Expression of ACKR4 demarcates the “peri-marginal sinus,” a specialized vascular compartment of the splenic red pulp

Highlights

- ACKR4 is expressed in the spleen by red pulp vessels surrounding the marginal zone

- Vascular shunts connect the marginal and the ACKR4+ “peri-marginal” sinuses

- Circulating T cells enter the spleen via the ACKR4+ “peri-marginal” sinuses

- ACKR4 supports spleen entry of T cells and their ensuing migration into the PALSs

Authors

Kathrin Werth, Elin Hub, Julia Christine Gutjahr, ..., Stefan Russo, Antal Rot, Reinhold Förster

Correspondence

a.rot@qmul.ac.uk (A.R.), foerster.reinhold@mh-hannover.de (R.F.)

In brief

Werth et al. describe an atypical chemokine receptor 4 (ACKR4)-positive “peri-marginal” sinus being a part of a closed segment of spleen microvasculature and the main site of T cell homing into the spleen. The expression of ACKR4 is required for optimal spleen entry of T cells and their subsequent migration into the periarteriolar lymphatic sheaths (PALSs).
Expression of ACKR4 demarcates the “peri-marginal sinus,” a specialized vascular compartment of the splenic red pulp

Kathrin Werth,1 Elin Hub,2,3,6 Julia Christine Gutjahr,2,6 Berislav Bosnjak,1 Xiang Zheng,1 Anja Bubke,1 Stefan Russo,2 Antal Rot,2,3,4,* and Reinhold Förster1,5,7,*

1Institute of Immunology, Hannover Medical School, 30625 Hannover, Germany
2Centre for Microvascular Research, The William Harvey Research Institute, Queen Mary University London, EC1M 6BQ London, UK
3Centre for Inflammation and Therapeutic Innovation, Queen Mary University London, EC1M 6BQ London, UK
4Institute for Cardiovascular Prevention, Ludwig-Maximilians University, 80336 Munich, Germany
5Cluster of Excellence RESIST (EXC 2155), Hannover Medical School, 30625 Hannover, Germany
6These authors contributed equally
7Lead contact
*Correspondence: a.rot@qmul.ac.uk (A.R.), foerster.reinhold@mh-hannover.de (R.F.)

https://doi.org/10.1016/j.celrep.2021.109346

SUMMARY

The spleen comprises defined microanatomical compartments that uniquely contribute to its diverse host defense functions. Here, we identify a vascular compartment within the red pulp of the spleen delineated by expression of the atypical chemokine receptor 4 (ACKR4) in endothelial cells. ACKR4-positive vessels form a three-dimensional sinusoidal network that connects via shunts to the marginal sinus and tightly surrounds the outer perimeter of the marginal zone. Endothelial cells lining this vascular compartment express ACKR4 as part of a distinct gene expression profile. We show that T cells enter the spleen largely through this peri-marginal sinus and initially localize extravascularly around these vessels. In the absence of ACKR4, homing of T cells into the spleen and subsequent migration into T cell areas is impaired, and organization of the marginal zone is severely affected. Our data delineate the splenic peri-marginal sinus as a compartment that supports spleen homing of T cells.

INTRODUCTION

The spleen is the largest secondary lymphoid organ equipped with an intricate microvasculature that enables an efficient survey and removal of potentially harmful blood constituents and the induction of humoral and cellular immune responses to blood-borne antigens (Mebius and Kraal, 2005). With regard to microanatomy, which determines the archetypal dichotomy of utilities, the spleen can be subdivided into the red pulp (RP), the site of innate host defenses, and the white pulp (WP), the site of adaptive immune responses, as well as the marginal zone (MZ), a specialized interface between the two main compartments. While the RP is composed of blood-filled sinuses and splenic cords, the WP consists of dense lymphocyte aggregates around the branches of the splenic artery. The lymphocytes in the WP are segregated into central T cell zones, also known as periarteriolar lymphatic sheaths (PALSs), and the surrounding B cell follicles. Such compartmentalization is achieved by the localized production of chemokines CXCL13 versus CCL19 and CCL21, attracting CXCR5-expressing B cells to the B cell follicles and CCR7-expressing cells to PALSs, respectively (Cyster, 2005; Förster et al., 2008). Besides CCR7, CCL19 and CCL21 can also ligate the atypical chemokine receptor 4 (ACKR4), which was also shown to bind CCL20, CCL22, and in human CXCL13, albeit with lower affinities (Bachererie et al., 2013, 2014; Gosling et al., 2000; Meyrath et al., 2021; Townsend and Nibbs, 2002). ACKRs do not couple to G proteins and fail to initiate downstream signaling cascades that characterize GPCRs but regulate the abundance and bioavailability of chemokines in defined tissue microenvironments by scavenging and either facilitating degradation of chemokines or their transport and retention (Nibbs and Graham, 2013; Pruenster et al., 2009; Rot, 2010; Ulvmar et al., 2011). The impact of ACKR4 on CCR7-mediated cell migration has been shown previously in skin and lymph nodes (LN) (Bryce et al., 2016; Ulvmar et al., 2014). Epidermal ACKR4 scavenges CCL19, thereby preventing desensitization of CCR7 on mature dendritic cells (DCs). This ensures their proper recruitment via lymphatic capillaries (Bryce et al., 2016). Within the LN, ACKR4 expression is restricted to the lymphatic endothelial cells lining only the ceiling of the subcapsular sinus. Scavenging its chemokine ligands in this microanatomical site shapes their gradients leading toward the LN parenchyma, thereby facilitating the CCR7-driven LN entry of incoming DCs (Ulvmar et al., 2014).

Despite the known contribution of ACKR4 to leukocyte migration into LNs, the expression and function of ACKR4 in the spleen
has not been investigated. Here, we describe a specific expression pattern of ACKR4 in splenic endothelial cells lining a previously non-delineated subset of interconnected blood vessels that form a dense three-dimensional (3D) sinusoidal network immediately surrounding the MZ, named here “peri-marginal sinus” (peri-MS). The venous origin of the peri-MS was confirmed by the retrograde injection of fluorescent microbeads into the spleen vein and profound transcriptomic profiling. We show that “peri-MS” receives blood directly from the MS via short vascular shunts and drains blood as vessels converge into larger veins, some of which also express ACKR4. Thus, ACKR4-positive peri-MS is an integral part of a closed segment splenic circulation connecting directly splenic artery to splenic vein with a distinct functional role and a point of T cell entry from blood into spleen. Adoptively transferred T cells home into spleen via the ACKR4-positive sinusoids to subsequently migrate into the PALSs through specialized bridging channels (SBCs) connecting the RP and the WP. Both steps of T cell homing into the spleen from blood and their subsequent migration into PALSs take place less efficiently in Ackr4-deficient mice, pinpointing the contribution of ACKR4 to the optimal T cell emigration and subsequent compartmentalization in the spleen. Additionally, in the absence of ACKR4, the area of the MZ is broadened and its cellular composition altered, highlighting the involvement of ACKR4 in the organization of MZ. We performed splenectomies and heterotopic spleen transplantations to show that ACKR4 expression on splenic endothelial cells is not required to regulate CCL19 levels in blood but control the release of spleen-derived CCL19 into the peripheral circulation.

RESULTS

ACKR4 is expressed in splenic vasculature in direct juxtaposition to the MZ

In order to detect ACKR4 expression in spleen, we stained frozen spleen sections of wild-type (WT) (C57BL/6N) mice with a validated specific antibody to ACKR4 (Ulvmar et al., 2014). Strikingly, an intense expression of ACKR4 was found on cells surrounding WP areas (Figure 1A), a signal clearly absent in spleen sections of Ackr4-deficient mice (Figure 1B). This expression pattern could be confirmed using Ackr4GFP reporter mice (Figure 1C), although ACKR4 expression in the spleen was not described in the initial characterization of these mice (Heinzel et al., 2007), likely because of the suboptimal tissue fixation. In our hands, acetone fixation also completely abolished the GFP signal in Ackr4GFP reporter mice, while this fixative was absolutely required for immunostaining with the validated anti-mouse ACKR1 antibody (Ulvmar et al., 2014). This antibody, however, when used within its specific concentration range, gave an overall lower signal as compared with the EGFP in Ackr4GFP reporter mice. Counterstaining with markers of endothelial cells (ECs), MECA-32 and CD31, readily identified ACKR4-expressing cells as parts of the splenic vasculature (Figures 1D and 1E). Remarkably, expression of ACKR4 was limited to a subset of blood vessels in the RP only and was not detectable in the WP vasculature or the MadCAM-1+ MS (Figure 1E).

Imaging of the entire spleen by two-photon microscopy identified few collecting and large veins localizing below the capsule of the spleen and those close to the hilus to express ACKR4 (Figure 1F; Video S1). To get better insight into areas within the deep spleen, we improved imaging by optical clearing of the spleen applying the OUBIC (Clear, Unobstructed Brain/Body Imaging Cocktail and Computational Analysis) protocol (Figure 1G) (Sussaki et al., 2014, 2015; Tainaka et al., 2014). Cleared spleen samples were counterstained with MEGA-32 to allow high-resolution tracing of splenic vessels up to 1 mm in depth (Figure 1H). In cleared samples, ACKR4 expression in large vessels below the spleen surface was abruptly lost in their smaller feeding branches. Importantly, two-photon imaging clearly showed that ACKR4-expressing vessels organize in a dense and convoluted 3D interconnected network tightly encompassing the MZ (Figure 1H; Video S1), thus forming a previously not recognized distinct vascular layer between the outer perimeter of the MZ and the RP proper.

ACKR4-expressing vasculature forms late during postnatal development

The vascularization in the spleen occurs within the first week after birth (Balázis et al., 2001; Balogh et al., 2007). Because of the positioning of the ACKR4+ vessels at the exact boundary between the WP and RP, we asked at which developmental stage the expression of ACKR4 appears and if it might play a role in the demarcation between these two compartments. An ACKR4-specific antibody and allelic Ackr4GFP/WT expression were used to determine ACKR4+ cells at different stages during post-natal development of the spleen. During the first days of life, when early spleen compartmentalization has already started, no ACKR4+ cells could be detected (data not shown). First ACKR4+ cells appeared between days 4 and 5 after birth and were predominantly found within large vessels in the central areas of the spleen (Figure 2A). This was in contrast with other defined subsets of splenic ECs, such as the MadCAM-1+ cells of the MS, reported to be present already at day 1 and build a nearly continuous cell layer as early as 1 week after birth (Balogh et al., 2007). On day 14, more ACKR4-expressing cells could be observed in a subset of elongated vessels. These vessels already localized in close proximity and juxtaposition to the MS, but notably, major parts of the MZ were still not surrounded by ACKR4+ vessels (Figure 2B). The encapsulations of MZ by ACKR4+ vessels became more prevalent, yet remained still incomplete on day 28 (Figure 2C). Only at the age of 42 days could the complex network of ACKR4+ vessels be observed in its entirety (Figure 2D). Thus, the development of the adult pattern of ACKR4 expression takes place after the main anatomical compartments have been formed, suggesting that ACKR4 is not required for the early development and functional compartmentalization of the spleen.

ACKR4 on splenic ECs regulates the release of CCL19 to peripheral circulation

To test the activity of ACKR4 on splenic ECs, we studied their uptake of a cognate fluorescent chemokine. To this end, splenic ECs from both Ackr4GFP/WT and Ackr4GFP/GFP mice were isolated and incubated with fluorescently labeled CCL19 (CCL19-AF647) for 90 min at 37°C. Flow cytometric analysis revealed a clear uptake only for cells expressing a functional allele of Ackr4 (Figures
while fluorescent signals in ACKR4-deficient cells remained at the background level seen in the CD31+ACKR4/C0 population (Figures 3A and 3B).

It has been well established that the continuous and efficient removal of extracellular chemokines by ACKRs affects the overall abundance of their respective ligands. For example, ACKR4 deficiency has been correlated with elevated serum levels of CCL21 (Comerford et al., 2010), and chemical inhibition of ACKR3-mediated scavenging leads to increased serum levels of CXCL12 (Berahovich et al., 2014). In line with these findings, we could show that ACKR4 deficiency also led to elevated serum levels of CCL19 (Figure 3C).

Figure 1. ACKR4-expressing vessels form a dense network in direct juxtaposition to the marginal zone (MZ)
(A) Positioning of ACKR4-expressing cells in the spleen was determined using antibodies against ACKR4, CD3, and B220 on frozen sections from WT mice as indicated.
(B) Lack of staining on sections from Ackr4−/−-deficient mice revealed specificity of the antibody.
(C) GFP expression in a heterozygous Ackr4GFP/WT reporter mouse; counterstaining with DAPI.
(D and E) counterstaining of WT sections with antibodies against ACKR4, CD169, Laminin, PLVAP (MECA-32), and MadCAM-1 as indicated.
(F) Performing two-photon microscopy on Ackr4GFP/WT spleens after counterstaining with MECA-32 (red) reveals large GFP-expressing vessels (green) on the spleen surface.
(G) Optical clearing of a whole spleen following the advanced CUBIC protocol.
(H) Two-photon micrograph of cleared spleen sample from (G) showing the complex network of GFP-expressing vasculature (green) surrounding WP and MZ. Red, counterstaining with MECA-32; blue, second harmonics generation. Scale bars: 1 mm (A and B); 200 μm (C); 100 μm (D, F and H); 50 μm (E). Length of squares: 10 mm (G).
Considering that the spleen is one of the best perfused organs of the body and, as we found, contains a dense network of ACKR4-expressing blood vessels, we tested whether ACKR4 expression in the spleen was sufficient to modify CCL19 serum levels. Interestingly, experimental splenectomy in WT mice did not result in increased serum levels of CCL19, and splenectomy in Ackr4−/− mice, in turn, failed to reduce them (Figure 3D). These findings indicate that serum levels of CCL19 are not controlled primarily by splenic expression of ACKR4 but by its overall expression in other organs and tissues. Next, we used a model of heterotopic spleen transplantations (Figures 3E and 3F) in which donor spleens from either WT or Ackr4−/− mice were implanted into the circulation of Ackr4−/− recipients and their CCL19 serum levels assessed 8 days later. In line with the notion that ACKR4 expressed within the splenic vasculature does not control chemokines in circulation, the implantation of an ACKR4-sufficient spleen failed to reduce elevated CCL19 serum levels in Ackr4−/− spleens (Figure 3G). However, we detected a highly significant increase in serum CCL19 after transplantation of Ackr4−/− spleens (Figure 3G). This suggests that ACKR4 in the spleen controls the release into the blood of CCL19 that is constitutively produced by splenic resident cells (Ngo et al., 1998). These findings might explain why Ackr4−/− spleens do not contain increased levels of CCL19 despite the putative lack of their ACKR4-mediated scavenging (Figure S1).

ACKR4 shapes MZ morphology

Although the overall architecture of ACKR4-deficient spleens was largely unaffected (Figures 1A and 1B), histological examination of the MZ revealed structural alterations developing in the absence of ACKR4. Most notably, staining with an anti-IgM antibody revealed a broadened MZ, with B cells outside the MS being less densely packed and lacking a clear demarcation toward the RP, thus giving MZ an overall blurred appearance (Figure 4A). Indeed, the MZ, defined as the area between MS and the F4/80-expressing RP macrophages (Figure 4B), was significantly enlarged in ACKR4-deficient mice (Figure 4C). Macrophages of the MZ are positioned as an inner layer of CD169+ marginal metallophilic macrophages (MMMs) and an outer layer of SIGN-R1-expressing MZ macrophages (MZMs) (Borges da Silva et al., 2015). Previously, CCR7 ligands have been suggested to impact on the positioning of MZMs and MMMs (Ato et al., 2004; Benedict et al., 2006). We used flow cytometric quantification to reliably enumerate MZMs and MMMs present in single-cell suspensions of WT and ACKR4-deficient spleens. Strikingly, the relative proportions, as well as the absolute counts of both MZMs and MMMs, were significantly increased in Ackr4−/− spleens as compared with their WT counterparts (Figures 4D and 4E). These changes and the altered MZ morphology might be because of an increased abundance of ACKR4 ligands in the spleen. However, ACKR4-deficient mice, in contrast with having increased levels of CCL19 in blood (Figure 3C), contained similar amounts of this chemokine in spleens as compared with those seen in WT mice (Figure S1). Naturally, CCL19 levels might still be increased locally within the MZ microenvironments, or alternatively, increased levels of other ACKR4-cognate chemokines might be responsible for cellular phenotypes observed in ACKR4-deficient spleens. We used confocal microscopy to ascertain whether ACKR4 expression also impacted on the relative positioning of MZMs and MMMs within the MZ, but we observed no apparent differences between WT and Ackr4−/−.
spleens (Figure 4F). The proportions and absolute counts of F4/80-positive RP macrophages were reduced in Ackr4<sup>−/−</sup> spleens as compared with their WT counterparts (Figures 4D and 4E), indicating a potential direct influence of ACKR4 on the resident cells of the RP. However, the relative microanatomical positioning of F4/80-positive macrophages within the RP was not affected by the expression of ACKR4 (Figure 4F).

**Transcriptome of ACKR4-positive ECs confirms their venular identity and suggests distinct function**

Based on its positioning within the RP, ACKR4-expressing endothelium most likely belongs to the venous segment of splenic vasculature. In order to better define the subpopulation of ACKR4-expressing vessels, we performed a transcriptome analysis on isolated splenic ECs. ECs were sorted into CD31<sup>+</sup>ACKR4<sup>+</sup> and CD31<sup>+</sup>ACKR4<sup>−/−</sup> populations (sorting strategy depicted in Figure S2A) routinely yielding 92%–98% cell purity (Figure S2B).

Although typical endothelial markers, such as VE-cadherin, ICAM-2, PECAM-1, and PLVAP (Hallmann et al., 1995; Lampugnani et al., 1992; Vecchi et al., 1994), were highly expressed in both populations (Figures S2C and S2D), we identified 1,875 probes (comprising 1,627 genes) to be differentially expressed (>2-fold change) (Figure S2E). Among them we found genes that are considered to be specifically or preferentially expressed in arterial ECs, such as EphrinB2 (<sup>Efnb2</sup>) (Gale et al., 2001; Shin et al., 2001), delta-like 4 (<sup>Dll4</sup>) (Krebs et al., 2001), notch 4 (<sup>Notch4</sup>) (Villa et al., 2001), neuropilin 1 (<sup>Nrp1</sup>) (Mukouyama et al., 2005), and hairy/enhancer-of-split related with YRPW motifs 1 and 2 (<sup>Nrp1</sup>) (Nakagawa et al., 1999). All these markers were expressed substantially higher within the ACKR4<sup>−/−</sup> population (Figures 5A and 5B), reflecting the venous belonging of ACKR4-positive ECs. On the other side, markers specifically expressed by venous ECs, such as ephrin receptor B4 (<sup>Ephb4</sup>) (Adams et al., 1999; Gerety et al., 1999), neuropilin 2 (<sup>Nrp2</sup>) (Yuan et al., 2002), and FMS-like tyrosine kinase 4 (<sup>Flt4</sup>) (Kaipainen et al., 1995; Thompson et al., 1998), were equally expressed in both populations (Figures 5A and 5B). Of note, beta 3 tubulin class III (<sup>Tubb3</sup>) suggested to be specifically expressed in venous valves, but not in the veins (Kang and Lee, 2006), was expressed higher in ACKR4-positive ECs (Figures 5A and 5B). These data confirm our histological observations that ACKR4 is expressed not only on ECs lining a subset of RP sinuses but also on a subset of large veins with valves.

Additionally, we performed Gene Ontology (GO) clustering for both populations. Among the top GO terms for biological processes within the ACKR<sup>−/−</sup> population, we found mostly terms...
Figure 4. Macrophage subsets in the MZ are significantly expanded in the absence of ACKR4 expression

(A) Immunohistological analysis of WT and Ackr4<sup>−/−</sup> spleens after counterstaining with antibodies as indicated reveals a loosened and scattered MZ in the absence of ACKR4.

(B) Definition of MZ area was based on counterstaining with anti-F4/80 and anti-Laminin antibody as depicted by shaded white area.

(C) Quantification of MZ area in relation to whole spleen area of WT and Ackr4<sup>−/−</sup> spleen sections; pooled data are from 11 mice.

(legend continued on next page)
Although i.a. injected splenocytes were found in the MS (Figure 5C, upper panel), in contrast, many of the biological processes significantly enriched within the ACKR4+ population were related to biosynthetic and metabolic processes (Figure 5C, lower panel), indicating that ACKR4 defines a subset that was also functionally different from other ECs in the spleen. The disparity of both populations was further reflected by the variety of genes clustering in the biological process “extracellular matrix organization,” a term that was significantly enriched in each of the populations (Figures 5C, highlighted in red, and 5D). On the one hand, this underlined the general importance of extracellular matrix components for vessel integrity; on the other hand, this emphasized the specific composition of the ACKR4-expressing vascular segment. Interestingly, ACKR1, ACKR2, and ACKR3 transcripts were also detected in the ACKR4+ population (Figures 5E and 5F). Expression of these ACKRs was not exclusively limited to but was significantly enhanced in this subset, reflecting its importance for excess chemokine removal in close proximity to the MZ.

ACKR4+ ECs define a novel segment of the venous system in the RP, the peri-MS

To functionally assess whether ACKR4+ vessels belong to the venous compartment, we injected either fluorescently labeled beads or splenocytes into euthanized Ackr4GFP/WT mice in the abdominal aorta or retrograde via the portal vein. Although i.a. injected beads localized in the MS, beads injected intravenously (i.v.) could be found within GFP-expressing vessels, but not in the MS (Figure 6A). It is important to note that some i.v. injected beads were also detected within the GFP-negative vasculature of the RP, indicating that ACKR4+ ECs line only a subpopulation of venous vessels in the RP draining into the main spleen vein. Although i.a. injected splenocytes were found in the MS (Figure 6B, left panel), some were localizing within ACKR4+ vessels (Figure 6B, right panel), indicating the existence of vascular shunts connecting the MS with the ACKR4+ venous sinuses. Indeed, confocal microscopy of spleen sections (Figure 6C) and 3D reconstructions of cleared spleens (Figure 6D) visualized the vascular shunts and showed that they are particularly prevalent in the areas of either particularly thin or non-existent MZ (Video S2). These are the sites where the B cell areas are remote, and the RP and PALSs are juxtaposed and directly connected by the SBCs (Bajenoff et al., 2008; Chauveau et al., 2020; Lyons and Parish, 1995). It has been traditionally thought that lymphocytes enter the spleen via the MS (Bajenoff et al., 2008; Lyons and Parish, 1995), which recently has been contested in favor of the vessels of the RP (Chauveau et al., 2020; Tadayon et al., 2019). We adoptively transferred fluorescently labeled T cells into Ackr4GFP/GFP and Ackr4GFP/WT mice and immediately afterward analyzed their positioning in the spleen. Already within 5 min after transfer, injected cells were found within the MS and GFP+ vessels, particularly in the areas with prominent shunts between the MS and ACKR4+ sinus. Moreover, the transferred cells observed within the GFP+ veins were traversing their walls or positioned extravascularly, in their immediate proximity (Figure 6E; Figure S3). This pattern of T cell localization characterized equally ACKR4-sufficient and -deficient spleens and was observed similarly after the adoptive transfer of CCR7-sufficient and -deficient T cells (Figure S4), indicating that CCR7 ligands are not involved in regulating T cell entry into the spleen. We performed flow cytometry to compare the homing of the adoptively transferred WT T cells into WT and ACKR4-deficient spleens at 10, 15, and 20 min after their i.v. injection. Overall, fewer T cells entered the spleens of ACKR4-deficient recipients, although their numbers were significantly different only at 15 min postinjection (Figure S5). This indicates that ACKR4 contributes to T cell emigration into the spleen, although the mechanism involved is not yet clear. However, based on our findings, we cannot rule out entirely the involvement of MS in T cell entry into the spleen. Thus, our data show that T cells enter the spleen prominently via the ACKR4+ peri-MSs, hence allowing them to immediately migrate via the SBCs from the RP to the PALSs, as shown recently (Chauveau et al., 2020). The analysis of the SBCs shortly after the T cells transfer indicated that they contain ample cells that have arrived via ACKR4+ vessels (Figure 6F).

ACKR4 affects T cell homing to splenic WP

T cell homing into the WP is mainly mediated by CCR7 with the corresponding ligands being expressed in the splenic T cell zone (Bajenoff et al., 2008; Förster et al., 1999). By modifying the bioavailability of CCR7 ligands, ACKR4 has been shown to modulate the migration behavior of CCR7-expressing cells in peripheral LNs and skin (Bryce et al., 2016; Ulvmar et al., 2014). Accordingly, we adoptively transferred labeled T cells (Figures 7A and 7B) into WT or Ackr4−/− recipients and analyzed the positioning of transferred T cells after 30 or 120 min, respectively (Figures 7C and 7D). For both time points investigated, we observed a clear reduction of the T cells that had successfully entered the PALSs in ACKR4-deficient as compared with WT recipients, with the difference being more pronounced at the earlier time point (Figure 7E). Importantly, at 120 min, i.e., 100 min after the spleen entry of transferred T cells plateaued and their numbers in spleen were practically the same for both mouse strains (Figure S5), T cell numbers in PALSs of ACKR4-deficient recipients remained significantly lower than those in WT mice. These data are consistent with ACKR4 expression in the peri-MS facilitating the migration of transferred T cells into PALSs. Irrespective of ACKR4 expression in the recipient spleens, transferred Ccr7−/− T cells failed to enter the PALSs (Figure 7F). Under steady-state conditions, the numbers of resident lymphocyte subsets in ACKR4-deficient and WT spleen remained similar (Figure S6). This suggests that the requirement of

(D and E) Representative plots (D) and proportion and absolute cell counts (E) of CD169+, SIGN-R1+, and F4/80+ macrophages analyzed in flow cytometry; cell aggregates and CD3+ or B220+ cells were excluded from analysis, and numbers indicate percentage of cells in gate.

(F) Staining of CD169+ MMs (red), SIGN-R1+ MZMs (green), and F4/80+ RP macrophages (blue) in WT and Ackr4−/− spleens.

Representative (D) or pooled (E) data from 8–11 mice obtained in three independent experiments. The comparative analyses of lymphocyte population in WT and ACKR4-deficient spleens obtained in the same experiments are shown in the Figure S8. Scale bars: 100 μm. Dots represent individual mice. Red bar, mean. Unpaired t test. *p < 0.05; **p < 0.001.
ACKR4 expression for T cell homing into the WP can be bypassed, also resulting in intact microanatomical structures of WP observed in ACKR1-deficient mice (Figure 1A). In conclusion, expression of ACKR4 supports optimal migration of T cells to the splenic T cell zone in response to CCR7 ligands but is not absolutely required for the functional microanatomical organization and overall cellularity of the PALSs.

**DISCUSSION**

The spleen is equipped with a unique and particularly complex microvasculature to support the functionality and the diverse features of its distinct compartments. Splenic arteries give rise to trabecular arteries, which, upon further branching, are cylindrically surrounded by the WP and are referred to as central arterioles (Schmidt et al., 1985). Smaller branches of the central arterioles feed the capillary bed of the WP and either terminate in the RP or converge in the MS, a dense, flat network of vascular spaces that delimits the MZ toward the WP (Schmidt et al., 1985, 1993). Blood flow continues through MS and MZ toward the RP and enters an open system of vascular spaces, which is a peculiar feature of splenic microvasculature. In mice, venous drainage is conducted by RP venules that flow together to form a superficial venous plexus and converge in splenic veins before forming the main splenic vein eventually draining into the portal vein (Schmidt et al., 1985).

Here, we describe previously not delineated venular structures in the RP marked by the expression of ACKR4. These vessels tightly encompass the outer edge of the MZ, are in a concentric juxtaposition and directly connected by vascular shunts to the MS, which, in turn, demarcates the inner edge of the MZ. We suggest to name the hitherto not yet described splenic vessels "peri-MSs."

**Figure 5.** ACKR4-expressing vessels belong to the venous segment of splenic vasculature

(A) Gene array heatmap showing relative signal intensities of selected genes preferentially expressed on ACKR4+ and ACKR4- splenic endothelial cells. (B) Relative mRNA expression levels of selected genes shown in (A) as indicated. (C) Negative log of p value showing the top terms clustering within “biological process” for ACKR4 (upper panel) and ACKR4- (lower panel) ECs as calculated using Enrichr. Of note, “extracellular matrix organization” was significantly enriched in both populations (highlighted in red). (D) Heatmap showing relative signal intensities of selected genes clustering in “extracellular matrix organization” as indicated. (E) Heatmap showing relative signal intensities of genes encoding for the four known ACKRs. (F) mRNA expression levels of genes shown in (E). RNA units represent expression intensity (arbitrary units). Mean and SD are shown. RNA for microarray analysis was isolated after sorting of populations in two independent experiments with two or three mice pooled per experiment.
Based on a differential staining pattern by anti-EC antibodies, heterogeneity among segments in murine splenic vasculature has been noted previously (Balázs et al., 1999, 2001). One antibody, IBL-7/1, stained the MS and a subset of sinuses in the RP, while another, IBL-9/2, preferentially marked the sinuses deep in the RP, which were only weakly stained by IBL-7/1 (Balázs et al., 1999). The molecular nature of antigens detected by these antibodies remains unknown, but based on the discrepant staining pattern with that observed here, neither of them detects ACKR4. Despite the fact that IBL-9/2 was later reported to stain

Figure 6. Function and positioning of ACKR4-expressing vessels

(A and B) Positioning of 1-μm microspheres (A) or splenocytes (B) after intraarterial (purple) or intravenous (orange) retrograde spleen injection into euthanized Ackr4GFP/WT reporter mice (green). Representative pictures from three to four mice injected in two to three independent experiments. Blue, VCAM-1. (C and D) Arrows indicate direct shunts between the MECA-32+ MS (red) and the ACKR4+ (green) peri-marginal sinus in the spleen; (C) confocal and (D) two-photon images. (E and F) Positioning of adoptively transferred T cells (white) in spleens 5 min after injection; confocal images stained with the antibodies indicated. Scale bars: 50 μm (A, B, F, left); 20 μm (C–E); 10 μm (F, right).
all splenic sinusoidal ECs in the RP (Berahovich et al., 2014), the immunoreactivity of these antibodies potentially pinpoint to further heterogeneity of the venous sinuses in the RP. Curiously, the aforementioned epitopes appear in spleen as early as 12 h after birth even preceding the formation of the vascular network, which takes about 3 post-natal weeks to develop completely (Balázs et al., 2001). Conversely, the onset of ACKR4 expression occurs late during post-natal development, suggesting that it is dispensable for appropriate organogenesis during early stages of post-natal spleen development.

The comparison of transcriptomes in ACKR4+ and ACKR4− splenic ECs revealed a difference in expression for more than 1,600 genes, indicating that ACKR4-positive ECs were a functionally distinct subset. Despite a low number of replicates for

Figure 7. ACKR4 is required for appropriate homing of T cells to the PALSs
5–10 × 10⁶ purified T cells from WT or Ccr7−/− donor mice were intravenously transferred into WT and Ackr4−/− recipients, and positioning of transferred cells was analyzed after 30 or 120 min.

(A–C) Exemplary plots for purity (A), labeling intensity (B), and positioning of transferred cells (C) after i.v. transfer of MACS-purified and eFl670-labeled T cells. (D) The T cell zone was defined based on counterstaining with anti-CD3 antibody (dotted white line). (E) Number of transferred WT T cells per mm² T cell zone at the time points indicated. (F) Number of transferred Ccr7−/− T cells per mm² T cell zone at the time points indicated. Representative (A–D) or pooled (E and F) data from three independent experiments with 5–8 mice analyzed per genotype and time point. Mean and SD are shown. Unpaired t test, *p < 0.05; **p < 0.01; ***p < 0.001.
this array (n = 2), potentially representing a source of some uncertainty, high consistency was observed in the independent experiments, thus underscoring the reliability of the study. In line with that, typical markers for ECs were abundantly expressed in both populations, while markers for B and T cells were constantly absent, demonstrating the high purity of the samples. In accord with prior reports, our transcriptomic profiling also revealed the co-expression of all known ACKRs in splenic vasculature. ACKR3 was previously on splenic ECs, which, based on their co-staining with IBL-9/2 mAb, were defined as sinusoidal (Berahovich et al., 2014). Similarly, ACKR2 expression was identified in ECs of the RP vessels with clear sinusoidal morphology (Nibbs et al., 2001). ACKR1 expression has also been directly demonstrated in the venous vasculature of murine RP (Duchene et al., 2017). Moreover, expression of ACKR1 by ECs has been described first in RP sinuses of human spleen (Peiper et al., 1995). Recently, ACKR1 has been shown as a viable marker of postcapillary and collecting venules in many murine organs (Thirot et al., 2017).

We show that ACKR4 is required for proper organization of the MZ but can only speculate on how its expression in the peri-MS, a compartment directly surrounding the MZ, might be involved. The absence of CCR7 ligands has been shown to affect MZM and MMM numbers (Ato et al., 2004; Benedict et al., 2006). Hence, in a diametrically opposite scenario, because of the lack of their scavenging by ACKR4 (Comerford et al., 2006; Haraldsen and Rot, 2006), the excess of CCL19 and CCL21 in ACKR4-deficient mice might lead to an increased recruitment or retention of MZMs. This, in turn, would subsequently affect the localization of MZ B cells, shown to be strongly interdependent on the macrophage positioning in the MZ (Karlsson et al., 2003; Nolte et al., 2004; You et al., 2011). The overall levels of CCL19 in Ackr4−/− spleens are comparable with those found in their WT counterparts. Nevertheless, the absence of ACKR4-mediated scavenging might still cause local tissue accumulation of CCR7 ligands in the microenvironments surrounding the MZ. It is also possible that the lack of scavenging of ACKR4-cognate chemokines other than CCR7 ligands might be responsible for the cellular phenotypes characterizing MZs of Ackr4−/− spleens.

The lack of chemokine scavenging in Ackr4−/− spleens and putative changes in chemokine milieu within their defined micro-anatomical structures potentially causing alterations of MZ morphology might also contribute to the reduced T cells spleen homing and subsequent migration into the PALs. Previously, it has been suggested that T cells enter the spleen via the MS into the MZ (Bajénoff et al., 2008), a notion recently contested, implicating instead vessels of the RP as the point of T cell entry. We show here that the ACKR4+ peri-MS is a key site of T cell trafficking into the spleen and argue that it is ideally suited to support this migratory step. Thus, there is considerable analogy in molecular compartmentalization of the splenic MS, MZ, and peri-MS with the subcapsular area in the LNs. The positioning of ACKR4-expressing peri-marginal vessels outside the MZ mirrors the expression of ACKR4 in lymphatic ECs lining the ceiling of the subcapsular sinus, but not its floor (Ulvmar et al., 2014). By such peculiar arrangement in peripheral LNs, ACKR4 shapes functional gradients of CCR7 ligands pointing toward the LN parenchyma. Mature DCs arriving via afferent lymphatics in the subcapsular sinus require these gradients to efficiently enter the parenchyma. Accordingly, lack of ACKR4 leads to an accumulation of these cells within the subcapsular sinus. We find that T cells transferred into Ackr4−/− mice home less efficiently into the spleen and show reduced subsequent migration into the T cell zone. Therefore, it is tempting to speculate that ACKR4 in the peri-MSs might shape chemokine gradients analogously to the mechanism described for LNs. However, clear depiction of such gradients currently remains elusive as histological staining of CCR7 ligands in the splenic RP is perturbed by multiple organ-characteristic unspecific signals, complicated by peculiarities of 3D sub-compartmentalization and difficulties of detecting soluble extracellular chemokines in native tissues (data not shown). Likewise, the relatively weak expression of Ackr4 promoter-driven GFP does not allow visualization of immune cells passing through Ackr4+ vessels in the spleen involving the use of modern multiphoton imaging (data not shown).

**Limitations of this study**

We detected ACKR4 in the spleen using the Ackr4GFP reporter mice as well as by immunostaining with an anti-ACKR4 antibody (Ulvmar et al., 2014). This antibody, when used within its specific concentration range, gave an overall lower signal as compared with EGFP in the reporter mice. To formally prove that the antibody and EGFP are marking the same vascular compartment, we attempted to use both techniques simultaneously. However, such double staining was not possible, because the uniquely specific anti-ACKR4 antibody requires acetone fixation and loses its immunoreactivity when PFA is used as a fixative, while the reporter EGFP, present as a soluble protein in the cytoplasm, requires crosslinking by PFA and cannot be visualized at all following acetone fixation.

We postulate that in the spleen, similarly to other sites of ACKR4 expression, this receptor is scavenging its cognate chemokines. We show chemokine internalization using isolated spleen ACKR4-expressing ECs, however, could not observe in ACKR4 deficient mice signs of reduced chemokine scavenging in situ when measuring the spleen levels of CCL19. It is likely that in the absence of ACKR4, the spleen-derived CCL19 that has successfully avoided scavenging is leached out from the spleen into the blood circulation because of the absence of ACKR4, as suggested by the spleen transplantation experiments. Despite ACKR4-deficient spleens showing no changes to their overall chemokine levels, it is still possible that chemokines are over-abundant in discreet spleen microenvironments. This might disrupt putative gradients and lead to an impaired migration of T cells into the PALs, broadening of the MZ, and increases in MMM and M2M, but decreases in F4/80 macrophage counts. Our conjecture implicating altered chemokine gradients responsible for these phenotypes, as observed in ACKR4-deficient spleens, currently remains only speculative. This is because we are not able to reliably image chemokine gradients in the spleen. Also, in our current study, we did not attempt to independently confirm the CCR7 dependency of MMM and M2M localization suggested before (Ato et al., 2004; Benedict et al., 2006).
The EGFP signal in the peri-MS was not sufficiently strong to allow live imaging using multiphoton microscopy. Thus, we were not able to observe in real time the homing of T cells to spleen and their subsequent migration into PALSs. Our data on spleen entry of T cells via the peri-MS, as well as their impeded homing and delayed subsequent migration in the ACKR4-deficient spleens, are snapshot time-course observations in immunohistochemistry, quantitative flow cytometry, and morphometry. Thus, it was not possible to seamlessly align the numeric data provided by these dissimilar methodologies, which were cumulatively consistent with the contribution of ACKR4 to both T cell spleen homing and migration into PALSs. However, the relative magnitude of ACKR4 impact on these two respective T cell migratory steps could not be reliably established. Furthermore, despite consistently observing T cells entering spleen by traversing the walls of the peri-MSs, but not even once of a MS, we are not able to formally exclude the involvement the latter route in T cell entry into the spleen.

In summary, our study of ACKR4 expression in the spleen allowed us to identify a complex 3D vascular network that tightly surrounds the MZ, runs concentrically and in parallel to the MS, and is connected to it directly via vascular shunts. Accordingly, we termed this vascular structure peri-MS. Based on its localization, genetic expression profile, and on the positioning of beads and cells injected into the splenic vein and artery, we provide evidence that the ACKR4+ peri-MS is a part of the venous sinus system and is directly connected to the main venous vessels draining the spleen, as well as via shunts to the arterial circulation and MS. In contrast with the human spleen, where most of the blood bypasses the reticular meshwork and directly enters venous sinuses, the circulation of the murine spleen, till now, lacked unequivocal evidence for similar shortcuts and was considered to be open (Schmidt et al., 1985).

Our discovery of the vascular shunts directly connecting the MS with the peri-MS shows that in mouse spleen a subset of the circulatory "bypasses" exists connecting the WP to the collecting veins in the RP. The description of the peri-MS and the circulatory shunts at the border between the WP and RP should entail studies addressing how molecules and cells might interfere with the circulatory shunts at the border between the WP and RP. The description of the peri-MS and the circulatory shunts at the border between the WP and RP should entail studies addressing how molecules and cells might interfere with the circulatory shunts at the border between the WP and RP. The description of the peri-MS and the circulatory shunts at the border between the WP and RP should entail studies addressing how molecules and cells might interfere with the circulatory shunts at the border between the WP and RP.

The EGFP signal in the peri-MS was not sufficiently strong to allow live imaging using multiphoton microscopy. Thus, we were not able to observe in real time the homing of T cells to spleen and their subsequent migration into PALSs. Our data on spleen entry of T cells via the peri-MS, as well as their impeded homing and delayed subsequent migration in the ACKR4-deficient spleens, are snapshot time-course observations in immunohistochemistry, quantitative flow cytometry, and morphometry. Thus, it was not possible to seamlessly align the numeric data provided by these dissimilar methodologies, which were cumulatively consistent with the contribution of ACKR4 to both T cell spleen homing and migration into PALSs. However, the relative magnitude of ACKR4 impact on these two respective T cell migratory steps could not be reliably established. Furthermore, despite consistently observing T cells entering spleen by traversing the walls of the peri-MSs, but not even once of a MS, we are not able to formally exclude the involvement the latter route in T cell entry into the spleen.

In summary, our study of ACKR4 expression in the spleen allowed us to identify a complex 3D vascular network that tightly surrounds the MZ, runs concentrically and in parallel to the MS, and is connected to it directly via vascular shunts. Accordingly, we termed this vascular structure peri-MS. Based on its localization, genetic expression profile, and on the positioning of beads and cells injected into the splenic vein and artery, we provide evidence that the ACKR4+ peri-MS is a part of the venous sinus system and is directly connected to the main venous vessels draining the spleen, as well as via shunts to the arterial circulation and MS. In contrast with the human spleen, where most of the blood bypasses the reticular meshwork and directly enters venous sinuses, the circulation of the murine spleen, till now, lacked unequivocal evidence for similar shortcuts and was considered to be open (Schmidt et al., 1985).

Our discovery of the vascular shunts directly connecting the MS with the peri-MS shows that in mouse spleen a subset of the circulatory "bypasses" exists connecting the WP to the collecting veins in the RP. The description of the peri-MS and the circulatory shunts at the border between the WP and RP should entail studies addressing how molecules and cells might interfere with the circulatory shunts at the border between the WP and RP.
vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. Dev. Biol. 230, 151–160.


Rot, A. (2010). Chemokine patterning by glycosaminoglycans and intercep-


## KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purified anti-mouse ACKR4 (polyclonal goat)</td>
<td>Santa Cruz</td>
<td>sc-204823</td>
</tr>
<tr>
<td>AlexaFluor 488 anti-mouse CD3 (clone 17A2)</td>
<td>Produced in the Förster lab</td>
<td>N/A</td>
</tr>
<tr>
<td>Cy3 anti-mouse CD3 (clone 17A2)</td>
<td>Produced in the Förster lab</td>
<td>N/A</td>
</tr>
<tr>
<td>Cy5 anti-mouse CD3 (clone 17A2)</td>
<td>Produced in the Förster lab</td>
<td>N/A</td>
</tr>
<tr>
<td>PE anti-mouse CD3 (clone 145-2C11)</td>
<td>(eBioscience) ThermoFisher</td>
<td>A14714</td>
</tr>
<tr>
<td>AlexaFluor647 anti-mouse CD3 (clone 17A2)</td>
<td>BioLegend</td>
<td>100209</td>
</tr>
<tr>
<td>Cy3 anti-mouse B220 (clone RA3-3A1)</td>
<td>Produced in the Förster lab</td>
<td>N/A</td>
</tr>
<tr>
<td>Cy5 anti-mouse B220 (clone RA3-3A1)</td>
<td>Produced in the Förster lab</td>
<td>N/A</td>
</tr>
<tr>
<td>BV510 anti-mouse B220 (clone RA3-6B2)</td>
<td>eBioscience</td>
<td>69-0452-82</td>
</tr>
<tr>
<td>PerCP-Cy5.5 anti-mouse B220 (clone RA3-6B2)</td>
<td>eBioscience</td>
<td>45-0452-80</td>
</tr>
<tr>
<td>Cy3 anti-mouse IgD (clone HB250)</td>
<td>Produced in the Förster lab</td>
<td>N/A</td>
</tr>
<tr>
<td>Cy5 anti-mouse IgD (clone HB250)</td>
<td>Produced in the Förster lab</td>
<td>N/A</td>
</tr>
<tr>
<td>BV510 anti-mouse IgD (clone 11-26c.2a)</td>
<td>BD Biosciences</td>
<td>563110</td>
</tr>
<tr>
<td>Purified anti-mouse laminin (polyclonal rabbit)</td>
<td>Cosmo Bio Co., Ltd.</td>
<td>N/A</td>
</tr>
<tr>
<td>PE anti-mouse F4/80 (clone BM8)</td>
<td>eBioscience</td>
<td>12-4801-82</td>
</tr>
<tr>
<td>purified anti-mouse SIGN-R1 (clone eBio22D1)</td>
<td>eBioscience</td>
<td>15340710</td>
</tr>
<tr>
<td>APC anti-mouse SIGN-R1 (clone eBio22D1)</td>
<td>eBioscience</td>
<td>17-2093-82</td>
</tr>
<tr>
<td>FITC anti-mouse CD169 (clone MOMA-1)</td>
<td>AbD serotec</td>
<td>MCA947F</td>
</tr>
<tr>
<td>AlexaFluor647 anti-mouse CD169 (clone MOMA-1)</td>
<td>(AbD serotec) BioRad</td>
<td>MCA947G</td>
</tr>
<tr>
<td>AlexaFluor647 anti-mouse PLVAP (clone MECA-32)</td>
<td>(AbD serotec) BioRad</td>
<td>MCA2539GA</td>
</tr>
<tr>
<td>AlexaFluor647 anti-mouse PLVAP (MECA-32)</td>
<td>BioRad</td>
<td>MCA2539GA</td>
</tr>
<tr>
<td>purified anti-mouse panendothelial cell antigen (clone MECA-32)</td>
<td>BioLegend</td>
<td>120501</td>
</tr>
<tr>
<td>purified anti-mouse MadCAM-1 (clone MECA-367)</td>
<td>BioLegend</td>
<td>120702</td>
</tr>
<tr>
<td>AlexaFluor647 anti-mouse VCAM-1 (clone 429 (MVCAM.A))</td>
<td>BioLegend</td>
<td>105711</td>
</tr>
<tr>
<td>APC anti-mouse IgM (clone RMM-1)</td>
<td>BioLegend</td>
<td>406509</td>
</tr>
<tr>
<td>FITC anti-GFP (goat polyclonal)</td>
<td>Abcam</td>
<td>ab6662</td>
</tr>
<tr>
<td>eFluor570 anti-mouse CD4 (clone RM4-5)</td>
<td>eBioscience</td>
<td>41-0042-82</td>
</tr>
<tr>
<td>APC anti-mouse CD8 (clone 53-6.7),</td>
<td>BD Bioscences</td>
<td>553035</td>
</tr>
<tr>
<td>AlexaFluor594 anti-mouse CD19 (clone 6D5)</td>
<td>BioLegend</td>
<td>115552</td>
</tr>
<tr>
<td>eFluor570 anti-mouse IgM (clone II/41)</td>
<td>eBioscience</td>
<td>41-5790-82</td>
</tr>
<tr>
<td>anti-IgM mAb (clone II/41, PE-Cy7)</td>
<td>eBioscience</td>
<td>25-5790-82</td>
</tr>
<tr>
<td>PE anti-mouse CD31 (clone MEC13.3)</td>
<td>eBioscience</td>
<td>12-0311-82</td>
</tr>
<tr>
<td>AlexaFluor647 anti-mouse CD31 (clone 390)</td>
<td>BioLegend</td>
<td>102416</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE anti-mouse CD45 (clone 30-F11)</td>
<td>BioLegend</td>
<td>103106</td>
</tr>
<tr>
<td>PerCP anti-mouse CD11b (clone M1/70)</td>
<td>eBioscience</td>
<td>45-0031-80</td>
</tr>
<tr>
<td>FITC anti-mouse CD1d (clone 1B1)</td>
<td>eBioscience</td>
<td>53-0011-82</td>
</tr>
<tr>
<td>AF688 anti-mouse CD169 (clone 3D6.112)</td>
<td>BioLegend</td>
<td>142419</td>
</tr>
<tr>
<td>PE-Cy7 anti-mouse CD3e (clone 145-2C11)</td>
<td>eBioscience</td>
<td>25-0232-81</td>
</tr>
<tr>
<td>eFluor660 anti-mouse CD23 (clone B3B4,</td>
<td>eBioscience</td>
<td>25-0232-81</td>
</tr>
<tr>
<td>PE anti-mouse CD21/35 (clone 7G6)</td>
<td>BD Biosciences</td>
<td>A9044-2ML</td>
</tr>
<tr>
<td>AlexaFluor647 mouse anti-goat</td>
<td>Jackson ImmunoResearch</td>
<td>205-602-176</td>
</tr>
<tr>
<td>Cy3 mouse anti-rat</td>
<td>Jackson ImmunoResearch</td>
<td>212-166-168</td>
</tr>
<tr>
<td>AlexaFluor647 mouse anti-rat</td>
<td>Jackson ImmunoResearch</td>
<td>212-606-168</td>
</tr>
<tr>
<td>FITC goat anti-rabbit</td>
<td>Jackson ImmunoResearch</td>
<td>111-096-045</td>
</tr>
<tr>
<td>Cy3 goat anti-rabbit</td>
<td>Jackson ImmunoResearch</td>
<td>111-165-144</td>
</tr>
<tr>
<td>AlexaFluor647 mouse anti-rabbit</td>
<td>Jackson ImmunoResearch</td>
<td>211-605-109</td>
</tr>
<tr>
<td>Cy3 goat anti-syrian hamster</td>
<td>Jackson ImmunoResearch</td>
<td>107-165-142</td>
</tr>
<tr>
<td>AlexaFluor647 goat anti-syrian hamster</td>
<td>Jackson ImmunoResearch</td>
<td>107-606-142</td>
</tr>
<tr>
<td>AlexaFluor 488 donkey anti-goat</td>
<td>Invitrogen</td>
<td>A-11055</td>
</tr>
<tr>
<td>anti-Fc receptor antibody culture supernatant (clone 2.4G2)</td>
<td>Produced in the Förster lab</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### Chemicals, peptides, and recombinant proteins

| Tissue-Tek® O.C.T. Compound | Sakura | Cat# 4583 |
| DAPI | Sigma-Aldrich | Cat# D9542 |
| Prolong Gold | ThermoFisher Scientific | P140144 |
| Heparin | Roth | 7692.1 |
| SeTau-647-NHS | SETA BioMedicals | K9-4142 |
| polyethylene glycol mono-p-isooctylphenyl ether (Triton X-100) | Sigma-Aldrich | X-100-500 ml |
| N,N,N’,N’-tetrakis(2-hydroxypropyl) ethylenediamine (Quadrol) | Sigma-Aldrich | 122262 |
| urea | Sigma-Aldrich | U5378 |
| sodium azide | Sigma-Aldrich | 71290 |
| FluoSpheres Carboxylate-modified Microspheres, red fluorescent, 1 μm diameter | Invitrogen | F8821 |
| Fluoresbrite® BB Carboxylate Microspheres, 1 μm diameter | Polysciences | 17458-10 |
| low melting point agarose | Invitrogen | 16520050 |
| Cell Proliferation Dye eFluor 670 | eBioscience | 65-0840-90 |
| CellTracker Violet | Thermo Fisher Scientific, Invitrogen | C10094 |
| CellTracker Orange | Thermo Fisher Scientific, Invitrogen | C34551 |
| CellTracker Yellow | Thermo Fisher Scientific, Invitrogen | C34567 |
| Dispase Grade I | Roche | 4942086001 |
| Collagenase D | Roche | 11088858001 |
| DNeasy | Roche | 11284932001 |
| RPMI 1640 | GIBCO | Cat# 31780-025 |
| Dynabeads Sheep Anti-Rat IgG | Invitrogen | Cat# 11055 |
| AF647 labeled CCL19 | Almac | Cat# CAF-06 |

### Critical commercial assays

| LUNARIS Mouse 11-Plex CHEMOKINE Kit | AYOXXA Biosystems | LMCK 20110S |
| MACS Pan T Cell Isolation Kit II | Miltenyi Biotech | 130-095-130 |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Prof. Reinhold Förster (foerster.reinhold@mh-hannover.de).

Materials availability
This study did not generate new unique reagents. Commercially available reagents are indicated in the Key resources table.

Data and code availability
The gene array data generated during this study are available at Gene Expression Omnibus - GEO at accession number: GSE171335.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice were bred at the Central Animal Facility of Hannover Medical School, Germany, Charles River, Manston Rd, Margate CT9 4LT, UK and BSU, Charterhouse Square, QMUL, EC1M 6BQ, UK and maintained under specific pathogen-free conditions. Knockout Ackr4<sup>−/−</sup> (Comerford et al., 2010), knockin reporter Ackr4<sup>GFP/wt</sup> and Ackr4<sup>GFP/GFP</sup> (Heinzel et al., 2007), and knockout Ccr7<sup>−/−</sup> mice (Förster et al., 1999) have been described previously. Male and female mice were used at the age of 8 - 12 weeks, unless otherwise specified. All animal experiments have been performed in accordance with institutional guidelines and have been approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit or the Institutional Ethics and Animal Welfare Committee and the Home Office, UK.

All animal experiments were performed according to the recommendations and guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) and Society of Laboratory Animals (GV-SOLAS) and approved by the institutional review board of the Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit and Animal Welfare Committee and the Home Office, UK.

METHOD DETAILS

Immunohistology
For immunohistology of spleens from Ackr4<sup>GFP/wt</sup> or Ackr4<sup>GFP/GFP</sup> mice, organs were harvested and fixed overnight in 2% paraformaldehyde (PFA) plus 30% (vol/vol) sucrose and rinsed in PBS before embedding. Spleens from other mice were directly embedded.
in Tissue-Tek OCT (Sakura Finetek), frozen on dry ice and fixed in ice-cold acetone for 10 min after cutting. Sections were rehydrated in Tris-buffered saline with 0.05% Tween20 (TBS/T). Subsequently, sections were blocked with 10% mouse, goat, or rat serum in TBS/T and stained for 45 min at room temperature with the following directly conjugated antibodies or with purified antibodies followed by secondary antibodies in TBS/T: Anti-ACKR4 antibody (polyclonal goat, purified, Santa Cruz), anti-CD3 mAb (clone 17A2, AlexaFluor488, Cy3, or Cy5), anti-B220 mAb (clone RA3-3A1, Cy3 or Cy5), anti-igD mAb (HB250, Cy3 or Cy5, all homemade), anti-Laminin antibody (polyclonal rabbit, purified, Cosmo Bio Co., Ltd.), anti-F4/80 mAb (clone BM8, PE), anti-SIGN-R1 mAb (eBio22D1, purified or APC, eBioscience), anti-CD169 mAb (MOMA-1, FITC or AlexaFluor647), anti-PLVP mAb (MECA-32, AlexaFluor647, both AbD serotec), anti-mouse panendothelial cell antigen mAb (MECA-32, purified), anti-MadCAM-1 mAb (clone MECA-367, purified), anti-VCAM-1 mAb (clone 429 (MVCAM.A), AlexaFluor647), anti-IgM mAb (clone RMM-1, APC, all from BioLegend), mouse anti-goat antibody AlexaFluor647, mouse anti-rat antibody Cy3 or AlexaFluor647, goat anti-rabbit antibody FITC or Cy3, mouse anti-rabbit antibody AlexaFluor647, goat anti-syrian hamster antibody Cy3 or AlexaFluor647 (all from Jackson ImmunoResearch). Additionally, DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich) was used to identify cell nuclei. High-resolution composite images were acquired with either a BX61 epifluorescent microscope (Olympus; UPlanSapo objectives: 10x/0.4 and 20x/0.75; camera: F-View II) or an AxioScan Z1 slide scanner (Zeiss; Plan-Apochromat objective: 10x/0.45 M27; camera: Axiocam 506 mono) and further processed with cellSens (Olympus) or ZenBlue software (Zeiss), respectively.

Confocal microscopy
Spleens from Ackr4GFP/wt, Ackr4GFP/GFP or WT mice were harvested, cut into 3 pieces each and fixed in 2% PFA/PBS for 4 hours followed by 10%, 15% and 30% sucrose/PBS for 3 hours or overnight. Tissues were subsequently frozen in OCT on dry ice and stored at −80 °C. 6 μm or 20 μm thin sections were cut on a cryostat (OTF5000, Bright Instruments Ltd), air-dried and then stored a −20 °C. Before staining, sections were rehydrated in 0.1% BSA/PBS and blocked with 10% goat serum for 30 minutes. The following antibodies were diluted in 0.1% BSA/PBS and incubated on the slides for 30 minutes at room temperature: anti-GFP antibody (goat polyclonal, FITC, Abcam), anti-ACKR4 antibody (polyclonal sc-46835, purified, Santa Cruz), anti-CD3 mAb (either clone 17A2, AlexaFluor647, BioLegend, or clone 145-2C11, PE, eBioscience), anti-CD4 mAb (clone RM4-5, eFluor570, eBioscience), anti-CD8 mAb (clone 53-6.7, APC, BioLegend), anti-CD19 mAb (clone 6D5, AlexaFlour594, BioLegend), anti-IgM (clone II/I41, eFluor570, eBioscience), anti-CD31 mAb (either clone MEC13.3, PE, BioLegend, or clone 390, AlexaFluor647, BioLegend) and anti-PLVP mAb (MECA-32, AlexaFluor647, BioRad). The unconjugated anti-ACKR4 antibody was detected with a secondary antibody donkey anti-goat AlexaFluor 488 (Invitrogen). After three washes in 0.1% BSA/PBS the slides were mounted with Prolong Gold (ThermoFisher Scientific) and cured overnight. High-resolution composite images were acquired with a Zeiss LSM800 confocal laser scanning microscope (Plan Apochromatic objectives: 20x/0.8 and 40x/1.3) and further processed with ZenBlue software (Zeiss).

Clearing of spleen and two-photon microscopy
Optical clearing of spleens was done based on the advanced CUBIC protocol (Susaki et al., 2014, 2015; Tainaka et al., 2014) with several modifications. Heterozygous Ackr4GFP/wt mice were sacrificed and perfused via the left ventricle with 25 mL PBS containing 20 U/ml heparin (Roth) to prevent thrombus formation in the spleen. An incision in the right ventricle allowed blood exit during perfusion. Afterward, a blunt cannula was inserted into the V. portae and fixed by tight ligation. The spleen and parts of the intestine were flushed slowly with another 100 mL PBS substituted with 10 U/ml heparin to remove remaining blood. For endothelial cell labeling 25 μg MECA-32 mAb (BioLegend) conjugated to SeTau647-NHS (SETA BioMedicals) were injected in 1 mL PBS. After 25 min incubation at room temperature, unbound antibody was removed by flushing with PBS. In some experiments, spleens were removed after this step, cut longitudinally with a scalpel and imaged as described below. In other experiments, the spleens were additionally fixed by slow perfusion with 75 mL 4% PFA followed by another washing step with PBS. Subsequently, spleens were flushed with 25 mL modified Sca.eCUBIC-1 (reagent-1A) containing 10% (wt/wt) polyethylene glycol mono-p-isooctylphosphoryl ether (Triton X-100), 5% (wt/wt) N,N,N’,N’-tetrais(2-hydroxypropyl)ethylene diamine (Quadrol), and 10% (wt/wt) urea as well as 0.15% sodium azide (all from Sigma-Aldrich) and 25 mM NaCl (Roth) in water. Finally, spleens were removed and placed in Sca.eCUBIC-1 (reagent-1A) at room temperature with gentle shaking. One to two days later, spleens were completely cleared and imaged with an upright Olympus BX51 microscope equipped with a W Plan-Apochromat 20x/1.0 DIC (Zeiss), TrimScope scanning unit (LaVision Biotech), and Mai Tai Titanium:sapphire pulsed infrared lasers (Spectra-Physics). GFP and SeTau647 were excited at a wavelength of 920 nm. Acquired stacks were processed and analyzed using Imaris 7.4 – 8.x (Bitplane).

Injection of beads or splenocytes
Heterozygous Ackr4GFP/wt mice were culled and both abdominal and thoracic cavities were opened. For intraarterial application of beads, the abdominal aorta was isolated and ligated cranial to the diaphragm. A small incision was made at the caudal aorta distal to the exit of the V. lienalis. Initially, warm PBS (37 °C) was flushed through both accesses before microsphere suspensions (106 /ml; 1 μm diameter; FluoSpheres Carboxylate-modified Microspheres, red fluorescent from Invitrogen or Fluoresbrite® BB Carboxylate Microspheres from Polysciences) in 1% low melting point agarose (Invitrogen) were injected simultaneously. Colors of microspheres were alternated between experiments. Alternatively, splenocytes from a C57BL/6N donor mouse were injected at a concentration of 4-5×107 /ml after labeling with the cell proliferation dye eFluor™ 670 (eBioscience) at 37 °C for 10 min. Following the injection, the spleen...
was removed and fixed overnight at 4°C in 2% PFA, 30% sucrose. Immunohistological counterstaining and imaging was performed as described above.

**Isolation of splenic endothelial cells**

*Ackr4<sup>GFp/wt</sup>* or *Ackr4<sup>GFP/GFP</sup>* mice were culled and the spleens were removed. 1 - 2 mL of a digestion cocktail containing 0.2 mg/ml Dispase Grade I, 0.2 mg/ml Collagenase D, and 0.025 mg/ml DNase I (all from Roche) in RPMI-1640 (GIBCO) were injected into spleens at multiple sites using a 27 G needle. After 45 min incubation at 37°C, spleens were cut into small pieces and transferred into another 2 mL of the same digestion cocktail. During the following 20 min incubation at 37°C, the suspension was mixed thoroughly every 7 to 10 min. Subsequently, digestion was quenched by addition of 25 mL FACS buffer (3% FCS and 4 mM EDTA in PBS) and suspension was filtered through a 100 μm cell strainer (BD Biosciences). Cells were washed with FACS buffer and red blood cells were lysed. For enrichment of endothelial cells, CD45<sup>+</sup> cells were depleted by 30 min incubation with anti-CD45 mAb (clone 30-F11, BioLegend) followed by 30 min incubation with 1.2 × 10<sup>8</sup> sheep anti-rat Dynabeads (Invitrogen) at 4°C with gentle tilting and collection of the supernatant in a DynaMag 15 (Invitrogen).

**Transcriptomic profiling**

Splenic endothelial cells were isolated from *Ackr4<sup>GFp/wt</sup>* mice as described above and counterstained with appropriate antibodies and DAPI. The following fluorescent-activated cell sorting was performed at the Core Facility Cell Sorting at Hannover Medical School using a FACSemia Fusion (BD Biosciences). Cell fractions were pre-gated on CD45<sup>-</sup> DAPI<sup>-</sup> single cells and sorted into one batch. Total RNA was extracted using an RNeasy Plus Micro kit (QUIAGEN) according to the manufacturer’s protocol. Further processing of samples and microarray experiments were performed at the Research Core Unit Genomics of Hannover Medical School.

The Microarray study was conducted by use of a refined version of the Whole Mouse Genome Oligo Microarray 4x44K v2 (design ID 026655, Agilent Technologies), called ‘02665AsQuadruplicatesOn180k’ (design ID 048306) developed by the Research Core Unit Genomics of Hannover Medical School. Microarray design was defined at Agilent’s eArray portal using a 4x180k design format for mRNA expression as template. All non-control probes of design ID 026655 have been selected to be printed four times onto one 180k Microarray. To properly run Feature Extraction software algorithms, control probes were determined and placed automatically by eArray using recommended default settings.

6-20 ng of total RNA were used to prepare aminoallyl-UTP-modified (aaUTP) cRNA (Amino Allyl MessageAmp II Kit, life technologies) applying one-round of amplification as suggested by the company, with the exception that a twofold downsampling of all reaction volumes was used.

aaUTP-cRNA was labeled with Alexa Fluor 555 Reactive Dye (Thermo Fisher Scientific) according to the manufacturer’s guidelines except a twofold downsampling of reaction volumes.

cRNA fragmentation, hybridization and washing steps were carried-out as recommended in the ‘One-Color Microarray-Based Gene Expression Analysis Protocol V5.7’, except that 150 ng of each fluorescently labeled cRNA population were used for hybridization.

Slides were scanned on the Agilent Micro Array Scanner G2565CA (pixel resolution 3 μm, bit depth 20). Data extraction was performed with the ‘Feature Extraction Software V10.7.3.1’ using the extraction protocol file ‘GE1_107_Sep09.xml’.

Measurements of on-chip replicates (quadruplicates) were averaged using the geometric mean of processed intensity values of the green channel, ‘gProcessedSignal’ (gPS) to retrieve one resulting value per unique non-control probe. Single Features were excluded from averaging, if they i) were manually flagged, ii) were identified as Outliers by the Feature Extraction Software, iii) lay outside the interval of ‘1.42 x interquartile range’ regarding the normalized gPS distribution of the respective on-chip replicate population, or, iv) showed a coefficient of variation of pixel intensities per Feature that exceeded 0.5.

Averaged gPS values were normalized by global linear scaling: All gPS values of one sample were multiplied by an array-specific scaling factor. This factor was calculated by dividing a ‘reference 75th Percentile value’ (set as 1500 for the whole series) by the 75th Percentile value of the particular Microarray to be normalized (‘Array i’ in the formula shown below). Accordingly, normalized gPS values for all samples (microarray datasets) were calculated by the following formula:

\[
normalized\; gPS_{Array\; i} = gPS_{Array\; i} \times \left(\frac{1500}{75^{th}\; Percentile_{Array\; i}}\right)
\]

Finally, a lower intensity threshold (surrogate value) was defined based on intensity distribution of negative control features. This value was fixed at 15 normalized gPS units. All of those measurements that fell below this intensity cutoff were substituted by the respective surrogate value of 15.

Differentially expressed genes were identified using the filtering option in the Excel-based analysis software RCUTAS (Research Core Unit Transcriptomics Analysis System). Gene sets were clustered with the gene set enrichment tool Enrichr (Chen et al., 2013; Kuleshov et al., 2016) and heatmaps were generated using Heatmapper (Babicki et al., 2016) with the normalized gPS values for selected genes.

**CCL19 Uptake**

Splenic endothelial cells from *Ackr4<sup>GFp/wt</sup>* or *Ackr4<sup>GFP/GFP</sup>* mice were isolated as described above and incubated with 15 nM CCL19-AF647 (Almac) at 37°C for 90 min with gentle shaking. Cells were washed three times with ice-cold FACS buffer, counterstained with appropriate antibodies and DAPI, and analyzed on a LSR II flow cytometer (BD Biosciences).
Splenic and sham laparotomy

C57BL/6N and Ackr4−/− mice were anesthetized with an intraperitoneal injection of Ketamin (100 mg/kg, Anesketin, Albrecht) and Xylazin (20 mg/kg, Rompun, Bayer). The abdominal cavity was accessed through an incision over the left upper quadrant. After the spleen was exposed, splenic vessels were isolated from surrounding tissue and firmly ligated. After dissecting the vessels, the spleen was removed and the surgical wound was closed in two layers. For sham laparotomies, the abdominal cavity was opened and the spleen was briefly exposed and repositioned. Analgesics were administered via the drinking water (0.5 mg/ml Metamizol-Natrium, Novaminsulfon-Ratiopharm, Ratiopharm) until mice were sacrificed on day 8 after surgery.

Spleen transplantation

Spleen transplantations were performed as described previously (Swirski et al., 2009). In brief, spleen donor mice were anesthetized with an intraperitoneal injection of Ketamin (100 mg/kg) and Xylazin (20 mg/kg). The thoracic cavity was opened and the mouse was perfused via the aorta with up to 20 mL PBS containing 20 U/ml heparin. Subsequently, the abdominal cavity was opened and the spleen together with the pancreas exposed. To that aim, splenic vessels were isolated up to the aorta or portal vein, respectively. Smaller incoming and outgoing vessels as well as aorta and portal vein were dissected after ligation and the remaining package containing spleen, pancreas, and connecting vasculature was removed.

Next, recipient mice were anesthetized and, after opening of the abdominal cavity, descending aorta and inferior vena cava were isolated, clamped, and opened. Portal vein and aorta from the donor were anastomosed to these incisions before clamps were opened and the blood flow was restored. The surgical wound was closed and analgesics were administered via the drinking water (0.5 mg/ml Metamizol-Natrium, Novaminsulfon-Ratiopharm, Ratiopharm) during the postoperative period.

Quantification of CCL19

Whole blood samples were obtained from euthanized mice by cardiac puncture and left for clotting at room temperature for approximately 30 min. After centrifugation, serum was collected and stored at −20 °C for later analysis. CCL19 levels in serum were determined using a LUNARIS Mouse 12-Plex CHEMOKINE Kit (AYOXXA Biosystems) according to the manufacturer’s protocol. Briefly, serum samples were incubated on the LUNARIS BioChip, which is equipped with immobilized beads that carry specific capture antibodies against particular target proteins, and the presence of chemokine in each sample was detected after incubation with secondary antibodies and a fluorescent label using the LUNARIS Reader (LRS-001, AYOXXA Biosystems). Readout quantification was performed using LUNARIS Analysis Suite (AYOXXA Biosystems). CCL19 levels in spleens were determined using an ELISA (RayBiotech, GA, USA). Spleens from WT and ACKR4-deficient mice were harvested, weighed and crushed through a 70 µm mesh in 5 mL cold PBS. Spleen supernatants were obtained by centrifugation of the single cell suspension at 2000 g for 15min at 4°C and stored at −80 degrees until ELISA was performed according to manufacturer instructions.

Flow cytometry

For single cell suspensions, spleens were homogenized between the frosted ends of two glass slides and filtered through a 100 µm cell strainer. Red blood cells were lysed and samples were blocked for 15 min on ice with anti-Fc receptor antibody culture supernatant (clone 2.4G2, homemade) prior to labeling with the following antibodies for 30 min on ice:

- Anti-CD45 mAb (clone 30-F11, PE), anti-CD31 mAb (clone 390, Alexa Fluor 647), anti-IgD mAb (clone 11-26c.2a, BV510), anti-B220 mAb (clone RA3-6B2, BV510), anti-CD11b (clone M1/70, PerCP), anti-CD1d mAb (clone 1B1, FITC), anti-CD169 mAb (clone D6.112, AF488), anti-F4/80 mAb (clone BM8, PE, all from BioLegend), anti-B220 mAb (clone RA3-6B2, PerCP-Cy5.5), anti-CD3e mAb (145-2C11, PE-Cy7), anti-CD23 mAb (clone B3B4, eFluor660), anti-IgM mAb (clone II/41, PE-Cy7), anti-SIGN-R1 mAb (CD209b, clone eBio22D1, APC, all from eBioscience), and anti-CD21/35 mAb (clone 7G6, PE, BD Biosciences).

In some experiments, DAPI was added to assess live/dead cells. Cells were analyzed with a LSR II (BD Biosciences) and data were processed with FlowJo 9.9 – 10 (TreeStar).

Adaptive T cell transfer

Untouched T cells were isolated from spleens and peripheral lymph nodes of C57BL/6N or Ccr7−/− mice using a MACS Pan T Cell Isolation Kit II and an AutoMACS separator (both from Miltenyi Biotec) according to the manufacturer’s protocol. Isolation yielded a purity of 90 – 98%. Purified cells were labeled with the cell proliferation dye eFluor™ 670 (eBioscience) or CellTracker Violet, Orange or Yellow (Thermo Fisher Scientific, Invitrogen) at 37°C for 10 min or 20 min, respectively, before depending on the experiment 1-10x10⁶ cells were intravenously injected into WT and Ackr4−/− mice. For competitive homing of WT and Ccr7−/− untouched T cells were isolated from respective spleens, stained with CellTrace Violet and Yellow, counted, mixed 1:1 and injected at 10⁶ into the tail veins of WT or ACKR4-deficient recipients. Recipients were sacrificed in different experiments 5, 10, 15, 20, 30 or 120 min after the injection and spleens harvested and either fixed in 4% buffered PFA, frozen and processed for fluorescent immunohistochemistry analysis or processed for the enumeration of transferred cells in flowcytometry using CountBright beads, as described above. The number of transferred T cells within the T cell zone was determined using the Analyze Particle function in the Fiji software package after manual definition of the TCZ based on counterstaining with an anti-CD3 mAb (clone 17A2, homemade).
QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis throughout the whole manuscript was performed using Prism 4 (GraphPad Software, Inc.). Statistical significance between groups was determined as indicated in figure legends. Statistical significance for CCl19 levels in serum was determined using an unpaired t test, comparing n = 8 mice per group (see details in legend for Figure 3). For MZ area, statistical significance was verified using an unpaired t test, comparing the data of n = 8 mice. Relative proportion and absolute cell counts of distinct cell populations in n = 8–11 mice were analyzed for statistical significance using an unpaired t test (see details in legend for Figure 4). Statistical significance for the number of transferred WT or Ccr7−/− T cells was demonstrated using an unpaired t test with n = 5–8 mice per group (see details in legend for Figure 7).