups, only littermates were used for both groups. Infection experiments were started with animals between 6 and 8 weeks of age. The Institutional Animal Care and Use Committees of Penn State College of Medicine and UMass Medical School approved all protocols used.

***Salmonella* infection**

Mice were pretreated with 20 mg of streptomycin (Thermo Fisher Scientific, Waltham, MA) by oral gavage 24 hours before infection with 2×10^6 CFU of WT *S. enterica* strain SL1344 in 200 µl of phosphate-buffered saline (PBS) (29,96) by oral gavage. For some experiments, mice were infected with 2×10^6 CFU of SL1344 without streptomycin before treatment to restrict the immune response to the organized GALT (29, 58). Infected mice were euthanized at indicated time points, or their survival and weight loss were monitored up to 5 to 6 weeks after infection. To assess *Salmonella*-specific antibody responses, fecal and blood samples were collected weekly from mice during the course of infection. Fecal pellets were homogenized in cold PBS at 100 mg/ml, and supernatants were used to assess antibody titers and lipocalin-2 levels. Serum was isolated by incubating blood samples overnight at 4°C until coagulated, followed by centrifugation at 12,000g for 10 min. Infected mice were given drinking water supplemented with ampicillin (1 mg/ml) after 5 days of infection where indicated.

For adoptive OT-II T cell transfer experiments, OVA expressing SL1344 (SL-OVA) was created by transforming SL1344 with an OVA-expressing plasmid (97) by electroporation, followed by selection with ampicillin. Mice were infected with 2×10^8 CFU of SL-OVA.

***Salmonella* translocation**

To determine *Salmonella* colonization of the mucosa and organized GALT, as well as its translocation to the MLNs and spleen, mice were euthanized on days 1, 2, or 6 of the infection, and SB, Peyer's patches, cecum, colon, cecal and colonic patches, MLNs, and spleen were collected. Different regions of the SB (without Peyer's patches), cecum, and colon (without patches) were processed as described (71) to remove luminal contents and the epithelial cell layer in the presence of gentamicin (100 µg/ml) to neutralize extracellular bacteria (Thermo Fisher Scientific) and homogenized in PBS using TissueRuptor (Qiagen). Luminal content from each region was homogenized in PBS using vortex. Peyer's patches, LB cecal and colonic patches, MLNs, and spleen were rinsed in PBS, followed by homogenizing using TissueLyser II (Qiagen). Homogenized samples were cultured in different dilutions on MacConkey agar plates (Sigma-Aldrich) containing streptomycin (100 µg/ml) for 16 hours at 37°C. As nonlactose fermenting Gram-negative bacteria, *Salmonella* forms white colonies on MacConkey agar. White colonies were counted, and total *Salmonella* CFU per organ were calculated.

Enzyme-linked immunospot (ELISpot)

LB of mice infected with SL1344 were collected on days 7 and 28 after infection and processed as described previously using Percoll gradient separation (98). A twofold dilution series starting with 10^6 cells was prepared in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Hyclone), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml), and gentamicin (100 µg/ml) (Thermo Fisher Scientific) and cultured in the wells of 96-well enzyme-linked immune absorbent spot (ELISpot) plates (Millipore Sigma, Burlington, MA) coated with 2×10^6 CFU of heat-killed (HK)z SL1344 (95°C for 15 min) for 18 hours at 37°C and 5% CO₂.

To isolate TLSs from the inflamed colon, optically dense areas (barely visible before day 14 after infection) were identified under a Stereomaster dissection microscope (Thermo Fisher Scientific), and about 1-mm² tissue pieces containing those areas were excised using a disposable biopsy punch (Integra, York, PA). MLNs, cecal and colonic patches, and tissue pieces from remaining mucosa were collected from the LB, homogenized with collagenase type IV (Thermo Fisher Scientific), and cultured on ELISpot plates coated with HK SL1344 in RPMI 1640 supplemented with 10% FBS, 5.5×10^{-5} M 2-ME (2-mercaptoethanol), 10 mM Hepes, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml), and gentamicin (100 µg/ml) along with recombinant IL-21 (100 ng/ml), recombinant BAFF (25 ng/ml; PeproTech, Rocky Hill, NJ), and CD40 mAb (5 µg/ml; BioLegend, San Diego, CA) at 37°C and 5% CO₂ for 2 days.

Salmonella-specific IgA-producing cells were detected using rat mAb against mouse IgA (SouthernBiotech, Birmingham, AL) and Vector Blue alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA). Plates were captured using ELISpot reader (Cellular Technology Limited, Cleveland, OH) and analyzed using ImmunoSpot software.

Enzyme-linked immunosorbent assay

To detect *Salmonella*-specific antibodies, 96-well high-binding enzyme-linked immunosorbent assay (ELISA) plates (Immulon, Thermo Fisher Scientific) were coated overnight with HK SL1344. To detect total antibody levels, plates were coated with either anti-mouse IgA or IgG antibodies (SouthernBiotech). After blocking with PBS containing 1% BSA, ELISA plates were incubated with a series of dilutions of fecal or serum samples prepared in PBS overnight at 4°C. Horseradish peroxidase-conjugated anti-mouse IgA or IgG (SouthernBiotech) and trimethylboron substrate reagent (Thermo Fisher Scientific) were used for detection. The reaction was stopped using 2 N of H₂SO₄ solution and optical density (OD) values read at 450 nm. Antibody titers are shown as dilution factor (DF) corresponding to an OD of 1 (OD, 0.5 for total IgA ELISA assay). To calculate DF values, a graph was prepared for each sample with OD values on *y* axis and dilution values on *x* axis on a logarithmic scale, followed by adding a trend line to the linear phase of the graph. To detect lipocalin-2 concentrations in fecal samples, a mouse lipocalin-2 DuoSet ELISA kit (R&D Systems) was used.

Lymph collection

For lymph collection, anesthetized mice were injected intravenously with 1% Evans Blue dye in physiological saline to visualize lymphatic vasculature, and after a midline laparotomy of the abdominal wall, the intestines were exteriorized to expose mesentery with prenodal branches of the mesenteric lymph duct running parallel to the superior mesenteric artery. Up to 30 µl of lymph was collected from the ileocolic branch (dilated due to infection) that collects lymph from terminal ileum, cecum, and proximal ascending colon of the mesenteric lymph duct, using a heparin-coated insulin syringe. Cell populations in the collected lymph were washed with PBS, stained with mAbs, and analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. Data are presented as means ± SEM. The statistical significance of differences between group means was determined with the two-tailed unpaired or paired Student's *t* test and two-way analysis of variance (ANOVA). Values of *P* < 0.05 were considered to indicate statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Inflammatory CX3CR1⁺ MPs are composed of CX3CR1^{lo}, CX3CR1^{int}, and CX3CR1^{hi} Mφ subsets.

Cx3cr1^{GFP/WT} (F1 of B6 × 129S1) and *Ccr2*^{RFP/WT} *Cx3cr1*^{GFP/WT} mice were infected with SL1344 as described in Materials and Methods. (A) Flow cytometry dot plots show phenotypes and percentages of MPs in the normal [day 0 (D.0)] and infected [day 10 after infection (p.i.)] LB. Gated on total MPs defined as viable CD45⁺CD11b^{lo/hi}CD11c^{lo/hi}MHCII⁺ populations (fig. S2A). (B) t-SNE flow cytometry plots define phenotypes of all MP subsets in the normal (day 0) and infected (day 10 after infection) colon. Gated on total MPs as in (A). (C) Flow cytometry dot plots show the

phenotype of MP populations identified using unbiased t-SNE analysis. **(D)** t-SNE heat maps show levels of CD103, CD11b, *Cx3cr1*-GFP, XCR1, CD101, CD64, and CD16/32 expression by MP subsets in normal (day 0) and inflamed LB (day 10 after infection) based on t-SNE analysis in **(B)**. **(E)** Pie charts show percentages of each MP subset among total MPs in the normal (day 0) and infected (days 3 and 8 after infection) LB. **(F)** Hierarchy of MP populations based on RNA-seq analysis of MP subsets isolated by FACS from the normal (day 0) and infected (day 5 after infection) LB as compared with controls: SB cDC2 and peritoneal (Per.) M ϕ s isolated from normal mice on the same background. The height at which any two samples are joined in the dendrogram indicates the distance between them. Samples clustered close to each other are more similar than others. **(G)** RNA-seq heat map shows levels of M ϕ - and cDC-specific genes expressed by MP subsets isolated by FACS from the normal (day 0) and infected (day 5 after infection) LB as compared with controls: SB cDC2 and peritoneal M ϕ s isolated from normal mice on the same background. **(H)** Uninfected (day 0) and *Salmonella*-infected mice were treated with CCR2 mAb to deplete monocytes or control IgG as described in Materials and Methods. Graphs show cell counts of LB MPs and percentages of blood Ly6C^{hi} monocytes ($n = 3$ to 6). **(I)** Percentages (%) of CCR2⁺ cells (identified as RFP⁺ cells) among blood monocyte and LB MP populations in *Ccr2*^{RFP/WT} *Cx3cr1*^{GFP/WT} mice infected with *Salmonella* (day 8 after infection) ($n = 3$ to 4). Graphs show means $\pm$ SEM from the combination of two independent experiments. Statistical analysis: two-way ANOVA, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, and ***** $P < 0.000001$.

Fig. 2. CX3CR1⁺ Mφ subsets are required for the development of the mucosal *Salmonella*-specific IgA response.

Cx3cr1^{GFP/WT} mice (F1 of B6 × 129S1) were infected with SL1344 (A). WT mice were injected with CSF1R mAb to deplete Mφs (CSF1R Ab) or control IgG before infection with SL1344 (B to G). *Batf3*^{-/-} mice were used as a cDC1-deficient model, and their WT littermates were used as control (B to G). Mice were rescued with ampicillin (E to G). (A) RNA-seq heat map shows expression levels of the indicated pathways by MP subsets isolated by FACS from normal (day 0) and infected (day 5 after infection) LB as compared with controls: SB cDC2 and peritoneal Mφs isolated from normal mice on the same background (see the Supplementary Materials for statistical analysis). AP, antigen presentation. (B) Survival curves of mice treated with IgG or CSF1R Ab (left) and WT and *Batf3*^{-/-} mice (right) ($n = 5$ to 10). (C) Weight loss as percentage of body weight at day 0 of

mice treated with IgG or CSF1R Ab (left) and WT and *Batf3*^{-/-} mice (right) ($n = 7$ to 9). (D) *Salmonella* CFU in the cecum (day 1 after infection), MLNs (day 2 after infection), and spleen (day 6 after infection) of mice treated with IgG or CSF1R Ab (left) and WT and *Batf3*^{-/-} mice (right) ($n = 4$ to 10). (E) Lipocalin-2 titers in fecal pellets of infected mice treated with IgG or CSF1R Ab (top) and WT and *Batf3*^{-/-} mice (bottom) ($n = 7$ to 10). (F) *Salmonella* (SL)-specific antibody titers in fecal pellets (mucosal IgA) and blood (serum IgA and IgG) in infected mice treated with IgG or CSF1R Ab (top) and WT and *Batf3*^{-/-} mice (bottom) ($n = 4$ to 8). (G) Numbers of *Salmonella*-specific IgA-producing cells in the LB of infected mice treated with IgG or CSF1R Ab (top) and WT and *Batf3*^{-/-} mice (bottom) as measured by ELISpot ($n = 5$ to 7). Graphs show means $\pm$ SEM and are representative or combination of at least two independent experiments. Statistical analysis: Student's t test or two-way ANOVA, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Fig. 3. CX3CR1⁺ Mφ subsets are required for the development of TLSs, which serves as a site for Salmonella-specific IgA response.

WT and *Cx3cr1*^{GFP/WT} mice (A to H), CSF1R Ab and control IgG treated mice (I to K), and *Batf3*^{-/-} and WT mice (L and M) were infected with WT *Salmonella* SL1344. (A) RNA-seq heat maps show expression levels of genes related to the indicated pathways by MP subsets isolated by FACS from infected (day 5 after infection) LB (see the Supplementary Materials for statistical analysis). (B) Representative images of cross sections of colonic Swiss rolls stained with B220 (B cells; red) mAb and 4',6-diamidino-2-

phenylindole (DAPI) (nuclei; blue). Colons were obtained from WT uninfected (day 0) and infected (day 10 after infection) mice. (C) Statistical summary of (B) shows number of TLS follicles (TLF) per section before and after infection ($n = 3$ to 4). (D) Top left: Colonic cross sections from WT mice (day 10 after infection) were stained with B220 (B cells; green) and MAdCAM1 [high endothelial venules (HEV); red] antibodies and DAPI (nuclei; blue). Bottom left: Colonic cross sections from *Cx3cr1*^{GFP/WT} mice (day 10 after infection) were stained with B220 (B cells; red) and CD4 (CD4T cells; blue) mAbs and DAPI (nuclei; gray). Top right: Colonic cross sections from WT mice (day 10 after infection) were stained with B220 (B cells; red) and GL7 (GC B cells; green) mAbs and DAPI (nuclei; blue). Bottom right: Colonic cross sections from WT mice (day 10 after infection) were stained with B220 (B cells; red) and IgA (IgA⁺ B cells; green) mAbs and DAPI (nuclei; blue). (E) Colonic cross sections from WT mice (day 10 after infection) were stained with B220 (B cells; red) and *Salmonella*-specific (*SL*; green) antibodies and DAPI (nuclei; blue). (F) Statistical summary of flow cytometry data shows proportions of B220⁺ B cells, GL7⁺CD95⁺ GC B cells, and CD4⁺ T cells among live cells in single-cell suspension prepared from macroscopic TLSs, remaining mucosa, and colonic and cecal patches of LB isolated from an infected WT mouse (day 14 after infection) ($n = 4$). (G) Numbers of *Salmonella*-specific IgA-producing cells [unstimulated or stimulated (Stim.) with anti-CD40 mAb, BAFF, and IL-21] in TLSs, mucosa, LB patches, and MLNs (per 10⁵ plated total cells) isolated from a WT mouse (day 14 after infection) ($n = 3$ to 6). (H) Images of ELISpot wells from a representative experiment described in (G); cells stimulated with anti-CD40 mAb, BAFF, and IL-21. (I) Representative images of cross sections of colonic Swiss rolls from infected (day 10 after infection) control (IgG) and CSF1R Ab mice stained with B220 (B cells; red) mAb and DAPI (nuclei; blue). (J) Numbers of follicles per colonic section in control (IgG) and CSF1R Ab mice before and after infection ($n = 3$ to 4). (K) Area of follicles in colonic sections of control (IgG) and CSF1R Ab mice (day 10 after infection) ($n = 3$ to 4). (L) Numbers of follicles per colonic section in WT and *Batf3*^{-/-} mice before and after infection ($n = 3$ to 6). (M) Area of follicles in colonic sections of WT and *Batf3*^{-/-} mice (day 10 after infection) ($n = 3$ to 6). Graphs show means $\pm$ SEM and are representative or combination of at least two independent experiments. Statistical analysis: Student's *t* test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Fig. 4. Antigen presentation by CX3CR1⁺ Mφ subsets is required for the development of TLSs and the mucosal *Salmonella*-specific IgA response.

H2-AbI^{fl/fl} (Cont.) and *Lyz2*^{Cre/WT} *H2-AbI*^{fl/fl} (*Lyz2:MHCII*^{-/-}) mice were infected with SL1344. Mice were rescued with ampicillin (D to F). (A) Survival curves (*n* = 4 to 7). (B) Weight loss as a percentage of body weight at day 0 (*n* = 7 to 10). (C) *Salmonella* CFU in the cecum (day 1 after infection), MLNs (day 2 after infection), and spleen (day 2 after infection) of Cont. and *Lyz2:MHCII*^{-/-} mice (*n* = 6 to 12). (D) Lipocalin-2 titers in fecal pellets of infected control and *Lyz2:MHCII*^{-/-} mice (*n* = 6 to 10). (E) *Salmonella*-specific

antibody titers in fecal pellets (mucosal IgA) and blood (serum IgA and IgG) in infected control and *Lyz2:MHCII*^{-/-} mice ($n = 6$ to 10). (F) Numbers of *Salmonella*-specific IgA-producing cells in the LB of control and *Lyz2:MHCII*^{-/-} mice as measured by ELISpot ($n = 4$ to 5). (G) Representative images of cross sections of colonic Swiss rolls stained with B220 (B cells; red) and DAPI (nuclei; blue), day 10 after infection. (H) Numbers of follicles per colonic section before and after infection in control and *Lyz2:MHCII*^{-/-} mice (day 10 after infection) ($n = 2$ to 7). (I) Area of follicles in colonic sections of control and *Lyz2:MHCII*^{-/-} mice (day 10 after infection) ($n = 7$). (J) Numbers of total B and GC B cells in the LB before and after infection in control and *Lyz2:MHCII*^{-/-} mice ($n = 4$ to 5). (K) Numbers of total CD4⁺ T and Tfh cells in control and *Lyz2:MHCII*^{-/-} mice (day 10 after infection) ($n = 4$). Graphs show means $\pm$ SEM and are representative or combination of at least two independent experiments. Statistical analysis: Student's *t* test or two-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Fig. 5. Migration of CX3CR1^{int} Mφs to the MLNs is not required for TLS formation and function.

Ccr7^{fl/fl} (Cont.) and *Lyz2*^{Cre/WT} *Ccr7*^{fl/fl} (*Lyz2:Ccr7*^{-/-}) mice and other indicated mouse strains were infected with SL1344. Mice were rescued with ampicillin in drinking water (F, K, and L). (A) Kinetics of *Ccr7* expression by MP subsets isolated by FACS from the normal and infected LB from *Cx3cr1*^{GFP/WT} mice (F1 of B6 × 129S1) as measured by quantitative polymerase chain reaction (qPCR). Data show fold change over CX3CR1^{hi} Mφs at day 0 (*n* = 3 to 6). (B) Flow cytometry zebra plots show percentages of cDC and Mφ subsets in the lymph collected from pre-MLN lymphatic vessels of WT mice (day 9 after

infection). Gated on total viable CD45⁺CD11b^{lo/hi}CD11c^{lo/hi}MHCII⁺ MPs. (C) Flow cytometry dot plots show percentages of cDC and M ϕ subsets in the lymph collected from pre-MLN lymphatic vessels of mice treated with control and CSF1R Ab (day 11 after infection). Gated on total viable CD45⁺CD11b^{lo/hi}CD11c^{lo/hi}MHCII⁺ MPs. (D) Survival curves of control and *Lyz2:Ccr7*^{-/-} mice (*n* = 4 to 7). (E) Weight loss as a percentage of day 0 in control and *Lyz2:Ccr7*^{-/-} mice (*n* = 8 to 9). (F) *Salmonella* CFU in the cecum and MLNs of control and *Lyz2:Ccr7*^{-/-} mice at day 1 after infection (*n* = 4 to 5). (G) Lipocalin-2 titers in fecal pellets of infected control and *Lyz2:Ccr7*^{-/-} mice (*n* = 8 to 9). (H) Flow cytometry dot plots show percentages of cDC and M ϕ subsets in the lymph collected from pre-MLN lymphatic vessels of infected control and *Lyz2:Ccr7*^{-/-} mice (day 4 after infection) mice. Gated on total viable CD45⁺CD11b^{lo/hi}CD11c^{lo/hi}MHCII⁺ MPs. (I) Statistical summary of (G) shows the ratio of percentage of CD103⁺ cDCs and CX3CR1^{int} M ϕ subsets among MHCII⁺ MPs in lymph from control and *Lyz2:Ccr7*^{-/-} mice (*n* = 5 to 6). (J) Numbers of follicles per colonic section in control and *Lyz2:Ccr7*^{-/-} mice before and after infection (*n* = 3 to 8). (K) Area of follicles in colonic sections of control and *Lyz2:Ccr7*^{-/-} mice (day 10 after infection) (*n* = 3 to 8). (L) *Salmonella*-specific antibody titers in fecal pellets (mucosal IgA) and blood (serum IgA and IgG) in infected control and *Lyz2:Ccr7*^{-/-} mice and negative control mice infected without streptomycin pretreatment (Cont. w/o strep.) (*n* = 6 to 9). (M) Numbers of *Salmonella*-specific IgA-producing cells as measured by ELISpot in the LB of infected control and *Lyz2:Ccr7*^{-/-} mice (*n* = 4 to 10). Graphs show means $\pm$ SEM and are representative or combination of at least two independent experiments. Statistical analysis: Student's *t* test or two-way ANOVA, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Fig. 6. Mucosa-resident CX3CR1^{hi} Mφs induce TLS associated T and B cell/IgA responses. Mice were infected with WT SL1344 (*SL*) or OVA-expressing SL1344 (*SL*-OVA) where indicated. (A) WT, *Ccr7*^{-/-}, *Ccr7*^{-/-} control (injected with IgG), and *Ccr7*^{-/-} mice injected with CSF1R mAb were infected with *SL*-OVA the day before receiving CFSE-labeled congenic CD45.1⁺ OT-II T cells. Representative flow cytometry dot plots show percentages of CFSE low cells among total CD45.1⁺ Vα2⁺CD4⁺CCR9⁻ T cells in the LB on day 5 after infection. (B) Statistical summary of (A) (*n* = 3 to 5). (C) Colonic sections (day 7 after infection) from WT and *Ccr7*^{-/-} mice described in (A) were stained with B220 (B cells;

green) and CD45.1 (OT-II T cells; red) mAbs and DAPI (blue). **(D)** Kinetics of *Il10*, *Tgfb*, *Tnfsf13b* (BAFF), and *Cxcl13* expression by MP subsets isolated by FACS from the normal (day 0) and infected LB as measured by qPCR. Data show fold change over control (day 0) cDC1 (*Il10* and *Tgfb*) or control (day 0) CX3CR1^{hi} Mφs (*Tnfsf13b* and *Cxcl13*) ($n = 3$ to 5). **(E)** Statistical summary of flow cytometry data shows proportions of B220⁺ cells among CX3CR1^{hi} Mφs in TLSs or mucosa of infected LB isolated from a WT mouse (day 14 after infection) ($n = 4$ to 6). **(F)** *Cxcl13* and *Il10* expression by B220⁺ and B220⁻ CX3CR1^{hi} Mφs subsets, B cells, and CD45⁻ stromal cells FACS-isolated from the infected LB (day 7 after infection) as measured by qPCR. Data show fold change over B220⁻CX3CR1^{hi} Mφs ($n = 3$ to 6). **(G)** Colonic cross sections from *Cx3cr1*^{GFP/WT} mice (day 10 after infection) were stained with CXCL13 (red) and B220 (B cells; blue) antibodies and DAPI (nuclei; gray). **(H)** Migration of B220⁺ B cells in transwell experiments cultured with CX3CR1^{hi} Mφs FACS-isolated from infected LB (day 12 after infection), in presence or absence of CXCL13-blocking antibody (CXCL13 Ab). Data are shown as a percentage of input ($n = 2$). **(I)** MP subsets isolated from LB of infected *Cx3cr1*^{GFP/WT} mice (day 5 after infection) were cocultured in vitro with CFSE-labeled OT-II CD4⁺ T cells in the presence of 2×10^6 CFU of HK *SL*-OVA. Representative flow cytometry dot plots show percentages of proliferating CFSE low cells among OT-II Vα2⁺CD4⁺ T cells on day 4 of coculture. **(J)** Statistical summary of (I) ($n = 5$). **(K)** B220⁺ B cells (negative control) or MP subsets isolated from LB of infected *Cx3cr1*^{GFP/WT} mice (day 5 after infection) were cocultured with naïve splenic B cells from an uninfected congenic CD45.1⁺ donor. Representative flow cytometry plots show percentages of GL7⁺ cells among total CD45.1⁺B220⁺ B cells. Histogram compares CD95 expression by GL7⁻ (gray) and GL7⁺ (red) CD45.1⁺B220⁺ B cells. **(L)** Summary of (K) ($n = 2$). **(M)** IgG and IgA levels in supernatants of naïve splenic B cells cultured alone (Cont.) or cocultured with CX3CR1^{hi} Mφs isolated from LB of infected *Cx3cr1*^{GFP/WT} mice (day 5 after infection) in the presence or absence of SL1344 (*SL*). Data show fold change over B cells cultured alone without *SL* ($n = 3$). **(N)** Statistical summary of flow cytometry data show proportions of MHCII⁺ cells among MP populations in the LB isolated from tamoxifen-treated *Cx3cr1*^{CreER/WT}*H2-Ab1*^{WT/fl} (Cre:WT/fl) or *Cx3cr1*^{CreER/WT}*H2-Ab1*^{fl/fl} (fl/fl) mice (Cre:fl/fl) (day 7 after infection) ($n = 4$). **(O)** Representative images of cross sections of colonic Swiss rolls from tamoxifen-treated *Cx3cr1*^{CreER/WT}*H2-Ab1*^{WT/fl} (WT/fl) or *Cx3cr1*^{CreER/WT}*H2-Ab1*^{fl/fl} (fl/fl) mice stained with B220 (B cells; red) and DAPI (nuclei; blue), day 7 after infection. **(P)** Numbers and area of follicles in colonic section of tamoxifen-treated *Cx3cr1*^{CreER/WT}*H2-Ab1*^{WT/fl} (Cre:WT/fl) or *Cx3cr1*^{CreER/WT}*H2-Ab1*^{fl/fl} (Cre:fl/fl) mice (day 7 after infection) ($n = 6$ to 8). **(Q)** Numbers of *Salmonella*-specific IgA-producing cells as measured by ELISpot in the LB of tamoxifen-treated *Cx3cr1*^{CreER/WT}*H2-Ab1*^{WT/fl} (WT/fl) or *Cx3cr1*^{CreER/WT}*H2-Ab1*^{fl/fl} (fl/fl) mice (day 7 after infection) ($n = 8$ to 12). Graphs show means $\pm$ SEM and are representative or combination of at least two independent experiments. Statistical analysis: Student's *t* test or two-way ANOVA, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Fig. 7. Proposed model of spatial organization of mucosal *Salmonella*-specific IgA response induced by intestinal inflammatory Mφ subsets.

Three functionally distinct Mφ populations are present in the inflamed colon:

Nos2⁺CX3CR1^{lo} with the bactericidal gene expression signature, lymph-migratory *Ccr7*⁺CX3CR1^{int}, and mucosa-resident *Cxcl13*⁺CX3CR1^{hi} populations. In infectious colitis, they collectively induce *Salmonella*-specific IgA response in two locations: MLNs where CX3CR1^{int} Mφs migrate and locally within the mucosa via formation of TLSs driven by CX3CR1^{hi} Mφs.