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COLONIC SILT MATURATION IS DEPENDENT ON AN INTACT CASPASE1-IL1R-MYD88 SIGNALING AXIS

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ABSTRACT

The mammalian gastrointestinal tract harbors a large community of symbiotic microorganisms, which is essential for the host’s fitness, but that must be tightly controlled at all times. Such control is provided by a vast array of lymphoid tissues present within the intestinal wall. In this study, we show that the maturation of colonic SILTs is dependent on caspase1 activation (IL1 processing), IL1R signaling and MyD88. These molecules regulated a chain of events that culminated in the accumulation of NKp46+ cells in colonic SILTs. The presence of NKp46+ cells correlated with BAFF expression, which in turn allowed the accumulation and organization of B cells in the B cell follicles that characterize mature colonic SILTs. Together, these data implicate, for the first time, the inflammasome and NKp46+ cells in the process of lymphoid tissue development.
INTRODUCTION

The gastrointestinal tract is home to a large community of symbiotic microorganisms. These microorganisms perform multiple functions for the host, such as digesting food, providing essential nutrients such as vitamins, and preventing colonization by pathogenic bacteria; in return, they receive from the host a sheltered environment stable in temperature and rich in nutrients. In spite of their symbiotic nature, however, commensal microorganisms retain the potential to become themselves pathogenic and therefore must be constantly kept in check by the mucosal immune system.

The organized lymphoid tissues present within the intestinal wall include Peyer’s patches, colonic patches and solitary isolated lymphoid tissues (SILTs). Collectively, they provide protection to the host by collecting antigens directly from the intestinal lumen and inducing appropriate immune responses against them, including IgA synthesis. In mice, Peyer’s patches and colonic patches develop during the embryonic period under sterile conditions. In contrast, SILTs, which consist of dynamic lymphoid clusters ranging from small accumulations of lineage-negative lymphoid tissue inducer (LTi) cells and CD11c+ dendritic cells known as cryptopatches to large aggregates of LTi cells, CD11c+ dendritic cells and B cells known as isolated lymphoid follicles (ILFs), develop within the first weeks after birth concurrently with the onset of microbial colonization.

In agreement with the differential temporal windows in which the development of these tissues occurs, i.e. either before or after birth, microbial stimulation seems to influence SILT, but not Peyer’s patch or colonic patch development. In the small intestine, bacterial recognition via the innate immune receptor nucleotide-binding oligomerization domain containing 1 (NOD1) drives the transition from cryptopatches to immature ILFs by inducing the production of CCL20 and β-defensin 3, which in turn recruit CCR6+ B cells. The subsequent transition from immature to mature ILFs, i.e. the organization of B cell follicles, further depends on the recognition of bacteria by toll-like receptors (TLR) 2 and 4 and signaling via the adaptor molecule MyD88. Such a detailed picture on how microbial stimulation might influence SILT development in the colon is currently not available. This is largely due, on the one hand, to the lack of studies directly assessing lymphoid tissue development in the colon; and, on the other hand, to the presence of contradictory evidence in the literature. Recent studies have either reported a non-redundant role for the intestinal microbiota in the promotion of colonic SILT maturation similar to that observed in the small intestine; or, alternatively, described colonic SILT maturation to progress independently of the intestinal microflora by comparing germ-free and antibiotic-treated mice to mice maintained in SPF conditions. In spite of these contradictions, MyD88 is a crucial player in the transition from immature to mature colonic ILFs.

Lymphoid tissue development critically depends on RORγt+-dependent LTi cells. The role of the gut microbiota in the development of RORγt+ innate lymphoid cells (ILCs) is currently unclear. Conclusive evidence that RORγt+ ILCs develop independently of the microbiota can be easily inferred from the presence of LTi cells and the generation of lymphoid tissues in the sterile environment of fetuses before birth. Regarding their post-natal development, however, while some groups reported a substantial reduction in the numbers of NKp46+RORγt+ cells in the absence of microbiota, others reported a normal development of all RORγt+ ILC subsets in both germ-free and antibiotic-treated mice. Such contradictory data is likely to have arisen...
from the critical role of adaptor molecule MyD88 in the functional differentiation of RORγt+ ILCs. As MyD88 transduces signals from TLRs as well as the IL1 receptor, it is often difficult to discriminate between bacterial and cytokine stimulation. In this context, the discrepancies described above regarding the role of commensal microorganisms in colonic SILT maturation are not entirely surprising.

In the present study, we readdressed the role of MyD88 in colonic SILT development. We confirmed the crucial role of MyD88-derived signals in colonic SILT maturation and placed it downstream of caspase1 activation (IL1 processing) and IL1R signaling. The requirements for caspase1, IL1R and MyD88 in colonic SILT development converged into BAFF expression and NKp46+ cell development. In the absence of either caspase1, IL1R or MyD88, colonic BAFF expression was reduced and the numbers of colonic SILT NKp46+ cells diminished. Given that BAFF expression and colonic SILT NKp46+ cells, whose human equivalents were recently shown to produce BAFF, were spatially associated in B cell follicles, we propose NKp46+ cells to contribute to the organogenesis of colonic SILTs by promoting B cell homeostasis.

**RESULTS**

**Impaired colonic SILT maturation in the absence of IL1R**

Recently, we reported a role for the adaptor protein MyD88 in the developmental maturation of colonic SILTs, which was independent of the intestinal commensal microflora. Specifically, accumulation of B cells within colonic SILTs was severely impaired in the absence of MyD88 signalling (MyD88−/− mice), but progressed normally in both germ-free and antibiotic-treated mice. Overall, this suggested that Toll-like receptor (TLR) signalling was not directly involved in colonic SILT maturation and that other MyD88-dependent mechanisms were at play. As MyD88 also conveys signals with origin on the IL1 receptor (IL1R), we hypothesized that colonic SILT maturation would be arrested in the absence of IL1R signalling. Supporting our hypothesis, IL1R−/− mice, similarly as to MyD88−/− mice, showed a reduced number of mature colonic SILTs, i.e. immature as well as mature isolated lymphoid follicles (ILFs) (fig.1a). In contrast, the number of immature colonic SILTs, i.e. colonic cryptopatches, was not affected by IL1R deficiency. This led to a highly distorted representation of the different SILT maturation stages within the colon, with cryptopatches representing a much larger fraction of the total colonic SILTs in IL1R−/− mice as compared to wild-type mice (fig. 1b). Altogether, these data suggested that colonic SILT maturation is dependent on IL1R signalling.
Importantly, however, the remaining IL1R-deficient mature ILFs were normally formed (fig. 1e).

Similarly to mature ILFs in wild-type (fig. 1c) and MyD88−/− (fig. 1d) mice, IL1R−/− mature ILFs had a central well-defined B cell follicle that contained CD35+ follicular dendritic cells; CD3−CD4−IL7R+ LTi cells and CD11c+ dendritic cells concentrated at the periphery of the ILF in a “ring-like” pattern; few CD3+ T cells sparsely distributed and not forming separate T cell areas; and VCAM1+gp38+ stromal cells present throughout their parenchyma (fig. 1e).

Impaired colonic SILT maturation in the absence of Caspase1

The cytokines of the IL1 family are synthesized as cytosolic precursors, which require processing by caspase1, in order to be secreted and become functionally active30. Hence, to confirm our data on the role of IL1R in colonic SILT development, we evaluated the development of colonic SILTs in caspase1-deficient mice. Similarly as to IL1R−/− mice, caspase1−/− mice had reduced numbers of both immature and mature colonic ILFs (fig. 1a). This, in concert with preserved numbers of colonic cryptopatches (fig. 1a), led to an abnormal representation of the different SILT maturation stages within the colon of caspase1−/− mice as compared to wild-type mice (fig. 1b).

Importantly, similarly to wild-type, MyD88−/− and IL1R−/− mice, the few mature ILFs that developed within the colon of caspase1−/− mice were normally shaped (fig. 1f). Caspase1-deficient mature ILFs had well-defined B cell follicles containing CD35+ follicular dendritic cells, which were surrounded by a ring of CD3−CD4−IL7R+ LTi cells and CD11c+ dendritic cells. The few CD3+ T cells present within mature ILFs were sparsely distributed among their parenchyma of VCAM1+gp38+ stromal cells.

Taken together, these data suggest that the proteolytic processing of IL1 family members by caspase1 is required for the maturation of colonic SILTs. In agreement with the data from IL1R−/− mice, caspase1-mediated processing of IL1 and not IL18 seems to be the essential prerequisite to the development of mature colonic SILTs.

Reduced accumulation of B cells in colonic SILTs is associated with reduced colonic BAFF expression

To gain insight into which caspase1/IL1R/MyD88-dependent mechanism might lead to B cell accumulation into colonic SILTs and therefore to their maturation, we compared the expression of gene transcripts potentially involved in B cell attraction, retention or survival between whole colon samples collected from day0 and day14 wild-type, MyD88−/−, IL1R−/− and caspase1−/− mice by real-time PCR.

First, we assessed the expression of transcripts for chemokines known to influence B cell migration. CXCL12, CXCL13, CCL20 and CCL21 mRNA levels were comparable between wild-type, MyD88−/−, IL1R−/− and caspase1−/− colons (fig. S1a). In contrast, CCL19 mRNA expression was significantly reduced in MyD88−/−, IL1R−/− and caspase1−/− colons as compared to wild-type colons at day 14 (fig. S1a). However, as CCL19 and CCL21 share the same chemokine receptor (CCR7), it is unlikely that defective CCR7-mediated B cell migration would contribute to defective B cell accumulation within the colonic SILTs of MyD88−/−, IL1R−/− and caspase1−/− mice. Altogether, these data suggest that B cell migration into and within the colon is per se not defective in the absence of IL1/IL18 processing and IL1R signalling.

Next, we assessed the expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Their mRNA levels were comparable
between all mice groups (fig. S1b), further suggesting that migration as well as retention of B cells within the colon of MyD88<sup>-/-</sup>, IL1R<sup>-/-</sup> and caspase1<sup>-/-</sup> mice is not disturbed.

Finally, we assessed the expression of transcripts for cytokines known to influence B cell survival and differentiation. At day 14 post-partum, colonic mRNA expression of a proliferation-inducing ligand (APRIL) was significantly reduced in IL1R<sup>-/-</sup> and caspase1<sup>-/-</sup> mice as compared to wild-type mice (fig. 2a). MyD88<sup>-/-</sup> mice, however, did not show the same trend (fig. 2a). Colonic B cell activating factor belonging to the TNF family (BAFF) expression, on the other hand, was reduced in all knockout mice as compared to wild-type mice (fig 2a). Furthermore, we found colonic BAFF mRNA levels, but not colonic APRIL mRNA levels, at day 14 post-partum, to significantly correlate with the colonic mRNA expression of the B cell-specific molecule CD19.
Taken together, these data suggest that impaired B cell accumulation within the colonic SILTs of mice with defective IL1 processing and signalling is likely due to impaired B cell survival caused by reduced BAFF expression within the colon.

**Colonic BAFF expression is restricted to colonic lymphoid tissues, where it becomes concentrated in the B cell follicles.**

To characterize colonic BAFF expression, we combined immunofluorescence and flow cytometry analysis. By immunofluorescence, we determined BAFF to be expressed exclusively in the lymphoid tissues of the colon (figs. 3a). BAFF was expressed throughout the entire lymphoid tissue; however, in keeping with its role in B cell homeostasis, it appeared to be present at higher levels in the B cell follicles of colonic patches (fig. 3a) and, in colonic SILTs, it was only evident when B cells were present and clustered (fig. 3a and data not shown). Importantly, these expression patterns were preserved in MyD88−/−, IL1R−/− and caspase1−/− mice (fig. S2). By flow cytometry, we

![Image](image-url)

**Figure 3. Colonic BAFF expression is restricted to CD45− and CD45+ cells present within the colonic lymphoid tissues.**

a. Immunofluorescence characterization of colonic patches and colonic solitary intestinal lymphoid tissues (SILT) present in the colon of 14 days-old wild-type mice, stained for CD4 (green), BAFF (red) and B220 (blue). Scale bars=100μm and 50μm for colonic patches and colonic SILTs, respectively; n=4.

b. Flow cytometrical characterization of BAFF expressing cells in the colon of 14 days-old wild-type mice. Representative contour plots of two independent experiments of 6-pooled colons each. The numbers on the plots represent the percentage of cells within the gates.
Figure 4. Reduced frequency of NKp46+ cells within MyD88−/−, IL1R−/− and Caspase1−/− colonic SILTs. Immunofluorescence characterization of colonic patches (a) and colonic SILTs (b) present in the colon of 14 days-old wild-type (wt), MyD88−/−, IL1R−/− and Caspase1−/− mice. Sections were stained for CD4 (green) and NKp46 (red). Scale bars=100μm and 50μm for colonic patches and colonic SILTs, respectively. n=4 wild-type mice, n=4 for MyD88−/− mice, n=3 for IL1R−/− mice and n=3 for Caspase1−/− mice.
determined BAFF to be expressed by both CD45+ hematopoietic cells as well as CD45- stromal cells (fig. 3b). Further phenotypic analysis of BAFF-expressing cells was, however, precluded due to the low frequencies of BAFF+ cells present within the colon (fig. 3b).

**Caspase1/IL1R/MyD88-dependent colonic SILT NKp46+ cells**

The functional differentiation of NKp46+ innate lymphoid cells (ILCs) in mice as well as in human was recently found to depend on IL1-IL1R-MyD88 signaling\(^{23, 29}\). Importantly, NKp46+ ILCs differentiate from LTi cells\(^{22}\), suggesting that they may retain some of the lymphoid tissue inducer properties of their precursors. We, therefore, hypothesized that BAFF-expressing NKp46+ cells could be involved in the organogenesis of colonic SILTs, namely in B cell maintenance and organization within these lymphoid tissues. To test this hypothesis, we characterized NKp46 expression in the colonic lymphoid tissues of wild-type, MyD88-/-, IL1R-/- and Caspase1-/- mice (fig. 4). In support of our proposition, we found NKp46+ cells to concentrate in the B cell follicles in wild-type colonic patches (fig. 4a) as well as wild-type colonic SILTs (fig. 4b). Indeed, in wild-type colonic SILTs, similarly as to BAFF expression, infiltrating NKp46+ cells were only found when B cells were also present (data not shown). NKp46+ cells were present in the B cell follicles of MyD88-/-, IL1R-/- and Caspase1-/- colonic patches (fig. 4a); this was in agreement with the general absence of any discernible colonic patch phenotype in these mice (fig. S3). In marked contrast, however, NKp46+ cells seemed to be mainly absent from their SILTs (fig. 4b). Altogether, our data suggest a role for the Caspase1/IL1R/MyD88 axis in the differentiation of colonic lamina propria NKp46+ cells, but not colonic patches’ NKp46+ cells. More importantly, they support a previously unrecognized role for NKp46+ cells in the organogenesis of lymphoid tissues.

**DISCUSSION**

Secondary lymphoid tissues develop at predetermined locations throughout the body as a result of a series of highly coordinated interactions between hematopoietic, mesenchymal and endothelial cells that must take place during a narrow temporal window, which varies depending on the particular lymphoid tissue\(^{31-33}\). In the murine intestine, such cellular interactions take place either during embryogenesis in the case of Peyer’s patch and colonic patch development or within the first few weeks after birth in the case of SILT development\(^{5-13}\). Importantly, the concurrent onset of microbial colonization and SILT formation has led researchers to evaluate the influence of commensal microorganisms on SILT development. Regarding the small intestine, it is now consensual that microbial stimulation promotes SILT maturation\(^{11-14}\); bacterial components must be sequentially recognized by the innate immunity receptors NOD1 and TLR2/4 in order to drive the transition from cryptopatches to immature ILFs and from immature towards mature ILFs, respectively\(^{13}\). In contrast, in the colon, despite the recognized role of the adaptor molecule MyD88 in driving SILT maturation\(^{5, 13}\), contradictory results regarding the influence of microbial recognition in this process still persist\(^{5, 13, 34}\). In the present study, we have readdressed the role of MyD88 in colonic SILT maturation. We show that the crucial role of MyD88 in colonic SILT maturation is located downstream of caspase1-mediated processing of IL1 family members and IL1R signaling. The requirements for caspase1, IL1R and MyD88 converged on BAFF expression,
which, consistent with its role in B cell homeostasis\textsuperscript{35}, was predominantly found within B cell follicles, and NKp46\textsuperscript{+} cell development. Given that NKp46\textsuperscript{+} ILCs, whose human equivalents were recently found to produce BAFF\textsuperscript{29}, were also located in B cell follicles and absent from caspase1-, IL1R- and MyD88-deficient SILTs, we propose NKp46\textsuperscript{+} cells to contribute to SILT development by regulating B cell homeostasis within the colonic lamina propria.

As previously mentioned, despite the recognized role for the adaptor molecule MyD88 in colonic SILT maturation\textsuperscript{5, 13}, the upstream origin of the signals transduced by it remains controversial. While some authors have reported a role for commensal microorganisms and TLR signaling in the development of colonic ILFs from colonic cryptopatches\textsuperscript{13}, we have previously shown in both germ-free as well as antibiotic-treated mice that microbial stimulation did not influence such transition\textsuperscript{5}. Such contradictory results may have arisen, on the one hand, from differences in the age of the mice analyzed in these studies (adult\textsuperscript{13} vs. 2 week-old mice\textsuperscript{5}), differences in the composition of their microbiota and/or differences in their diet. On the other hand, however, they might have emerged from the dual role of MyD88 as an adaptor molecule in TLR as well as IL1R signaling\textsuperscript{28}. In this context, the analyses of caspase1\textsuperscript{-/-} and IL1R\textsuperscript{-/-} mice presented in this study, represent an important advance by providing conclusive evidence of the major role of IL1 processing and IL1R signaling in colonic SILT maturation, respectively.

The requirements for caspase1, IL1R and MyD88 in colonic SILT maturation were neither associated with disturbed chemokine nor adhesion molecule expression, suggesting that neither defective attraction nor defective retention of B cells within colonic SILTs was the underlying cause of their developmental arrest in Caspase1\textsuperscript{-/-}, IL1R\textsuperscript{-/-} and MyD88\textsuperscript{-/-} mice. Instead, they converged on the expression of the B cell survival factor BAFF. BAFF expression was reduced in Caspase1\textsuperscript{-/-}, IL1R\textsuperscript{-/-} and MyD88\textsuperscript{-/-} mice as compared to wild-type mice and at 2 weeks of age strongly correlated with the accumulation of the B cell specific transcript CD19 in the colon. Furthermore, in keeping with its role in B cell homeostasis\textsuperscript{35}, BAFF was expressed only in colonic SILTs containing B cell follicles. SILTs support T cell-independent B cell activation, somatic hypermutation and class switching towards IgA-producing plasmablasts\textsuperscript{18}. As colonic SILTs fail to form B cell follicles in the absence of either caspase1, IL1R or MyD88, it is therefore not surprising that caspase1\textsuperscript{-/-}, IL1R\textsuperscript{-/-} and MyD88\textsuperscript{-/-} mice have poor IgA responses\textsuperscript{36, 37}, which predispose them to both viral and bacterial mucosal infections\textsuperscript{36, 38-40}.

The requirements for caspase1, IL1R and MyD88 in colonic SILT maturation were also associated with NKp46\textsuperscript{+} cells, which in wild-type colonic SILTs co-localized with B cell follicles. In the absence of either caspase1, IL1R or MyD88, the few mature colonic ILFs that developed were almost completely devoid of NKp46\textsuperscript{+} cells. Supporting a role for these cells in the formation of B cell follicles, the human equivalents of mouse NKp46\textsuperscript{+} ILCs, ILC22 cells, were recently found to produce BAFF\textsuperscript{29}. Cytokine production by NKp46\textsuperscript{+} ILCs is driven by an IL1R-MyD88 pathway independent of commensal microorganisms\textsuperscript{23}. Taken together, as NKp46\textsuperscript{+} ILCs differentiate from ROR\textgreekgamma\textsuperscript{+} LTi cells\textsuperscript{22}, these cumulative evidences suggest that NKp46\textsuperscript{+} cells retain lymphoid tissue inducer properties, namely in the organization of B cell follicles. NKp46\textsuperscript{+} cell differentiation per se, however, can occur independently of MyD88\textsuperscript{23} as well as of IL1R and caspase1, as evidenced by their presence in MyD88\textsuperscript{-/-}, IL1R- and caspase1-deficient colonic patches. On the one hand, this suggests that the requirements for NKp46\textsuperscript{+} cell differentiation may differ between distinct
anatomical compartments, with caspase1 activation and IL1R and MyD88 signaling being necessary for NKp46+ cell differentiation in the colonic lamina propria, but not in the colonic submucosa. On the other hand, it may mean that NKp46+ cells in colonic SILTs and colonic patches are developmentally unrelated, i.e. NKp46+ type 3 ILCs vs. NKp46+ NK cells. Alternatively, it may also denote that attraction to and retention within the colonic SILTs requires caspase1, IL1R and MyD88. This is, however, less likely given that the overall expression of chemokines and adhesion molecules in the colon of caspase1−/−, IL1R−/− and MyD88−/− mice did not differ significantly when compared to wild-type colons.

Strikingly, recent reports have linked the RORγt+ ILC maintenance, SILT development and dietary components. Specifically, AHR ligands present in the diet were shown to promote the survival of RORγt+ ILCs and, with that promote the development of SILTs in the small intestine and colon. It is, therefore, tempting to speculate that such ligands could turn on the inflammasome and drive caspase1 activation, which would provide a mechanistic link on how AHR signaling drives SILT development. However, AHR signaling is known to induce the expression of plasminogen activator inhibitor-2 (Pai-2), an inhibitor of caspase1 activation that prevents the processing of the cytosolic precursor forms of IL1β. Other dietary components, however, can activate the inflammasome and therefore additional signals provided by the diet are likely to drive further steps in colonic SILT development. Importantly, inflammasome and caspase1 activation are critical in intestinal epithelial cell repair, via the induction of the IL1 family member IL18. IL18 decreases the expression of the antagonistic protein IL22 binding protein (IL22BP), thereby increasing the ratio of IL22 to IL22BP and consequently the concentration of bioactive IL22. IL22, which is produced in large amounts by RORγt+ ILCs, is the effector cytokine of this pathway directly stimulating tissue repair mechanisms on intestinal epithelial cells. Taken together, these data conclusively support a role for inflammasome activation in the regulation of RORγt+ ILC function, which is critical to the homeostasis of the intestinal mucosa. With the current study, we expand the understanding on how the inflammasome and IL1 family members contribute to the homeostasis of the intestinal barrier by linking it to development of colonic SILTs. Given the crucial role of SILTs in intestinal IgA production, our data helps clarifying the emergence of dysbiosis and pathogenic microflora in mice deficient in several components of the inflammasome.

Importantly, the data presented and discussed in this study seems to apply exclusively to colonic SILTs, as colonic patches in caspase1−, IL1R− and MyD88-deficient mice did not exhibit any discernible defect. In all animals, they contained T and B cells, which were segregated into distinct compartments; T cell areas were infiltrated by conventional CD11c+ dendritic cells; B cell follicles contained CD35+ follicular dendritic cell networks and expressed BAFF; and non-hematopoietic gp38−VCAM-1+ stromal cells were distributed throughout the colonic patch parenchyma.

In conclusion, we described a chain of events starting with caspase1 activation, IL1R and MyD88-signaling that specifically drives colonic SILT maturation. These early events induce NKp46+ cell accumulation in colonic SILTs, which by virtue of BAFF production lead to the development of B cell follicles. Together, these findings extend our understanding of the crucial roles of the inflammasome and NKp46+ cells in the maintenance of intestinal homeostasis, by directly implicating both in lymphoid tissue formation.
MATERIALS AND METHODS

**Mice.** Wild-type C57BL/6, MyD88-/-, IL1R-/- and Caspase1-/- mice were bred and housed under SPF conditions. Animals were analyzed at day of birth (day 0) and at day 14 post-partum. All procedures were approved by the local Scientific and Ethics Committees.

**Immunofluorescence.** Colons for histological analysis were dissected at day 14 post-partum, embedded in OCT (Sakura Finetek) and snap frozen in liquid nitrogen. Frozen blocks were cut into 7μm thick serial sections. Detection of organized lymphoid tissues and SILT enumeration and classification was performed as previously described. Briefly, detection of colonic lymphoid tissues was performed on every 10th section; and SILT enumeration on every 50th section. These sections were stained with antibodies recognizing CD4, α smooth muscle actin (αSMA) and B220. Cryptopatches (CPs) were defined as clusters of CD4+ cells; immature isolated lymphoid follicles (ILFs) as clusters of CD4+ cells containing scattered B220+ cells; and mature ILFs as CD4+ cell clusters containing organized B220+ B cell follicles. Noteworthy, the procedure to enumerate colonic SILTs provides only a rough estimation of their number; and, it is likely to give a biased representation of their maturation status as small SILTs (cryptopatches) are more likely to be missed than larger SILTs when screening only every 50th section. Relevant immunofluorescence stainings were subsequently performed on the sections adjacent to those containing organized lymphoid tissues by incubation with the appropriate antibodies for periods of 45 minutes at room temperature. Pictures were taken on a DM6000 Leica immunofluorescence microscope (Leica Microsystems).

**Flow cytometry.** Colons for flow cytometrical analysis were dissected at day 14 post-partum from wild-type C57BL/B6 mice. Single-cell suspensions were prepared in a three step approach. First, the colons were cut open along their length and faeces washed away by rinsing with Ca2+/Mg2+-free Hank’s balanced salt solution (HBSS; Gibco); subsequently, the mucus was removed by 2 successive 15 minutes incubations at 37°C in Ca2+/Mg2+-free HBSS supplemented with 15mM HEPES, 5mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), 10% fetal calf serum (FCS; Sigma-Aldrich), 1% penicillin-streptomycin (Sigma-Aldrich), 0.1% dithiothreitol (DTT; Promega) and 0.1% β-mercaptoethanol (Sigma-Aldrich) interspersed with thorough washes in Ca2+/Mg2+-free HBSS supplemented with 15mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco) and 0.5% gentamycin (Sigma-Aldrich); finally, the colons were enzymatically digested for 20 minutes at 37°C with 0.2 mg.ml-1 Liberase 2 (Roche) and 0.15 mg.ml-1 DNAse I (Roche) in Ca2+/Mg2+-containing HBSS (Gibco) supplemented with 15mM HEPES and 1% FCS. Following digestion, the resulting cell suspensions were thoroughly washed, resuspended in PBS and filtered over 100μm nylon meshes. Extracellular stainings were performed on ice for 30 minutes in PBS/2%FCS/5mM EDTA; subsequently, cells were fixed, permeabilized and intracellularly stained on ice for 30 minutes in permeabilization buffer (eBioscience). Live/dead cells were discriminated by staining with the live/dead Near-IR fixable dead cell stain from Invitrogen, according to the manufacture’s instructions. Fluorescence minus one (FMO) control stain sets were used to determine the detection thresholds of each individual marker. Cells were analyzed on a Cyan ADP flow cytometer (DakoCytomation) with FlowJo software (TreeStar).

**Antibodies.** The following antibodies were used for immunofluorescence: protein G-Sepharose (Pharmacia) purified rat α-mouse B220 (clone 6B2) and rat α-mouse CD4 (GK1.5) directly labelled.
with AlexaFluor488 or AlexaFluor647 (Invitrogen), respectively; protein G-Sepharose (Pharmacia) purified rat α-mouse CD35 (8c12) and rat α-mouse CD127 (A7R34) indirectly developed with goat α-rat AlexaFluor555 (Invitrogen); purified mouse α-mouse alpha smooth muscle actin (αSMA; 1A4; Sigma-Aldrich) developed with goat α-mouse IgG2a AlexaFluor555 (Invitrogen); purified rat α-mouse VCAM-1 (429; BD Biosciences) developed with goat α-rat AlexaFluor555 (Invitrogen); unpurified supernatant containing hamster α-mouse gp38 (8.1.1) visualized with goat α-hamster AlexaFluor647 (Invitrogen); biotin-labelled α-mouse CD11c (N418; BioLegend) visualized with streptavidin conjugated to AlexaFluor647 (Invitrogen); and directly labelled α-mouse BAFF PE (121808, R&D systems), α-mouse CD3 eFluor660 (17A2; eBioscience) and α-mouse NKp46 eFluor660 (29A1.4, eBioscience). The antibodies used for flow cytometry were: α-mouse BAFF PE (121808, R&D systems) and α-mouse CD45 PE.Cy7 (30-F11; eBioscience).

mRNA isolation, cDNA synthesis and real-time-PCR. Colons for gene/transcript expression analysis by real-time PCR were dissected at days 0 and 14 post-partum and stored in Trizol (Gibco) at -80°C. RNA was isolated and cDNA synthesized as previously described5. Real-time PCR was performed on an ViiA7 Sequence Detection System (Applied Biosystems). Primer efficiency was corrected by running standard curves generated by serial dilution of pooled steady-state peripheral lymph nodes cDNA for each primer set in every run. The expression level of each transcript was normalized to the expression of selected housekeeping genes with geNORM v3.4 software.

Statistics. Statistical analysis was performed with GraphPad Prism version v.4.00 (GraphPad Software Inc.). Kruskal-Wallis analysis and Dunn’s multiple comparison tests were used to compare multiple groups; Mann-Whitney’s tests used to compare two groups; and Spearman’s correlations used to associate variables. For mRNA transcript analysis, the mean value for day 0 samples of each mouse genotype was arbitrarily set at 1 (the absolute values for these samples did not differ significantly for the different mice). Results are expressed as mean ± standard error of the mean (SEM). p values ≤ 0.05 were considered significant.

REFERENCES


CASPASE1-IL1R-MYD88-MEDIATED COLONIC SILT MATURATION


Figure S1. MyD88/IL1R/Caspase1-independent colonic chemokine and adhesion molecule expression. Chemokine (a) and adhesion molecule (b) mRNA expression in whole colon samples of wild-type (wt), MyD88−/−, IL1R−/− and Caspase1−/− mice at days 0 and 14 after birth. The expression level of each transcript in every sample was normalized to the expression of the housekeeping gene transcripts Cyclophilin and Ubiquitin. The mean mRNA expression level of all day 0 samples was arbitrarily set at 1. n = 3-6 mice per group. * p < 0.05, ** p< 0.01 *** p < 0.001 between the considered groups.
Figure S2. Colonic BAFF expression in MyD88<sup>-/-</sup>, IL1R<sup>-/-</sup> and Caspase1<sup>-/-</sup> mice is restricted to lymphoid tissues. Immunofluorescence characterization of colonic patches (a) and colonic solitary intestinal lymphoid tissues (SILTs; b) present in the colon of 14 days-old MyD88<sup>-/-</sup>, IL1R<sup>-/-</sup> and Caspase1<sup>-/-</sup> mice, stained for CD4 (green), BAFF (red) and B220 (blue). Scale bars=100μm and 50μm for colonic patches and colonic SILTs, respectively. n=4 for MyD88<sup>-/-</sup> mice, n=3 for IL1R<sup>-/-</sup> mice and n=3 for Caspase1<sup>-/-</sup> mice.
Figure S3. Caspase1/IL1R/MyD88-independent colonic patch development. Quantification (a) and histological characterization (b-e) of colonic patches in wild-type (wt), MyD88⁻/⁻, IL1R⁻/⁻ and Caspase1⁻/⁻ mice. Serial sections were stained for: (I) CD4 (green), αSMA (red) and B220 (blue); (II) CD4 (green), CD35 (red) and CD3 (blue); (III) CD4 (green), IL7Rα (red) and CD11c (blue); and (IV) CD4 (green), VCAM-1 (red) and gp38 (blue). Scale bars=100μm. n=4 wild-type mice, n=4 for MyD88⁻/⁻ mice, n=3 for IL1R⁻/⁻ mice and n=3 for Caspase1⁻/⁻ mice.