Inflammation-induced formation of fat-associated lymphoid clusters

Cécile Bénézech1,12, Nguyet-Thin Luu1,13, Jennifer A Walker2,13, Andrei A Kruglov3–5, Yunhua Loo6, Kyoko Nakamura1, Yang Zhang1, Saba Nayar1, Lucy H Jones7,12, Adriana Flores-Langarica1, Alistair McIntosh1, Jennifer Marshall1, Francesca Barone1, Gurdyal Besra8, Katherine Miles9, Judith E Allen7, Mohini Gray9, George Kollias10,11, Adam F Cunningham1, David R Withers1, Kai Michael Toellner1, Nick D Jones1, Marc Veldhoen6, Sergei A Nedospasov3–5, Andrew N J McKenzie2 & Jorge H Caamaño1

Fat-associated lymphoid clusters (FALCs) are a type of lymphoid tissue associated with visceral fat. Here we found that the distribution of FALCs was heterogeneous, with the pericardium containing large numbers of these clusters. FALCs contributed to the retention of B-1 cells in the peritoneal cavity through high expression of the chemokine CXCL13, and they supported B cell proliferation and germinal center differentiation during peritoneal immunological challenges. FALC formation was induced by inflammation, which triggered the recruitment of myeloid cells that expressed tumor-necrosis factor (TNF) necessary for signaling via the TNF receptors in stromal cells. Natural killer T cells (NKT cells) restricted by the antigen-presenting molecule CD1d were likewise required for the inducible formation of FALCs. Thus, FALCs supported and coordinated the activation of innate B cells and T cells during serosal immune responses.

The peritoneal and pleural cavities support rapid immune responses when the integrity of the intestine or the lungs is compromised or lost. They contain innate-like B cell populations that produce natural antibodies vital for the early control of infection and provide protection against autoimmunity and to adapt immunity1–7. These B-1 cells recirculate between the peritoneal space and the omentum8, a sheet of intra-abdominal adipose tissue (AT) containing lymphoid structures called ‘milky spots’9–12. When peritoneal inflammation occurs, the number and size of milky spots increase and the recruitment of lymphocytes and macrophages that phagocytose particles and pathogens is substantially augmented9,11,12. The omentum also acts as a secondary lymphoid structure that promotes immunity to peritoneal antigens10,12.

The existence of B cell–rich clusters in AT has been extended to the rest of the visceral fat in the peritoneal and pleural cavity11,14. These have been called ‘fat-associated lymphoid clusters’ (FALCs)14. Their existence is associated with the presence of group 2 innate lymphoid cells (ILC2 cells)14–17 in visceral AT; yet no direct evidence has shown that ILC2 cells induce the formation of FALCs14. The exact composition of these clusters and their relative distribution in AT, as well as their function and the mechanisms that regulate their formation, remain unknown.

Here we found that the distribution of lymphoid structures in AT was very heterogeneous, with the omentum, the pericardium and mediastinum being the tissues that contained the largest number of FALCs. We report that the development of FALCs was regulated by unique cellular and molecular mechanisms that, in contrast to the mechanisms used by other secondary lymphoid tissues, did not involve lymphoid tissue–inducer (LTi) cells, ILC3 cells or the lymphtoxin β-receptor (LTβR) pathway18–20. Their postnatal formation was partly dependent on signaling via the tumor-necrosis factor receptors (TNFRs) and the presence of the commensal flora. FALC stromal cells had high expression of the chemokine CXCL13 that was crucial for the recruitment and retention of B cells in the clusters. Inflammation-induced formation of FALCs required TNF expression by myeloid cells and TNFR signaling in stromal cells. Peritoneal immunization with T cell–independent and T cell–dependent antigens induced the differentiation of B cells into plasma cells and germinal center (GC)–like B cells in FALCs, indicative of an important function for these clusters during immune responses. Finally, we found a key role for antigen-presenting molecule CD1d–restricted natural killer T cells (NKT cells), a subset of T cells for which AT shows enrichment, and interleukin 13 (IL-13) in the inflammation-induced formation of FALCs.
RESULTS
Visualization and characterization of FALCs
Whole-mount immunofluorescence staining of the main visceral AT, and a fluorescence stereomicroscope, allowed the visualization (Fig. 1a) and quantification (Fig. 1b) of CD45+ cell clusters present in the omental, gonadal, mesenteric, mediastinal and pericardial fat. In the peritoneal cavity, the omentum was the fat depot with the highest density of lymphoid clusters (8,000 clusters per gram), with a mean of 80 milky spots per omentum (Fig. 1b). The mesenteric fat depot contained a median of 120 clusters per gram, with a mean of 16 clusters per mesentery, while gonadal AT had 8 clusters per gram, with a mean of 1–2 clusters per depot (Fig. 1b). In the pleural cavity, the pericardium had the highest density of lymphoid clusters (5,400 clusters per gram), with a mean of 40 clusters per tissue (Fig. 1b). The mediastinum, with a density of 2,100 clusters per gram and a mean of 9 clusters per mediastinum, accounted for the rest of the FALCs in the pleural cavity (Fig. 1b). This analysis revealed the considerable heterogeneity in the lymphoid cluster content of ATs.

We characterized the cellular composition of the clusters by whole-mount immunofluorescence staining of mouse mesenteric tissues with antibodies specific for the T cell–specific coreceptor CD4, the hematopoietic marker CD45, immunoglobulin M (IgM) and the common myeloid marker CD11b (integrin αMβ2), followed by confocal microscopy. In resting conditions, FALCs were composed mostly of IgM+ B cells, with low numbers of CD4+ T cells and CD11b+ myeloid cells (Fig. 1c and Supplementary Fig. 1). We observed clusters of various sizes and degrees of B cell–T cell segregation in individual mice (Supplementary Fig. 1). A fraction of B cells expressed CD11b and had abundant surface IgM (data not shown), which indicated that these were probably B-1 cells. We did not detect any difference in the hematopoietic composition of FALCs from the mesentry, mediastinum or pericardium that resembled the milky spots of the omentum (data not shown). FALCs seemed to be highly vascularized, as shown by the presence of CD31+ blood vessels (Fig. 1d). However, we were unable to detect any distinct connection with Lyve-1+ lymphatic vasculature (Fig. 1d). Only scattered Lyve-1+ macrophages were present in close proximity to FALCs (Fig. 1d).

We used mesenteric ATs, in which FALCs were easier to identify and quantify, for the rest of our study. FALC formation was initiated after birth, with the first clusters identified in the mesenteric tissues of 2- to 3-week-old mice, and their number increased to reach a plateau at around 18 weeks of age (Fig. 1e). These results showed that FALCs were present in various visceral ATs in variable numbers and contained mainly B cells, with low numbers of T cells and myeloid cells.

Figure 1 Distribution of FALCs in visceral AT. (a) Whole-mount immunofluorescence staining of mesenteric tissues for visualization of CD45+ FALCs (green). Original magnification, ×4. (b) Density of hematopoietic clusters in the main fat deposits of the peritoneal cavity (omentum AT (n = 8 mice), gonadal AT (n = 7 mice) and mesenteric AT (n = 6 mice)), the pleural cavity (mediastinal AT (n = 13 mice) and pericardial AT (n = 8 mice)), and in subcutaneous fat (n = 7 mice). (c) Whole-mount immunofluorescence staining of a mesenteric FALC with CD11b+ myeloid cells (blue), CD45+ hematopoietic cells (green), IgM+ B cells (red) and CD4+ T cells (white). Scale bar, 50 μm. (d) Whole-mount immunofluorescence staining of a mesenteric FALC with CD45+ hematopoietic cells (green), CD31+ blood endothelial cells (red) and Lyve-1+ cells (blue). Scale bar, 50 μm. (e) Quantification of mesenteric clusters of newborn mice (NB) and mice 1–32 weeks of age (n = 5, 5, 5, 7, 18, 7 or 7 mice per group, left to right). Each symbol (b,e) represents an individual mouse; small horizontal lines indicate the mean. Data are representative of two independent experiments with eight clusters from four mice (a,c,d) or three independent experiments (e) or are pooled from two independent experiments (b).

High expression of CXCL13 by stromal cells in FALCs
To understand how B cells and T cells were recruited to FALCs, we used quantitative PCR to assess the expression of genes encoding the homeostatic chemokines CXCL13, CCL21 and CCL19 and the cytokine IL-7 in isolated clusters. Cxcl13 expression was higher in FALCs than in the associated AT (>100-fold) or lymph nodes (LNs) (10-fold) (Fig. 2a). In contrast, FALCs did not show enrichment for Ccl21, Ccl19 and Il7 transcripts relative to their abundance in the other tissues analyzed (Fig. 2a). In agreement with those findings, the number and composition of FALCs in CCL21- and CCL19-deficient mice (of the plt/plt (‘paucity of lymph node T cells’) strain) and Cerf7−/− mice showed no difference relative to that in their wild-type counterparts (Fig. 2b,c). Transcripts encoding TNF and lymphotoxin-β both had high expression in FALCs (Fig. 2b), which might have reflected the considerable abundance of B cells in these clusters.

Immunofluorescence staining revealed the presence of CD45+CXCL13+ stromal cells with an elongated morphology (Fig. 2d) that resembled follicular dendritic cells. Signaling via LTβR and/or TNFR is involved in inducing Cxcl13 expression in spleen and LN follicular dendritic cells21–23. However, CXCL13+ cells were present in FALCs of Lbr−/− mice, mice doubly deficient in TNFR1 and TNFR2 (Tnfrsf1a−/− Tnfrsf1b−/−) and lymphocyte-deficient Rag2−/− mice (Fig. 2d), which demonstrated that CXCL13 expression by FALC stromal cells was independent of signals induced by these receptors and the presence of lymphocytes24,25.

To determine whether CXCL13 expression was required for the recruitment of B cells into FALCs, we analyzed mice deficient in CXCR5, the receptor for CXCL13. A normal number of FALCs formed in the mesenteric tissues of these mice, but the number of B cells present in the clusters was markedly reduced (Fig. 2b,c). Thus, CXCL13 was not important for FALC formation but was essential for the recruitment of B cells. These data were consistent with reports...
Figure 2  CXCL13 expression by FALC stromal cells is necessary for B cell recruitment. (a) Real-time PCR analysis of Cxcl13, Tnf, Ccl21, Ltbr (encoding lymphoquotin-β), Ccl19 and Il7 mRNA in LNs (n = 5 mice), mesenteric FALCs (n = 4 mice) and adjacent fat (n = 5 mice) of wild-type mice; results are presented as the mean of technical duplicates of the ratio of the gene of interest to the control gene Actb. (b) Quantification of mesenteric clusters in wild-type (WT) mice (n = 7), plt/plt mice (n = 6), Ccr7−/− mice (n = 6) and Cxcr5−/− mice (n = 6). (c) Whole-mount immunofluorescence staining of FALCs from mice as in b, with CD11b+ myeloid cells (blue), CD45+ hematopoietic cells (green), IgM+ B cells (red) and CD4+ T cells (white). Scale bar, 50 μm. (d) Whole-mount immunofluorescence staining of mesenteric FALCs from wild-type, Ltbr−/−, Tnfrsf1a−/− Tnfrsf1b−/− and Rag2−/− mice (left margin), with CD45+ hematopoietic cells (green) and CXCL13+ stromal cells (red). Outlined areas at left are enlarged 2x at right. Scale bar, 50 μm. Each symbol (a,b) represents an individual mouse; small horizontal lines indicate the mean. NS, not significant; *P < 0.05 and **P < 0.01 (Mann-Whitney nonparametric two-tailed test). Data are representative of two independent experiments (a,b) or at least two independent experiments with eight clusters from four mice (c,d).

showing that mesenteric and omentum tissues are important sources of CXCL13 required for the retention of peritoneal B cells. Overall, we demonstrated that the presence of CXCL13 in the mesenteric tissues was due to its specific expression by FALC stromal cells and not by the surrounding visceral AT.

FALCs support the proliferation and differentiation B of cells

B-1 cells are important for immune responses to T cell–independent antigens25,36–31. To assess B cell responses in FALCs, we used the T cell–independent antigen NP-Ficoll (4-hydroxy-3-nitrophenylacetyl (NP) conjugated to the hydrophilic polysaccharide Ficoll) to immunize QM/QM (IghNPNP/Igk−/−) mice, which have a quasi-monoclonal primary B cell repertoire specific for NP32. Flow cytometry of the proliferation marker Ki67 revealed that both B-1 cells and B-2 cells were proliferating in the mesenteric tissues 24 h after intraperitoneal immunization (Supplementary Fig. 2a,b). The frequency of Ki67+ (proliferating) B-1 cells was three times higher in the mesenteric tissues than in the peritoneal cavity lavage or the spleen and reached 70% for CD5+CD11b+ B-1a cells, 50% for CD5−CD11b+ B-1b cells and 40% for CD5−CD11b− B-1c cells (Supplementary Fig. 2a,b). Up to 40% of B-2 cells also proliferated in response to NP-Ficoll (Supplementary Fig. 2a,b). Whole-mount staining of the mesenteric tissues 24 h after immunization showed considerable enlargement of FALCs and an increased frequency of IgM+ cells (Supplementary Fig. 2c).

To confirm that FALC B cells secreted IgM, we quantified antibody-forming cells in QM × C57BL/6 (B6) (IghNPNP/Igk−/−) mice33, in which 5% of the total B cell population is NP-specific, at 4 d after intraperitoneal immunization with NP-Ficoll. Enzyme-linked immunospot assays confirmed the population expansion of NP-specific B cells expressing IgM in the immunized mice (Fig. 3a, top), as well as a significantly greater number of IgG+ antibody-forming cells in the FALCs and spleen of immunized mice than in that of PBS-treated mice (Fig. 3a, bottom, and b). We obtained further evidence of immunoglobulin class switching in FALCs by immunization of C57BL6J/Cr(Cre × QM)ACTB−/−tdTomato,EGFP mice (IghNPNP/CrCreIgk−/−ACTB−/−tdTomato,EGFP) with NP-Ficoll. These mice carry one copy of the gene encoding Cre recombinase in the region encoding constant region Cγ1 in the immunoglobulin heavy-chain locus (Igh), one copy of the gene encoding NP-specific immunoglobulin heavy chain from QM mice, and the gene encoding the reporter mTmG (membrane Tomato membrane Green). Immunization of these mice with NP-Ficoll induced B cell activation and immunoglobulin class switching with concomitant expression of Cre recombinase that resulted in switching of the membrane reporter from IgM (red) to IgG (green). Whole-mount immunofluorescence analysis revealed that immunization with NP-Ficoll induced the appearance of clusters of class-switched (green) B cells in the mesenteric tissues (Fig. 3b). The early and ‘preferential’ proliferation of B-1 cells in FALCs, as well as the presence of IgM- and IgG-producing cells, demonstrated that these clusters contributed to peritoneal cavity B-1 cell immune responses to type II antigens.

We assessed the role of FALCs in T cell–dependent immune responses by adoptive transfer of peritoneal lavage cells from QM/QM(Gt(Rosa)26SorEYFP/+ mice (QM/QM mice heterozygous for the gene encoding enhanced yellow fluorescent protein (eYFP)) into C57BL/6 recipients that we subsequently immunized by intraperitoneal injection of alum-precipitated NP-ovalbumin (NP-OVA). Flow cytometry of cells isolated 8 d after immunization showed a significantly greater frequency and number of antigen-specific B cells and CD38+ GL7+ GC-like B cells in FALCs, spleen and mesenteric LNs (mLNs) of mice immunized with NP-OVA than in that of their counterparts treated with PBS (Fig. 3c,d and data not shown). An endogenous immune response was elicited by intraperitoneal immunization of C57BL/6J mice with alum-precipitated phycoerythrin. In agreement with the results reported above, flow cytometry of cells from the FALCs, spleen and mLNs showed a 100-fold greater proportion and number of phycoerythrin-specific B cells and GC-like B cells in mice immunized with phycoerythrin than in their PBS-treated counterparts (Fig. 3e,f and data not shown). Together these results indicated that FALCs functioned as lymphoid tissues by supporting early activation of B cells during inflammation and differentiation toward plasma cells and GC cells during adaptive immune responses in the peritoneal cavity.

Cells and signals required for FALC development

The development of LNs and Peyer’s patches is dependent on the activity of LTδ1 cells and engagement of LTβR18–20. To determine whether the formation of FALCs followed the same cues, we assessed their presence in mouse strains deficient in LTβR (Ltbr−/−), lymphoquotin-α (Ltα−/−) and the transcription factor RORγt (Rorc−/−), in which LN development is fully impaired34–36. The number and composition of FALCs in these strains was identical to that in wild-type mice.

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Representative of (QM/QM) isolated from the mesenteric tissues (Mes), spleen and mLN of C57BL/6J host mice given intravenous injection of peritoneal-lavage cells from NP-Ficoll (which results in the generation of IgG1-switched B cells (green; arrows) in FALC). Original magnification, ×4. (as specific cells per 1 × 10⁶ total cells. (formation, we analyzed whether any other lymphoid cell type was involved in FALC formation of FALCs, we assessed the role of TNF, another key regulator of lymphoid tissue organization. We observed fewer and smaller FALCs in Tnfrsf1a⁻/⁻ Tnfrsf1b⁻/⁻ mice compared with those in wild-type mice (Fig. 4a,b). Conversely, we observed an eightfold greater number of FALCs and larger clusters in Tnfrsf1a⁻/²iRE mice (which carry a deletion that results in the accumulation of Tnf mRNA and TNF protein) than in their wild-type littermates (Fig. 4a,b). Thus, we concluded that TNF signaling promoted the formation of FALCs.

Colonization by the commensal flora is involved in the postnatal development and maturation of the gut-associated lymphoid tissues. Germ-free mice had twofold fewer FALCs than specific pathogen–free (wild-type) mice had (Fig. 4a,b), which indicated that the commensal flora partly contributed to the formation of the FALCs. Overall, these analyses revealed that the formation of FALCs was dependent on a type of ILC different from LTi cells and other RORγ+ ILC3 cells and was partly dependent on TNF signaling and colonization by the commensal flora.

Peritoneal inflammation leads to rapid formation of FALCs

Since the formation of FALCs was enhanced in Tnfrsf1a⁻/²iRE mice, which develop inflammation in the colon and joints, we assessed the effect of acute sterile peritonitis on cluster formation. Peritoneal inflammation
driven by zymosan led to a much greater size and number of FALCs at day 3 than did treatment with PBS, with the clusters becoming visible under the dissecting microscope without counterstaining (Fig. 4c), and their numbers doubling (Fig. 4d). Immunofluorescence staining showed that this was associated with the recruitment of CD11b+ myeloid cells (Fig. 4e) that were mostly Gr-1lo-CD11b+Ly6ClowF4/80+ macrophages (Supplementary Fig. 4). Thus, peritoneal inflammation led to a general expansion of FALCs.

**TNF signaling induces FALC formation**

To test the hypothesis that TNF contributed to FALC formation, we immunized Tnfrsf1a−/−Tnfrsf1b−/− mice with zymosan. Even though existing FALCs were larger in mice immunized with zymosan than in their PBS-treated counterparts and recruited CD11b+ myeloid cells, zymosan failed to induce new formation of FALCs at day 3 after injection in this Tnfrsf1a−/−Tnfrsf1b−/− strain (Fig. 5a). We then sought to determine whether signaling via TNFR on stromal cells was required for induction of the formation of FALCs following injection of zymosan. We lethally irradiated wild-type and Tnfrsf1a−/−Tnfrsf1b−/− recipient mice, reconstituted these mice with wild-type bone marrow donor cells and gave them an intraperitoneal injection of zymosan. Reconstitution of Tnfrsf1a−/−Tnfrsf1b−/− recipient mice with wild-type donor bone marrow did not restore FALC formation when inflammation developed at day 3 after injection, as it did in the control wild-type chimeras that received wild-type bone marrow (Fig. 5b). Thus, our data indicated that radiation-resistant stromal cells responded to TNF and induced FALC formation.

To determine the cellular origin of the TNF, we used mouse strains with specific deletion of Tnf in CD4+ T cells (T-TNF; Cd4-Cre), B cells (B-TNF; Cd19-Cre) or macrophages and neutrophils (M-TNF; Ly6m-Cre). Specific ablation of Tnf in T cells or B cells did not impair the induction of cluster formation upon inflammation, as shown by the threefold and twofold greater number of FALCs observed in T-TNF mice and B-TNF mice that received zymosan than in their PBS-treated counterparts (Fig. 5c). In contrast, the loss of Tnf expression in neutrophils and macrophages in M-TNF mice resulted in the absence of new FALC formation following injection of zymosan, even though CD11b+ myeloid cells were recruited to the clusters (Fig. 5c). Intracellular flow cytometry confirmed that the TNF+ cells were of myeloid origin (data not shown), with 40% of F4/80+ macrophages and Ly6C hi/F4/80+ monocytes displaying intracellular TNF expression in the mesenteric tissues after injection of zymosan (Fig. 5d). Once correlated to their cell number, macrophages represented 60% of all TNF+ myeloid cells in the mesenteric tissues (Fig. 5d).

Together these results demonstrated the importance of macrophages as a key source of signaling via TNF and TNFR in stromal cells for the new formation of FALCs when inflammation developed.

**FALC formation after inflammation requires invariant NKT cells**

Given the prevalence of B cells in FALCs, we evaluated their role in the inducible formation of these clusters. To do so, we analyzed the effect of intraperitoneal injection of zymosan into T cell– and B cell–deficient Rag2−/− mice and into B cell–deficient µMT (Ighm−/−) mice. B cells were not involved in FALC formation, as shown by the threefold greater number of clusters in mice given injection of zymosan than in mice that received PBS (Fig. 6a), as well as the recruitment of CD11b+ myeloid cells (Fig. 6b). However, peritoneal inflammation failed to induce cluster formation in Rag2−/− mice (Fig. 6a), which indicated a requirement for T cells. We confirmed that result with Tg(CD3E)26Cpt mice, in which the development of T cells, NK cells and NKT cells is blocked. Inflammation induced the recruitment of myeloid cells into FALCs in both Rag2−/− mice and Tg(CD3E)26Cpt mice as it did in wild-type mice (Fig. 6a,b). γδT cells were not involved in FALC formation after the development of peritoneal inflammation, as shown by the fivefold greater number of FALCs in mice deficient in the T cell antigen receptor (TCR) γδ-chain (Tcdd−/−) mice after injection of zymosan than after injection of PBS (Fig. 6a,b).

We next assessed the role of CD1d-restricted NKT cells in inducible FALC formation. Zymosan failed to induce cluster formation at day 3 after injection in Cd1d−/− mice (Fig. 6a,b). This result indicated a role for NKT cells in FALC formation.

**Activation of invariant NKT cells induces FALC formation**

NKT cells recognize lipid-based antigens presented by CD1d. The best-characterized NKT cell population is the CD1d-restricted...
Figure 5  TNF is required for FALC formation after inflammation is elicited. (a) Quantification of clusters in mesenteric tissues of \(Tnfrsf1a^{-/-} Tnfrsf1b^{+/-}\) mice 72 h after injection of PBS or zymosan (left), and whole-mount immunofluorescence staining of FALCs from those mice (right), with CD11b+ myeloid cells (blue), CD45+ hematopoietic cells (green), IgM+ B cells (red) and CD4+ T cells (white). Scale bar, 50 µm. (b) Quantification of clusters in the mesenteric tissues of wild-type reconstructed with wild-type bone marrow (WT→WT) or \(Tnfrsf1a^{-/-} Tnfrsf1b^{+/-}\) mice reconstructed with wild-type bone marrow (WT→KO), followed by injection of PBS or zymosan (left), and whole-mount immunofluorescence staining (as in a) of FALCs from those mice (right). Scale bar, 50 µm. (c) Quantification of clusters in the mesenteric tissues of wild-type, T-TNF, B-TNF and M-TNF mice after injection of PBS or zymosan (left), and whole-mount immunofluorescence (as in a) of FALCs from those mice (right). Scale bar, 50 µm. (d) Flow cytometry analyzing intracellular staining of TNF in cells obtained from wild-type mice 2 h after injection of zymosan (left). Numbers adjacent to outlined areas indicate percent TNF+ cells among mesenteric eosinophils (top left; gated as CD45+CD11b+Siglec-F−), neutrophils (top right; gated as CD45+CD11b+Siglec-F−Ly6-Ghi), macrophages (bottom left; gated as CD45+CD11b+Siglec-F−Ly6-Ghi), and monocytes (bottom right; gated as CD45+CD11b+Siglec-F−Ly6-Clo). Right, frequency of TNF+ cells among mesenteric eosinophils (eos), neutrophils (Neu), macrophages (Mφ) and monocytes (Mo) in wild-type mice 2 h after injection of PBS or zymosan. Each symbol represents an individual mouse; small horizontal lines indicate the mean. **P < 0.01 and ***P < 0.001 (Mann-Whitney nonparametric two-tailed test). Data are pooled from two independent experiments with six mice per group (a, left), five wild-type or six \(Tnfrsf1a^{-/-} Tnfrsf1b^{+/-}\) recipients per group (b, left), seven wild-type mice (PBS or zymosan), six (PBS) or eight (zymosan) T-TNF mice, four (PBS) or six (zymosan) B-TNF mice, or eight (PBS) or thirteen (zymosan) M-TNF mice (c, left), or eight mice per group (d, right), or are representative of two independent experiments with eight clusters from four mice per group (a-c, right) or two independent experiments (d, left).

Invariant NKT cell (iNKT) population, due to its use of the TCRα chain (containing α-chain variable region 14 and α-chain joining region 18 \(V_{\alpha}14-J_{\alpha}18\) in mice). iNKT cells recognize and strongly respond to the synthetic glycolipid α-galactosylceramide (α-GalCer) presented on CD1d28. Published studies have reported the presence of NKT cells in AT and their role in the regulation of metabolism42–46. Flow cytometry with antibody to the invariant signaling protein CD3ε (anti-CD3ε) and tetramers of CD1d and the α-GalCer analog PBS-57 (CD1d–PBS-57) confirmed that ATs showed particular enrichment for iNKT cells, which represented up to 6% of all lymphocytes, relative to their abundance in the spleen or mLNs, which contained approximately 0.03% or 0.1% iNKT cells, respectively (Fig. 7a). To detect iNKT cells in situ, we labeled iNKT cells with the division-tracking dye CFSE, isolated them through the use of CD1d–PBS-57 tetramers and transferred them into wild-type mice and found that these cells were indeed recruited to FALCs (Fig. 7b).

We next assessed the effect of the activation of iNKT cells on FALC formation by intraperitoneal injection of α-GalCer into wild-type mice. Injection of α-GalCer elicited twofold more clusters than did injection of vehicle (Fig. 7c). This observation demonstrated that direct activation of iNKT cells via the TCR was sufficient to induce cluster formation. We then assessed whether TNFR pathways were also required for cluster formation following activation of iNKT cells via the TCR. The formation of FALCs after injection of α-GalCer was abrogated in \(Tnfrsf1a^{-/-} Tnfrsf1b^{+/-}\) mice (Fig. 7d), which indicated a need for signaling through the receptors TNFR1 and TNFR2.

To determine whether iNKT cells were sufficient to restore inflammation-induced FALC formation in \(Rag2^{-/-}\) mice, we transferred sorted splenic iNKT cells or CD3hi cells by intraperitoneal injection into these mice, 24 h before immunization of the mice with zymosan. Only mice that received iNKT cells showed an increase in the number of FALCs after injection of zymosan (Fig. 7e).

We next assessed the effect of the selective loss of iNKT cells on the formation of FALCs after the development of zymosan-induced inflammation. In mice lacking the gene segment encoding the TCRα chain \(J_{\alpha}18\) region, FALC numbers were twofold greater after injection of zymosan than after treatment with PBS (Fig. 7f), which indicated that iNKT cells were not absolutely required for the formation of these clusters. Together these results indicated that even though iNKT cells were sufficient to restore cluster formation in \(Rag2^{-/-}\) mice, the function of iNKT cells and other non-invariant CD1d-restricted NKT cells seemed to overlap during inflammation-induced FALC formation.
**Figure 6** CD1d-restricted NKT cells are required for FALC formation. (a) Quantification of mesenteric clusters mice of various strains (above plots) 72 h after injection of PBS or zymosan. Each symbol represents an individual mouse; small horizontal lines indicate the mean. (b) Whole-mount immunofluorescence staining of FALCs from the mice in a, with CD11b+ myeloid cells (blue), CD45+ hematopoietic cells (green), IgM+ B cells (red) and CD4+ T cells (white). Scale bar, 50 μm. *P < 0.01 (Mann-Whitney nonparametric two-tailed test). Data are pooled from two independent experiments with six Rag2−/− mice per group, five Ighm−/− or Tg(CD3E)26Cpt mice per group, four (PBS) or six (zymosan) Tcrd−/− mice per group, or five (PBS) or six (zymosan) Cd1d−/− mice per group (a), or are representative of two independent experiments with eight clusters from four mice per group (b).

**FALC formation requires the receptor IL-4Rα**

Upon being activated, NKT cells very rapidly produce large amounts of T helper type 1 (Th1) cytokines (interferon-γ (IFN-γ)) or T helper type 2 (Th2) cytokines (IL-4), which link innate and adaptive immune responses and serve important immunoregulatory functions. To understand the involvement of Th1 and Th2 cytokines in inflammation-induced FALC formation, we assessed the effect of

**Figure 7** Activation of NKT cells induces FALC formation. (a) Flow cytometry of cells from the mesenteric tissues (visceral AT (VAT)), spleen and mLN. Numbers adjacent to outlined areas indicate percent CD3+ CD1d–PBS-57 tetramer-positive NKT cells in the lymphocyte gate. (b) Whole-mount immunofluorescence staining of mesenteric FALCs from wild-type recipient mice 24 h after transfer of CFSE-loaded NKT cells (green), with CD11b+ myeloid cells (blue) and IgM+ B cells (red). Outlined area at left enlarged 4x at right. (c,d) Quantification of mesenteric clusters in wild-type mice (c) or Tnfrsf1a−/−Tnfrsf1b−/− mice (d) 72 h after injection of vehicle (Veh) or α-GalCer (left), and whole-mount immunofluorescence staining of FALCs from those mice (right), with CD11b+ myeloid cells (blue), CD45+ hematopoietic cells (green), IgM+ B cells (red) and CD4+ T cells (white). (e) Quantification of mesenteric clusters (left plot) in Rag2−/− mice 72 h after injection of PBS or zymosan alone (left half) or after injection of CD3hi T cells (CD3hi) or CD3ε+ Cd1d-α-GalCer tetramer-positive NKT cells (NKT), sorted to high purity, followed by injection of zymosan (right half) and whole-mount immunofluorescence staining of FALCs from those mice (right), with CD11b+ myeloid cells (blue) and CD45+ hematopoietic cells (green). (f) Quantification of mesenteric clusters in mice lacking the gene segment encoding the TCRα chain Jα18 region (Traj18−/−) 72 h after injection of PBS or zymosan (left), and whole-mount immunofluorescence as in c (right). Each symbol (c–f, left) represents an individual mouse; small horizontal lines indicate the mean. Scale bars (b–e), 50 μm. *P < 0.01 and **P < 0.001 (Mann-Whitney nonparametric two-tailed test). Data are representative of three independent experiments (a) or are pooled from two independent experiments with eight clusters from four mice per group (b, and c–f, right), two independent experiments with six (vehicle) or nine (α-GalCer) mice per group (c, left), three independent experiments with nine (vehicle) or seven (α-GalCer) mice per group (d, left), four independent experiments with four (PBS), five (zymosan), six (CD3hi T cells) or five (NKT cells) mice per group (e, left), or two independent experiments with seven (PBS) or six (zymosan) mice per group (f, left).
Figure 8 Formation of FALCs is dependent on signaling via IL-4Rα. (a) Quantification of mesenteric clusters mice of various strains (below plots). 72 h after injection of PBS or zymosan. (b) Whole-mount immunofluorescence staining of FALCs from mice as in a, with CD11b+ myeloid cells (blue), CD45+ hematopoietic cells (green), IgM+ B cells (red) and CD4+ T cells (white). Scale bar, 50 μm. (c) Quantification of mesenteric clusters in Il13−/− mice 72 h after injection of PBS or zymosan alone (left) or after injection of CD3ε+ T cells or CD3ε+ CD16α-GalCer tetramer–positive NKT cells (as in Fig. 7e), followed by injection of zymosan (right). (d) Quantification of mesenteric clusters in C57BL/6J (WT) and Il4ra−/− mice 72 h after injection of vehicle or α-GalCer. Each symbol (a, c, d) represents an individual mouse; small horizontal lines indicate the mean. *P < 0.01 and **P < 0.001 (Mann-Whitney nonparametric two-tailed test).

Data are pooled from two independent experiments (a, d; n = 7, 6, 5, 5, 6, 4, 6 (left to right) mice per group (a), n = 7, 6, 6, 6 (left to right) mice per group (c) or (n = 9, 5, 7, 5 (left to right) mice per group (d)) or two independent experiments with eight clusters from four mice per group (b).

zymosan-induced inflammation in mice deficient in either of these cytokines. FALC formation induced by the injection of zymosan was not impaired in Ifng−/− mice (Supplementary Fig. 5), which indicated that IFN-γ was not required for this.

In contrast, zymosan failed to induce the formation of new clusters at day 3 after injection in mice deficient in the cytokine receptor IL-4Rα (Il4ra−−/− mice), in which signaling via IL-4 and IL-13 is impaired, while BALB/c wild-type mice responded with a two-fold greater number of FALCs than that of their counterparts treated with PBS (Fig. 8a.b). The number of FALCs was two times greater in untreated mice of the the BALB/c strain than in their C57BL/6J counterparts (data not shown), which suggested that the Tg2 bias of the former contributed to their greater number of FALCs.

To determine the respective contributions of IL-4 and IL-13, we studied FALC formation in Il4−/− and Il13−/− mouse strains. In both mutant strains, injection of zymosan led to the formation of new clusters (Fig. 8a,b), which suggested that IL-4 and IL-13 served redundant functions on the BALB/c background. However, IL-13 had a critical role in inflammation-induced FALC formation on the C57BL/6 background, as shown by the absence of new cluster formation in Il13−/− mice at day 3 after injection of zymosan (Fig. 8c). To determine whether IL-13 produced by iNKT cells was sufficient to restore inducible FALC formation in Il13−/− mice, we transferred sorted splenic wild-type iNKT cells into host mice 24 h before immunizing these mice with zymosan. We found that IL-13-deficient iNKT cells induced slightly more FALCs in Il13−/− mice that received zymosan than in their counterparts treated with PBS, but this result did not reach statistical significance (Fig. 8c).

These results indicated that the production of IL-13 by iNKT cells contributed to inflammation-induced FALC formation but that additional sources of IL-13 might be required. We finally assessed the role of signaling via IL-4Rα in the formation of FALCs driven by direct activation of iNKT cells. Injection of α-GalCer failed to induce FALCs formation in Il4ra−/− mice (Fig. 8d). These findings indicated that the formation of FALCs through direct activation of iNKT cells also required signaling via IL-4Rα.

IL-4 partially restores FALC formation in the absence of TNF

Our results showed that signaling via TNFRs and IL-4Rα was necessary for inflammation-induced FALC formation. To investigate whether crosstalk between these receptors was necessary for this, we performed a series of combinatorial and rescue experiments. Injection of TNF and a complex of IL-4 and anti-IL-4, either alone or in combination, was not sufficient to induce cluster formation (Supplementary Fig. 6a); this indicated that other factors were involved in this process. Intraperitoneal injection of TNF did not restore the zymosan-induced formation of FALCs in Il4ra−/− mice (Supplementary Fig. 6a). In contrast, intraperitoneal injection of the IL-4–anti-IL-4 complex resulted in a small but significantly greater number of zymosan-induced FALCs in Tnfrsf1a−/−/Tnfrsf1b−/− mice than in their littermates that received zymosan only (P = 0.0029; Supplementary Fig. 6b). This indicated that IL-4 was able to partially restore cluster formation in the absence of TNFR signaling and thus might act downstream of TNF.

DISCUSSION

In this report we have defined the requirements for the formation of an additional type of lymphoid tissue, the FALC. In the peritoneal cavity, mesenteric FALCs and omental milky spots accounted for almost all (>96%) of the lymphoid clusters associated with AT. In the pleural cavity, the pericardium and mediastinum showed a density of lymphoid clusters approaching the density in the omentum. FALCs contained a large proportion of B-1 cells with no apparent segregation of B cells and T cells, comparable to what has been reported for the milky spots of the omentum2. FALCs had the high expression of CXCL13 required for the efficient recruitment of B cells, which upon immunological challenge with T cell–dependent or T cell–independent antigens actively proliferated and underwent differentiation into plasma cells or GC cells in these clusters. The function of FALCs and milky spots probably overlap in the peritoneal cavity, but FALCs present in the mediastinal and the pericardial fat are probably the main sites for the organization of immune responses and for the retention of B-1 cells by CXCL13 in the pleural cavity.

FALC development in mice was constitutive and its initiation coincided with weaning. Establishment of a normal number of FALCs

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in adult mice was dependent on TNFR signaling and colonization by commensal flora, which would suggest a role for pattern-recognition receptors. FALCs were totally absent from Rag2−/−Il2rg−/− mice, and immunofluorescence and flow cytometry of cells from Rag2−/− mice indicated that the cells present in FALCs in the absence of T cells and B cells were ILCs. Notably, FALC formation was independent of the presence of LTi cells and other ILC3 cells, as well as lymphotixin-α–lymphotoxin-β–LTBR signaling, which resembles the formation of nasal associated lymphoid tissues at7.

Peritoneal inflammation induced rapid new formation of a large number of FALCs, which reached a two- to threefold greater abundance than their abundance in resting conditions. This effect was dependent on TNF expression by myeloid cells and TNFR signaling on stromal cells. The coupling of inflammation and rapid FALC formation provides a platform for linking innate and adaptive immune responses that support rapid B cell proliferation and natural antibody secretion in the body cavities following infection. The new formation of FALCs also necessitated the activation of INKT cells and signaling through IL-4Rα. Notably, triggering of INKT cells via ligation of the TCR was sufficient to induce formation of FALCs that was also dependent on signaling via TNFR and IL-4Rα. Therefore, the induction of FALC formation seemed to require both an inflammatory signal, delivered by TNF, and a T1/2 or resolution signal transduced by IL-4Rα. How FALC formation is timed with the initiation of the resolution of inflammation and whether FALCs have a role in the resolution phase remain to be investigated.

ATs constitute an important reservoir of stem cells for the formation of lymphoid stroma in LNs during development22,28 and immune responses49. It is thus conceivable that FALC stromal cells originate from AT stem cells or perivascular cells, as has been shown for follicular dendritic cells at22.

We have reported here a lymphoid organogenic activity for NKT cells. Not only were NKT cells necessary for the formation of FALCs during inflammation, but also their sole activation was sufficient to induce new formation of FALCs. Published studies have emphasized the role of NKT cells in the regulation of AT metabolism in normal nutrient homeostasis and during obesity22–46. Whether the metabolic activity of NKT cells is linked to their ability to induce FALC formation and alter the homeostasis of other hematopoietic cells present in ATs remains to be investigated.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.B., N.-T.L., I.A.W., A.A.K., Y.L., K.N., Y.Z., S.N., L.H.J. and J.H.C. designed and performed the research and collected and analyzed the data; A.F.-L., A.M., J.M., E.B., K.M., J.E.A., M.G., G.K., A.F.C., D.R.W., K.M.T., N.D.J., M.V., S.A.N. and A.N.J.M. facilitated the research; and C.B. and J.H.C. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Silverman, G.J., Gronwall, C., Vas, J. & Chen, Y. Natural autoantibodies to apoptotic cell membranes regulate fundamental innate immune functions and suppress inflammation. Discov. Med. 18, 151–156 (2009).

2. Alugupalli, K.R. et al. B1b lymphocytes confer T cell-independent long-lasting immunity. Immunity 21, 379–390 (2004).

3. Ochsenbein, A.F. et al. Control of early viral and bacterial distribution and disease by natural antibodies. Science 286, 2156–2159 (1999).

4. Baumgarth, N. et al. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. J. Exp. Med. 192, 271–280 (2000).

5. Haas, K.M., Poe, J.C., Steuber, D.A. & Tedder, T.F. B1a and B1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. Immunity 23, 7–18 (2005).

6. Boes, M., Prodeus, A.P., Schmidt, T., Carroll, M.C. & Chen, J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J. Exp. Med. 188, 2381–2386 (1998).

7. Martin, F., Oliver, A.M. & Kearney, J.F. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity 14, 617–629 (2001).

8. Piatelli, C., Cooper, D., Papadimitriou, J.M. & Hall, J.C. The omentum. World J. Gastroenterol. 6, 169–176 (2000).

9. Arslan, K.M., Harris, R.B. & Cyster, J.G. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. Immunity 16, 67–76 (2002).

10. Carlow, D.A., Gold, M.R. & Ziltener, H.J. Lymphocytes in the peritoneum home to the omentum and are activated by resident dendritic cells. J. Immunol. 183, 1155–1165 (2009).

11. Sa S.A. et al. Regulation of B1 cell migration by signals through Toll-like receptors. J. Exp. Med. 203, 2541–2550 (2006).

12. Rangel-Moreno, J. et al. Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens. Immunity 30, 731–743 (2009).

13. Elewa, Y.H., Ichi, O., Otsuka, S., Hashimoto, Y. & Kon, Y. Characterization of mouse mediastinal fat-associated lymphoid clusters. Cell Tissue Res. 357, 731–741 (2014).

14. Moro, K. et al. Innate production of Th1 cytokines by adipose tissue-associated c-KIT+Sca-1+ lymphoid cells. Nature 463, 540–544 (2010).

15. Price, A.E. et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. Proc. Natl. Acad. Sci. USA 107, 11489–11494 (2010).

16. Neill, D.R. et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature 464, 1367–1370 (2010).

17. Spits, H. et al. Innate lymphoid cells—a proposal for uniform nomenclature. Nat. Rev. Immunol. 13, 145–149 (2013).

18. Randall, T.D., Caraglio, D.M. & Rangel-Moreno, J. Development of secondary lymphoid organs. Annu. Rev. Immunol. 26, 627–650 (2008).

19. Roозendaal, R. & Mebius, R.E. Stromal cell-immune cell interactions. Annu. Rev. Immunol. 29, 23–43 (2011).

20. Ruddle, N.H. & Akirav, E.M. Secondary lymphoid organs: responding to genetic and environmental cues in ontology and the immune response. J. Immunol. 183, 2205–2212 (2009).

21. Ngu, V.N. et al. Lymphotoxin α/β and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. J. Exp. Med. 189, 403–412 (1999).

22. Krautler, N.J. et al. Follicular dendritic cells emerge from ubiquitous perivascular precursors. Cell 150, 194–206 (2012).

23. Matsumoto, M. et al. Distinct roles of lymphotixin α and the type I tumor necrosis factor (TNF) receptor in the establishment of follicular dendritic cells from non-bone marrow-derived cells. J. Exp. Med. 186, 1997–2004 (1997).

24. Arslan, K.M. et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature 406, 309–314 (2000).
25. Fu, Y.X., Huang, G., Wang, Y. & Chaplin, D.D. B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin α-dependent fashion. J. Exp. Med. 187, 1009–1018 (1998).

26. Yang, Y. et al. Antigen-specific memory in B-1a and its relationship to natural immunity. Proc. Natl. Acad. Sci. USA 109, 5388–5393 (2012).

27. Yang, Y. et al. Antigen-specific antibody responses in B-1a and their relationship to natural immunity. Proc. Natl. Acad. Sci. USA 109, 5382–5387 (2012).

28. Brennan, P.J., Brigl, M. & Brenner, M.B. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat. Rev. Immunol. 13, 101–117 (2013).

29. Marshall, J.L. et al. The capsular polysaccharide Vi from Salmonella typhi is a B1b antigen. J. Immunol. 189, 5527–5532 (2012).

30. Gil-Cruz, C. et al. The porin OmpD from nontyphoidal Salmonella is a key target for a protective B1b cell antibody response. Proc. Natl. Acad. Sci. USA 106, 9803–9808 (2009).

31. Foote, J.B. & Kearney, J.F. Generation of B cell memory to the bacterial polysaccharide α-1,3 dextran. J. Immunol. 183, 6359–6368 (2009).

32. Cascalho, M., Ma, A., Lee, S., Masat, L. & Wabl, M. A quasi-monoclonal mouse. Science 272, 1649–1652 (1996).

33. Marshall, J.L. et al. Early B blasts acquire a capacity for Ig class switch recombination that is lost as they become plasmablasts. Eur. J. Immunol. 41, 3506–3512 (2011).

34. De Togni, P. et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science 264, 703–707 (1994).

35. Fütterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H. & Pfeffer, K. The lymphotoxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity 9, 59–70 (1998).

36. Sun, Z. Requirement for RORγt in thymocyte survival and lymphoid organ development. Science 288, 2369–2373 (2000).

37. Koni, P.A. & Flavell, R.A. A role for tumor necrosis factor receptor type 1 in gut-associated lymphoid tissue development: genetic evidence of synergism with lymphotoxin β. J. Exp. Med. 187, 1977–1983 (1998).

38. Pabst, O. et al. Adaptation of solitary intestinal lymphoid tissue in response to microbiota and chemokine receptor CCR7 signaling. J. Immunol. 177, 6824–6832 (2006).

39. Bouskra, D. et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 456, 507–510 (2008).

40. Hamada, H. et al. Identification of multiple isolated lymphoid foci on the antimesenteric wall of the mouse small intestine. J. Immunol. 168, 57–64 (2002).

41. Kamada, N., Seo, S.U., Chen, G.Y. & Nunez, G. Role of the gut microbiota in immunity and inflammatory disease. Nat. Rev. Immunol. 13, 321–335 (2013).

42. Ji, Y. et al. Short term high fat diet challenge promotes alternative macrophage polarization in adipose tissue via natural killer T cells and interleukin-4. J. Biol. Chem. 287, 24378–24386 (2012).

43. Ji, Y. et al. Activation of natural killer T cells promotes M2 macrophage polarization in adipose tissue and improves systemic glucose tolerance via interleukin-4 (IL-4)/STAT6 protein signaling axis in obesity. J. Biol. Chem. 287, 13561–13571 (2012).

44. Lynch, L. et al. Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. Immunity 37, 574–587 (2012).

45. Schipper, H.S. et al. Natural killer T cells in adipose tissue prevent insulin resistance. J. Clin. Invest. 122, 3343–3354 (2012).

46. Wu, L. et al. Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice. Proc. Natl. Acad. Sci. USA 109, E1143–E1152 (2012).

47. Fukuyama, S. et al. Initiation of NALT organogenesis is independent of the IL-7R, LTβR, and NIK signaling pathways but requires the Id2 gene and CD3−CD4+CD45+ cells. Immunity 17, 31–40 (2002).

48. Bénézech, C. et al. Lymphotoxin-β receptor signaling through NF-κB/RelB pathway reprograms adipocyte precursors as lymph node stromal cells. Immunity 37, 721–734 (2012).

49. Gil-Ortega, M. et al. Native adipose stromal cells egress from adipose tissue in vivo: evidence during lymph node activation. Stem Cells 31, 1309–1320 (2013).
**ONLINE METHODS**

**Mice.** All mice (C57BL/6J (H-2b), Ltr-b+/-, Lyt-2+, Ror-γt−/−, Rag2−/−, Il2rg−/−, Tnfrsf1α−/− Tnfrsf1b−/−, Tnfα−/−) were injected of recombinant mouse IL-4 (Peprotech) and monoclonal antibody to IL-4 injection of 5 ml cold PBS in the peritoneal cavity. Isolation of splenic CD3+strainers before staining. Lavage of the peritoneal cavity was performed by injection of 1 mg zymosan on day 0 with IL-4c on days 0 and day 2. Mice were treated with 1 mg zymosan on day 0 and TNF on day 0 and day 2. In all experiments with day 4. For experiments with co-administration of zymosan, mice were given 2 × 10⁶ cells obtained from QM-eYFP + donor mice by peritoneal lavage were transferred intraperitoneally into C57BL/6J recipient mice, before immunization of the recipients with NP-OVA (50 µg/ml). After 24 h of culture, the plates were washed and samples were incubated for 2 h at 22 °C with isotype-specific alkaline phosphatase–conjugated antibody IgM-Ap1 or IgG-Ap1 (SouthernBiotech) diluted in 1% BSA in PBS and plates were developed with Fast BCIP/NBT alkaline phosphatase substrate (Sigma). Spots were counted with an AID ELISpot reader (Autoimmune Diagnostika) and Eli4 software (Autoimmune Diagnostika).

**RT-PCR and real-time RT-PCR.** mRNA was isolated from tissues with an RNeasy mini kit (Qiagen), and reverse transcription was performed with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative RT-PCR was performed with an ABI PRISM 7900HT instrument and primers and probes from Applied Biosystems for Cxcl13 (Mm00445353_m1), Ccl21 (Mm03646971_gH), Ccl19 (Mm00839676_g1), Tnf (Mm00443258_m1), Ltb (Mm00434774_g1) and Il7 (Mm01295803_m1).

**Image acquisition and analysis of confocal images.** Confocal images were acquired with a Zeiss LSM 510 laser-scanning confocal head with a Zeiss Axio Imager Z1 microscope.

**Statistical analysis.** Statistical significance was determined for all analyses with a Mann-Whitney nonparametric two-tailed test. Power calculations showed that for our most commonly measured parameters (lymphoid cluster number and cell numbers), six mice per group provided sufficient power (90%) to detect a difference between groups of at least a twofold, which we regard as an acceptable cutoff for identifying important biological effects. In all figures, the Mann-Whitney non-parametric test was used so as not to assume Gaussian distribution. No randomization methods or blinding approaches were used. Whenever possible, the investigator was partially ‘blinded’ for assessing the outcome (cluster counts). No animals were excluded from the analysis. Samples for which we failed to isolate cells from a given tissue were omitted.