ALK1 signaling is required for the homeostasis of Kupffer cells and prevention of bacterial infection

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**Graphical abstract**

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Macrophages are highly heterogeneous immune cells that fulfill tissue-specific functions. Tissue-derived signals play a critical role in determining macrophage heterogeneity. However, these signals remain largely unknown. The BMP receptor activin receptor-like kinase 1 (ALK1) is well known for its role in blood vessel formation; however, its role within the immune system has never been revealed to our knowledge. Here, we found that BMP9/BMP10/ALK1 signaling controlled the identity and self-renewal of Kupffer cells (KCs) through a Smad4-dependent pathway. In contrast, ALK1 was dispensable for the maintenance of macrophages located in the lung, kidney, spleen, and brain. Following ALK1 deletion, KCs were lost over time and were replaced by monocyte-derived macrophages. These hepatic macrophages showed significantly reduced expression of the complement receptor VSIG4 and alterations in immune zonation and morphology, which is important for the tissue-specialized function of KCs. Furthermore, we found that this signaling pathway was important for KC-mediated *Listeria monocytogenes* capture, as the loss of ALK1 and Smad4 led to a failure of bacterial capture and overwhelming disseminated infections. Thus, ALK1 signaling instructs a tissue-specific phenotype that allows KCs to protect the host from systemic bacterial dissemination.

**Introduction**

Kupffer cells (KCs) are resident macrophages in the liver and constitute 80%–90% of the tissue macrophages present in the body. They are predominantly derived from fetal liver monocytes (1–4). Embryonic KCs (em-KCs) are self-maintained and are not replaced by circulating monocytes under homeostatic conditions (1). However, if these em-KCs are deleted, blood monocytes also generate self-renewing KCs (5). Furthermore, regardless of their origin, embryonically derived and monocyte-derived KCs (Mo-KCs) have nearly identical transcriptional signatures, implying that shared signals control the specific transcriptional program of KCs (5). Recently, Bonnardel et al. and Sakai et al. proposed that during monocyte differentiation into KCs, these cells are sequentially programmed by signals derived from surrounding cells in the liver (6, 7). However, the signals provided by the liver niche that maintain the phenotype and survival of KCs remain to be investigated.

The liver is a primary site for the clearance of circulating bacteria, given its highly vascular architecture combined with a unique network of intravascular KCs (8–10). Unlike tissue-resident macrophages in other organs, KCs are strategically positioned in liver sinusoids and are directly exposed to slow-flowing sinusoidal blood, where they constantly trap and phagocytose circulating bacteria including gram-positive *Staphylococcus aureus* and *Listeria monocytogenes* (11–14), thus suggesting that KCs form an intravascular immune defense that prevents bacterial dissemination by capturing and clearing bacteria. However, little is known about the role of the liver environment in maintaining the integrity of KC-mediated intravascular defenses.

Activin receptor–like kinase 1 (ALK1, also known as ACVRL1) is a type I receptor of the TGFβ receptor superfamily with 2 ligands, BMP9 and BMP10 (15). ALK1 is predominantly expressed in endothelial cells and plays a critical role in regulating developmental and pathological angiogenesis (16). However, unlike TGFβR2 signaling, the role of ALK1 in the immune system has not been reported to date. Here, we found that BMP9/BMP10/ALK1 signaling controlled the specific gene expression program and survival of KCs through a Smad4-dependent pathway. Functionally, the loss of ALK1 resulted in impaired capture of *L. monocytogenes* and overwhelming disseminated infections. Taken together, our data reveal a previously unappreciated role of ALK1 signaling in maintaining KC homeostasis and function.

**Results**

Loss of Alk1, rather than of Tgfbir2, Alk2, or Alk3, leads to an altered phenotype of KCs. Recently, Clec4F was identified as a specific surface marker for KCs (5). To specifically target KCs, we first generated *Clec4fflcre/DTR* mice (hereafter referred to as *Clec4fflcre*), in which an expression cassette encoding an internal ribosomal entry site (IRES), the Cre enzyme, a self-cleaving 2A peptide, and the human
diphtheria toxin receptor (DTR) was inserted into the 3’-UTR of the Clec4f gene. We crossed Clec4f<sup>Cre</sup> mice with a conditional reporter strain (R26-tdTomato) to determine the efficiency and specificity of Cre-mediated recombination using flow cytometry. In the liver, the Clec4f<sup>Cre</sup> strain efficiently recombined in CD64<sup>+</sup>F4/80<sup>+</sup> KCs (>90%), and almost all tdTomato<sup>+</sup> cells were KCs (Supplemental Figure 1, A and B, and Supplemental Figure 2A; supplemental material available online with this article; https://doi.org/10.1172/JCI10489/DS1). Furthermore, we did not detect tdTomato expression in other tissues (including CD45<sup>+</sup> and CD45<sup>+</sup> cells) (Supplemental Figure 1, C and D). Immunostaining experiments also confirmed that the reporter gene tdTomato was exclusively expressed in KCs and not in hepatocytes (Supplemental Figure 1E). In addition, 24 hours after diphtheria toxin (DT) administration, KCs were efficiently deleted in Clec4f<sup>Cre</sup> mice, as determined by immunostaining and flow cytometry (Supplemental Figure 1, F and G). Thus, the Clec4f<sup>Cre</sup> strain is a useful tool to specifically target KCs.

Recently, the expression of both the Id1 and Id3 genes was reported to be restricted to KCs compared with other tissue-resident macrophages, and Id3 deficiency impairs the differentiation of KCs (17). Loss of Id3 results in reduced numbers of KCs (17). Interestingly, Id1 is upregulated in Id3-deficient KCs, suggesting that Id1 may compensate for the function of Id3. The transcription factors Id1 and Id3 are target genes of BMP signaling (18), and thus an intriguing speculation is that BMPs present in the liver environment might be one of the tissue-derived signals that regulates KCs.

We first analyzed the expression of genes encoding BMP receptors and their coreceptors using data from the ImmGen Consortium to investigate which BMP signaling pathway regulates KCs and found that genes encoding BMPR2 and endoglin were expressed at high levels in KCs compared with expression levels in other tissue-resident macrophages (Supplemental Figure 3). Endoglin is required for BMP9/ALK1 signaling (19), and BMP9 is specifically expressed in the liver; therefore, a reasonable hypothesis is that ALK1 signaling might be important for KCs.

We generated Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice to test this hypothesis. We also prepared Alk2<sup>fl/fl</sup> Alk3<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice. ALK1, ALK2, and ALK3 belong to the superfamily of TGFβ receptors, and TGFβ signaling has been proposed to be important for KCs (7). Thus, we also generated Tgfr2<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice. Alk2, Alk3, and Tgfr2 were efficiently deleted in KCs from Alk2<sup>fl/fl</sup> Alk3<sup>fl/fl</sup> Clec4f<sup>Cre</sup> and Tgfr2<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice, respectively, but the deficiency of these genes did not affect the expression of Id1 and Id3 (Supplemental Figure 4, A and B). In contrast, ablation of Alk1 resulted in a dramatic reduction in the expression of Id1 and Id3 in CD64<sup>+</sup>F4/80<sup>+</sup> hepatic macrophages from Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice, although the expression of Alk2 was reduced by approximately 60% in these macrophages (Figure 1A). We further analyzed the total hepatic macrophage population in Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice. We observed no difference in the number of KCs between Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> and Alk1<sup>fl/fl</sup> mice (Figure 1B), but the KC surface phenotype in Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice was altered, with a reduced population of Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs and increased populations of Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs, Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs, and Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs (Figure 1C). In addition, no difference was observed in the cell number and surface phenotype of KCs between Alk2<sup>fl/fl</sup> Alk3<sup>fl/fl</sup> Clec4f<sup>Cre</sup>, Tgfr2<sup>fl/fl</sup> Clec4f<sup>Cre</sup>, and their littermate controls (Supplemental Figure 4, C-F). Taken together, these results suggested that ALK1, rather than ALK2, ALK3, and TGFβR2, is responsible for regulating the expression of Id1 and Id3 in KCs and plays an important role in maintaining the KC surface phenotype.

ALK1 is required for the identity of KCs. During homeostasis, nearly all KCs are Clec4f<sup>+</sup>Tim4<sup>+</sup> cells. However, upon KC loss, newly arrived monocyte-derived KCs (MoKCs) are initially Clec4f<sup>+</sup>Tim4<sup>+</sup> cells and then differentiate into Clec4f<sup>+</sup>Tim4<sup>+</sup> cells (5). Among these Clec4f<sup>+</sup>Tim4<sup>+</sup> MoKCs, only some acquire the expression of Tim4 (5). Thus, if KCs are constantly replaced by monocytes, 3 hepatic macrophage subsets are usually detected, based on the expression of Clec4f and Tim4, including Clec4f<sup>+</sup>Tim4<sup>+</sup>, Clec4f<sup>+</sup>Tim4<sup>+</sup>, and Clec4f<sup>+</sup>Tim4<sup>+</sup> macrophages (20, 21). Interestingly, in addition to these macrophage subpopulations, we identified a population of Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs in Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice (Figure 1C) that, to our knowledge, has not been reported previously. Indeed, Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs were also detected in the livers of tamoxifen-treated Alk1<sup>fl/fl</sup> UBC<sup>Clec4fCre</sup> mice (Figure 1D), in which we assessed Clec4f and Tim4 expression 5 days after tamoxifen treatment. Thus, these results suggested that the presence of Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs may have been caused by Alk1 loss.

We next performed single-cell RNA-Seq (scRNA-Seq) analysis of sorted CD64<sup>+</sup>F4/80<sup>+</sup> Tim4<sup>+</sup> KCs from Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice compared with their controls to understand how ALK1 affected KCs. After sequencing, aggregation of the samples, and removal of poor-quality and contaminating cells, 13,690 cells remained (6295 cells from Alk1<sup>fl/fl</sup> mice and 7395 cells from Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice). We identified 5 clusters by generating a uniform manifold approximation and projection (UMAP) from the transcriptome data using the Seurat pipeline (Figure 2A). Cluster 0 was predominantly composed of cells originating from Alk1<sup>fl/fl</sup> mice, whereas cells in clusters 1 and 2 mainly originated from Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice (Figure 2A). Because we were unable to determine which cells expressed full-length or floxed mRNA using the S’ Assay from 10X Genomics, we next analyzed the differentially expressed (DE) genes between these clusters to find markers that could distinguish the different cell populations by flow cytometry. scRNA-Seq analysis revealed 243 DE genes in cluster 0, 347 DE genes in cluster 1, 182 DE genes in cluster 2, 189 DE genes in cluster 3, and 383 DE genes in cluster 4 (Supplemental Table 1) and showed that Clec4f was expressed in clusters 0, 2, 3, and 4, but not in cluster 1 (Figure 2A and Supplemental Figure 5A). Interestingly, we found that expression of Id1 and Id3 was also substantially reduced in cluster 1 (Figure 2A). Given that Id1 and Id3 are target genes of ALK1 signaling in KCs, these results suggested that Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs (cluster 1) may be deficient in Alk1. We performed quantitative PCR (qPCR) and genomic PCR on sorted Clec4f<sup>+</sup>Tim4<sup>+</sup> and Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs from Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice to verify this result and found that Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs efficiently deleted Alk1, whereas Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs were heterozygous for the Alk1 deletion (Supplemental Figure 5, B and C). KCs from Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice did not display a phenotype similar to that of Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice (Supplemental Figure 5D), suggesting no obvious effect of Alk1 haploinsufficiency on KCs.

In Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice, Cre recombines is expressed under the control of the Clec4f promoter, implying that Clec4f was once expressed in Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs. We prepared Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> R26<sup>+</sup> reporter mice to examine this possibility and observed high expression of the YFP reporter gene in Clec4f<sup>+</sup>Tim4<sup>+</sup> KCs and
Clec4F−Tim4+ KCs, but very low YFP expression in Tim4+ KCs (Figure 2B), suggesting that Clec4F−Tim4+ KCs once expressed Clec4F. Moreover, upon ALK1 deletion, KCs no longer expressed Clec4F. We further prepared Alk1fl/fl Clec4fCre mice (homozygous for Cre) to increase the recombination frequency and to support this hypothesis and found that KCs from these mice did not express Clec4F. We identified Alk1+/− KCs from these mice (homozygous for Cre) to increase the recombination frequency and to support this hypothesis and found that KCs from these mice did not express Clec4F and Tim4 in KCs (Figure 2C).

Based on the results described above, we identified cluster 1 as Alk1+/− KCs from Alk1fl/fl Clec4fCre mice and cluster 0 as Alk1+/− KCs from Alk1fl/fl mice. Cells in cluster 2 from Alk1fl/fl Clec4fCre mice were identified as Alk1+/− KCs. Clusters 3 and 4 were proliferating cells expressing DNA replication–associated genes such as Mcm2-7 and Mki67 (Supplemental Figure 5A). Then, we compared the transcriptional profiles between clusters and found that among the 25 top core genes described previously (20), the expression of Tim4 in KCs from Alk1fl/fl mice and cluster 0 as Alk1+/− KCs. Clusters 3 and 4 were proliferating cells expressing DNA replication–associated genes such as Mcm2-7 and Mki67 (Figure 2D). Representative flow cytometric data and total number of CD64+F4/80+ KCs (pregated on CD45+Ly6C−) from Alk1fl/fl and Clec4fCre/Cre control mice at the age of 8 weeks (n = 6 per group). The gating strategy for KCs is shown in Supplemental Figure 2A. 

**Figure 1. ALK1 controls the KC surface phenotype.** (A) qPCR analysis of Alk1, Id1, and Id3 expression in sorted KCs (CD45+Ly6C−CD64+F4/80+ from Alk1fl/fl mice and Alk1fl/fl controls (n = 5–6 per group). **Figure 1. ALK1 controls the KC surface phenotype.** (B) Representative flow cytometric data and total number of CD64+F4/80+ KCs from Alk1fl/fl mice (pregated on CD45+Ly6C−) and Alk1fl/fl controls (n = 7–10 per group). (C) Flow cytometric expression of Clec4F and Tim4 in KCs (pregated on CD45+Ly6C−CD64+F4/80+) and the percentage of Clec4F+Tim4+, Clec4F−Tim4+, Clec4F+Tim4−, and Clec4F−Tim4− KCs from Alk1fl/fl Clec4fCre mice and Alk1+/− control mice at the age of 8 weeks (n = 6 per group). (D) Alk1fl/fl UBCCreERT2 mice were treated or not with tamoxifen (10 mg) 2 times every other day via oral gavage, and Clec4F and Tim4 expression in KCs was assessed 5 days after the last treatment. Data are representative of at least 3 independent experiments. Results represent the mean ± SEM. ***P < 0.001 and ****P < 0.0001, by 2-tailed Student’s t test (A–C).

The transcription factors Zeb2 and Nrrl3 are required for the identity of KCs (20). We found that the expression of Nrrl3, but not Zeb2, was significantly decreased in Alk1+/− KCs (Figure 2D and Supplemental Table I). The transcription factor SPI-C is required for the development of splenic red pulp macrophages (RPMs), and its expression is induced by heme (22, 23). SPI-C expression was significantly upregulated in the absence of ALK1 (Figure 2E), but the CD163 and CD91 (encoded by Lrp1) receptors that uptake circulating hemoglobin-haptoglobin and heme-hemopexin complexes, respectively, were downregulated (Figure 2E). Based on these results, the ALK1 signaling pathway is required for KC identity and may negatively regulate Spic expression in KCs.

The maintenance of KCs requires ALK1 signaling. Decreased expression of Tim4 in KCs from Alk1+/− Clec4fCre mice suggested that circulating monocytes might replenish liver macrophages. We generated shielded chimeras in which the livers of Alk1+/− Clec4fCre mice and Alk1+/− littermate controls were shielded during irradiation, and these mice were reconstituted with congenic CD45.1 WT BM to examine this possibility (Figure 3A). As expected, partial shielding resulted in mixed chimerism in blood Ly6C− monocytes in all groups (Figure 3B). KCs in Alk1+/− mice were not chimeric, as KCs were self-maintained under steady-state conditions independent of circulating monocytes (Figure 3B). However, hepatic macrophages from Alk1+/− Clec4fCre mice displayed chimerism (Figure 3B), suggesting that the ALK1 deficiency may have led to a loss of KCs and that circulating monocytes repopulated the empty niche to maintain the macrophage pool in the liver. Consistent with this result, the number of Clec4F+Tim4+ KCs decreased with age, and Tim4− Mo-KCs expanded significantly over time (Figure 3C). 5-ethyl-20-deoxyuridine (EdU) incorporation assays revealed that Clec4F+Tim4+ KCs had a reduced capacity to proliferate compared with their counterparts (Figure 3D), indicating that a decrease in the proliferation of Alk1-deficient KCs leads to a severe disadvantage of these cells under competitive conditions. Maf and Mafb function as negative regulators of KC proliferation (24). Consistent with the impaired proliferation, we found that

**Figure 1. ALK1 controls the KC surface phenotype.** (A) qPCR analysis of Alk1, Id1, and Id3 expression in sorted KCs (CD45+Ly6C−CD64+F4/80+) from Alk1fl/fl Clec4fCre mice and Alk1fl/fl controls (n = 5–6 per group). The gating strategy for KCs is shown in Supplemental Figure 2A. (B) Representative flow cytometric data and total number of CD64+F4/80+ KCs (pregated on CD45+Ly6C−) from Alk1fl/fl mice and Alk1fl/fl controls (n = 7–10 per group). (C) Flow cytometric expression of Clec4F and Tim4 in KCs (pregated on CD45+Ly6C−CD64+F4/80+) and the percentage of Clec4F+Tim4+, Clec4F−Tim4+, Clec4F+Tim4−, and Clec4F−Tim4− KCs from Alk1fl/fl Clec4fCre mice and Alk1+/− control mice at the age of 8 weeks (n = 6 per group). (D) Alk1fl/fl UBCCreERT2 mice were treated or not with tamoxifen (10 mg) 2 times every other day via oral gavage, and Clec4F and Tim4 expression in KCs was assessed 5 days after the last treatment. Data are representative of at least 3 independent experiments. Results represent the mean ± SEM. ***P < 0.001 and ****P < 0.0001, by 2-tailed Student’s t test (A–C).
Maf was expressed at higher levels in Alk1-deficient KCs than in their counterparts (Figure 2E). Thus, these results suggested that ALK1 may be required for the maintenance of KCs.

To determine whether ALK1 deficiency results in KC disappearance, we established BM chimeras in which CD45.1+ mice were lethally irradiated and injected with congenic CD45.2+ Alk1fl/fl UBCCreERT2 BM, in which tamoxifen administration leads to deletion of Alk1 in a wide range of cells (25). The chimeric mice were treated with tamoxifen 8 weeks after reconstitution. Based on a previous report (5), Mo-KCs are able to differentiate into mature Clec4F+ KCs, but only some of these cells acquire Tim4 expression. Our results also confirmed this finding (Figure 3E). Consistent with the aforementioned observation that ALK1 is important for Clec4F expression, we observed reduced expression of Clec4F in Tim4+ and Tim4- KCs after tamoxifen administration (Figure 3E). Because a good antibody is unavailable to stain ALK1 for flow cytometry and we cannot exclude the possibility that Clec4F–Tim4– KCs were derived from newly arrived WT MoKCs, we used Clec4F–Tim4+ KCs to represent Alk1-deficient cells and determined Clec4F expression in Tim4+ KCs originating from CD45.2+ donor cells 5, 10, and 25 days after the last treatment with tamoxifen. Chimeras not treated with tamoxifen were used as controls. Approximately 30% of donor-derived Tim4+ KCs were Clec4F+ on days 5 and 10 after the last treatment, whereas Clec4F + Tim4+ KCs were no longer detected in the liver 25 days after the last treatment (Figure 3E), indicating that Alk1-deficient KCs were lost over time.

ALK1 is dispensable for the maintenance of macrophages located in the lung, kidney, brain, and spleen. To assess whether ALK1...
reconstitution following transplantation (26). Recipient mice (CD45.1) were lethally irradiated and reconstituted with equal amounts of WT (CD45.1/CD45.2) and Alk1fl/fl Vav1Cre (CD45.2) BM or WT (CD45.1/CD45.2) and Alk1fl/fl (CD45.2) BM (Figure 4A). After

signaling is required for the maintenance of other tissue-resident macrophages, we generated mixed BM chimeras. We used Alk1fl/fl Vav1Cre mice, in which Cre recombinase is expressed at high levels in hematopoietic stem cells and maintains robust activity during

Figure 3. Maintenance of KCs requires ALK1 signaling. (A) Schematic of the experimental setup. (B) Expression of CD45.1 (donor) and CD45.2 (recipient) in blood monocytes and total KCs from Alk1fl/fl and Alk1fl/fl Clec4f cre chimeras. Plot shows the percentage of total chimerism of KCs in Alk1fl/fl and Alk1fl/fl Clec4f cre chimeras (n = 6 per group). The experimental setup was as indicated in A. (C) Expression of Clec4F and Tim4 and percentage of Clec4F–Tim4+ and Tim4– cells in KCs from Alk1fl/fl Clec4f Cre mice and Alk1fl/fl control mice at 2, 5, and 12 weeks of age (n = 7–12 per group). (D) Representative flow cytometric data and quantification of EdU incorporation in Clec4F–Tim4+, Clec4F+Tim4+, and Tim4– KCs from Alk1fl/fl Clec4f Cre mice and Alk1fl/fl controls (n = 6–7 per group). (E) The chimeric mice were treated with tamoxifen (10 mg) 2 times every other day via oral gavage. Clec4F and Tim4 expression in KCs originated from CD45.2+ donor cells was assessed 5, 10, and 25 days after the last treatment (n = 4–8 per group). Results represent the mean ± SEM. **P < 0.01, ***P < 0.001, and ****P < 0.0001, by 2-tailed Student’s t test (B) and 1-way ANOVA (C–E).
In 8 weeks, we examined the origin of KCs. In this competitive setting, approximately all KCs originated from WT (CD45.1/CD45.2) BM in WT (CD45.1/CD45.2) and *Alk1*fl/fl*Vav1Cre* (CD45.2) chimeras compared with that seen in WT (CD45.1/CD45.2) and *Alk1*fl/fl (CD45.2) chimeras (Figure 4B), suggesting that liver macrophages deficient in *Alk1* are outcompeted by their WT counterparts. In contrast, blood monocytes and other tissue macrophages, including lung macrophages, kidney macrophages, brain macrophages, and splenic macrophages, were reconstituted at an equal ratio in WT (CD45.1/CD45.2) and *Alk1*fl/fl*Vav1Cre* (CD45.2) chimeras (Figure 4, C–G). Taken together, these results suggested that ALK1 is dispensable for the survival of macrophages located in the lung, brain, kidney, and spleen.

**BMP9 and BMP10 instruct KC signature gene expression.** Both BMP9 and BMP10 are ligands of ALK1. In the results described above, we showed that ALK1, rather than ALK2 and ALK3, was required for the expression of *Id1*, *Id3*, and *Clec4f*. Consistent with these results, BMP9 and BMP10 treatment maintained higher expression of *Id1*, *Id3*, and *Clec4f* in cultured KCs than did BMP2 (ALK3 ligand) or BMP6 (ALK2 ligand) (Figure 5A). However, *Clec4f* expression in KCs from *Bmp9*-KO mice was unaltered compared with that in KCs from WT littermate controls (Figure 5B). In fact, it has been reported that BMP10 is able to compensate for the loss of BMP9 (27, 28). To verify this, we administered an anti-BMP10 neutralizing antibody to *Bmp9*-KO mice and found that it resulted in decreased expression of *Clec4f*, whereas an injection of this antibody into WT mice did not affect its expression (Figure 5B). qPCR analysis revealed that KC-specific genes, such as *Id1*, *Id3*, *Clec4f*, *Fabp7*, *Cd5l*, and *Cdh5*, were most significantly...
downregulated in KCs from Bmp9-KO mice treated with the anti-BMP10 antibody compared with their controls (Figure 5C). Thus, both BMP9 and BMP10 are important for maintaining KC identity.

**ALK1 signaling functions in KCs through the canonical Smad pathway**

Smad4 functions as a common Smad required for transcriptional regulation in response to BMPs. We generated Smad4fl/fl Clec4fCre mice to assess whether ALK1 signaling functions in KCs via the Smad pathway. Similar to the effect of Alk1 deletion, KCs from Smad4fl/fl Clec4fCre mice displayed altered expression of Clec4F and Tim4 (Figure 6A). In protected chimeras, Smad4 deficiency led to the replenishment of KCs from monocytes (Figure 6B). Similar to our findings in Alk1fl/fl UBCCreERT2 mice, Clec4F+Tim4+ cells were also detected in KCs from tamoxifen-treated Smad4fl/fl UBCCreERT2 mice (Figure 6C). We sorted Clec4F+Tim4+ and Clec4F–Tim4+ KCs and examined the expression of Smad4, Id1, and Id3. qPCR analysis revealed that Clec4F+Tim4+ KCs had efficiently deleted Smad4, whereas Clec4F–Tim4+ KCs maintained the expression of Smad4 at levels comparable to those in KCs isolated from untreated Smad4fl/fl UBCCreERT2 mice (Figure 6D). Accordingly, its target genes, Id1 and Id3, were significantly reduced in Clec4F+Tim4+ KCs but not in Clec4F–Tim4+ KCs. In summary, we found that ALK1 signaling regulated KCs through the canonical Smad pathway.

**The functional phenotype of KCs is maintained by ALK1 signaling.**

To investigate the functional consequences of ALK1 deletion in KCs, we generated ALK1 conditional-KO mice, in which Alk1 was completely targeted in these cells. Alk1 was not efficiently deleted in KCs from Alk1fl/fl Clec4fCre mice, so we analyzed Alk1fl/fl Vav1Cre mice. qPCR analysis revealed that Alk1 was successfully deleted in the total population of CD64+F4/80+ hepatic macrophages (Supplemental Figure 6A). Moreover, Id1 and Id3 expression was significantly reduced in KCs from Alk1fl/fl Vav1Cre mice compared with those from Alk1fl/fl mice (Supplemental Figure 6A), and Clec4F expression was not detected in either Tim4+ or Tim4– KCs from Alk1fl/fl Vav1Cre mice (Supplemental Figure 6A). We also analyzed the number of hepatic myeloid cells, including KCs, neutrophils, monocytes, plasmacytoid DCs (pDCs), and conventional DCs (cDCs), and found that these cell counts were normal in Alk1fl/fl Vav1Cre mice (Supplemental Figure 6, C–F). Similarly, Smad4 was also efficiently targeted in KCs from Smad4fl/fl Vav1Cre mice (Supplemental Figure 6, G and H).

In addition, we examined E-cadherin and glutamine synthetase expression and interactions between KCs, liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs) in Alk1fl/fl Vav1Cre and Smad4fl/fl Vav1Cre mice to determine whether the liver architecture was affected by ALK1 and Smad4 deficiency. We found...
confirmed this significant reduction in KCs from Alk1fl/fl Vav1Cre and Smad4fl/fl Vav1Cre mice (Figure 7, A–D).

In addition to the cell surface phenotype, KC function is also governed by the cell’s 3D morphology within the vasculature, as its elongated and branched shape increases the intravascular surface area available for interacting with circulating pathogens (9). KCs are strategically enriched near periportal regions. This positional asymmetry (immune zonation) is important for KCs to protect against the systemic dissemination of pathogens from local infection sites such as the digestive tract (29). Interestingly, Alk1fl/fl Vav1Cre and Smad4fl/fl Vav1Cre mice lacked the periportal polarization of KCs observed in their controls (Figure 8, A and B). In addition, KCs from Alk1fl/fl Vav1Cre and Smad4fl/fl Vav1Cre mice were generally smaller, with a decreased cell surface area and volume compared with KCs from Alk1fl/fl control mice (Figure 9, A and B). Taken

that E-cadherin and glutamine synthetase were expressed in the perportal and central vein regions of the liver lobules, respectively. The regional localization of E-cadherin and glutamine synthetase in the liver lobules of Alk1fl/fl Vav1Cre and Smad4fl/fl Vav1Cre mice was not affected (Supplemental Figure 7, A and C), suggesting that the liver architecture was intact. VSIG4 (also known as CRIg), a new family of complement receptors, was reported to be expressed by KCs at high levels (13) and plays an important role in KC-mediated capture of gram-positive bacteria (11, 13, 14). qPCR analysis revealed that Vsig4 was obviously upregulated after BMP9 and BMP10 treatment (Figure 5A). The transcriptomic analysis revealed that Vsig4 expression was significantly decreased following the deletion of Alk1 in KCs (Figure 2D). Confocal microscopy and flow cytometry also
together, ALK1 signaling plays an important role in maintaining the functional phenotype of KCs.

The ALK1 signaling pathway in KCs protects the host from infection with *L. monocytogenes*. Next, we infected Alk1fl/fl Vav1Cre and Alk1fl/fl mice with *L. monocytogenes* and found that approximately 80% of Alk1fl/fl Vav1Cre mice died within 7 days of infection, whereas no Alk1fl/fl mice succumbed to the infection (Figure 10A). Similarly, Smad4fl/fl Vav1Cre mice exhibited higher mortality following *L. monocytogenes* infection than did Smad4fl/fl control mice (Supplemental Figure 8A). Vav1-Cre not only deletes floxed genes in KCs, but also in other cell types, such as hematopoietic stem cells and their progenies. To examine whether the influence of ALK1 on controlling *L. monocytogenes* infection is KC intrinsic, we infected Alk1fl/fl Clec4fCre/Cre and Alk1fl/fl mice with *L. monocytogenes* and found that Alk1fl/fl Clec4fCre/Cre mice were more susceptible to *L. monocytogenes* infection than were their controls (Figure 10B), suggesting that ALK1 has a KC-intrinsic role in protecting the host from *L. monocytogenes* infection. Moreover, we used intravital microscopy (IVM) to visualize and quantify the bacterial capture within the liver and to understand the mechanism underlying the increased susceptibility to infection and found that Alk1/Smad4-deficient mice showed a significant reduction in the capture of circulating *L. monocytogenes* by KCs (Figure 10, C and D, Supplemental Figure 8B, and Supplemental Videos 1–3), despite having a similar number of KCs compared with their controls (Supplemental Figure 7, E and F). As a result, rapid systemic bacterial dissemination occurred in Alk1fl/fl Vav1Cre mice, with less *L. monocytogenes* in the liver but significantly more bacteria in the lung and blood (Figure 10E). These results show that the ability of KCs to capture bacteria from the bloodstream was significantly impaired in Alk1fl/fl Vav1Cre mice, resulting in systemic bacterial dissemination and increased mortality of the host.

KCs are essential for host survival in *L. monocytogenes* infection, but most *L. monocytogenes* are not killed by the KCs (30). Elimination of the pathogen in *L. monocytogenes* infection requires the recruitment of neutrophils to the liver and the specific binding of these neutrophils to KCs (30). We found that neutrophil recruitment to the liver occurred 2 hours after infection, and we observed no difference in their recruitment and interaction with KCs between Alk1fl/fl Vav1Cre and Alk1fl/fl mice (Supplemental Figure 9).

IFN-γ is essential for the innate defense against *L. monocytogenes* infection (31). We observed increased expression of intracellular IFN-γ in NK cells, CD4+ T cells, and CD8+ T cells from Alk1fl/fl Vav1Cre mice 24 hours after infection (Supplemental Figure 10A), which coincided with higher bacterial counts in the livers and spleens of Alk1fl/fl Vav1Cre mice than in Alk1fl/fl mice (Supplemental Figure 10B). Recently, it has been shown that IL-17A plays a critical role in innate defense against *L. monocytogenes* infection in the liver (32). Consistent with the previous report (32), we found that IL-17A

![Figure 7. ALK1/Smad4 signaling is important for KCs to maintain the expression of VSIG4.](https://doi.org/10.1172/JCI150489)
their tissue of residence, they undergo extensive differentiation according to molecular cues provided by their tissue-specific niche (33). This process enables these progenitors to develop into specialized tissue-resident macrophages with a unique transcription profile. However, the precise signals governing this process remain largely unknown. Here, we identified a critical role for BMP9 /BMP10 /ALK1 signaling in imprinting KC identity. Loss of ALK1 impaired the ability of KCs to proliferate. Notably, we found that ALK1 was dispensable for the survival of macrophages in many organs, which demonstrated a specific role in the maintenance of KCs.

Clec4F is selectively expressed in mature KCs. In contrast to F4/80, which is a constitutively expressed surface marker for resident macrophages (34, 35), Clec4F is inducible in the liver microenvironment (36). Embryonic progenitors and monocytes progressively acquire Clec4F expression upon entry into the liver (5, 36). Thus, studies aiming to understand what types of signals or molecules are necessary to regulate Clec4F expression in the liver microenvironment would be interesting (36). We and other researchers (6) showed that BMP9 stimulation induces the expression of Clec4F. In the present study, in vivo blockade of ALK1 signaling by injecting anti-BMP10–blocking antibody into Bmp9 KO mice resulted in decreased expression of Clec4F in KCs from Alk1 fl/fl Vav1 Cre mice. Together, these results suggested that ALK1 signaling is essential for Clec4F expression in KCs.

Discussion

Most tissue-resident macrophages were derived from yolk sac macrophages or fetal liver monocytes. Once progenitors arrive at their tissue of residence, they undergo extensive differentiation according to molecular cues provided by their tissue-specific niche (33). This process enables these progenitors to develop into specialized tissue-resident macrophages with a unique transcription profile. However, the precise signals governing this process remain largely unknown. Here, we identified a critical role for BMP9/BMP10/ALK1 signaling in imprinting KC identity. Loss of ALK1 impaired the ability of KCs to proliferate. Notably, we found that ALK1 was dispensable for the survival of macrophages in many organs, which demonstrated a specific role in the maintenance of KCs.
BMP9 is preferentially expressed in the liver. In the present study, in addition to BMP9, BMP10 was critical for controlling the identity of KCs. BMP10 is mainly expressed in the heart and present in blood (27). However, this circulating BMP10 is unable to activate the ALK1 signaling pathway (27). In fact, it is also weakly expressed in the liver (37). The source of BMP9 and BMP10 was reported to be HSCs (38, 39), indicative of a paracrine loop that regulates KC identity and self-maintenance.

KCs are enriched near periportal regions. This asymmetric localization also has a critical role in protecting against systemic bacterial dissemination (29). Interestingly, we found that hepatic macrophages lost their tissue-specific localization in Alk1fl/fl and Smad4fl/fl mice. These KCs were not affected (our unpublished observations), suggesting that the alteration of anatomical localization was not due to the lack of CXC3 expression. In fact, monocytes constantly replenished the macrophage pool in the livers of Alk1fl/fl mice and Smad4fl/fl mice. CXCR3 expressed on KCs has been reported to play a role in shaping the positioning of resident immune cells in the liver (29). However, CXCR3 expression in hepatic macrophages from Smad4fl/fl Vav1Cre mice was not affected (our unpublished observations), suggesting that the alteration of KCs during liver injury or infection in mice, KC homeostasis was also disrupted, with loss of resident KCs and replenishment of monocyte-derived macrophages (43–45). More important, the alteration of KC homeostasis also occurs in human liver diseases, as the number of liver macrophages is significantly reduced in patients with liver fibrosis (46). It has been reported that patients with acute liver failure and advanced cirrhosis have a high risk of bacterial infections, and dysfunction of liver macrophages may play a role (46, 47). This suggests that therapeutic interventions aimed to prevent the loss of KCs and/or promote the maturation of newly arrived monocyte-derived macrophages might help reduce susceptibility to infection in these patients.

Recently, Bonnardel et al. and Sakai et al. proposed a 2-step model in which Notch signaling drives the initial commitment of BM monocytes to the KC lineage, and TGF-β family ligands, including TGF-β and BMP9, establish KC identity (6, 7). In addition, Sakai et al. documented an important role for Smad4 in maintaining KC identity (7). The 2 reports provided the background for understanding how engrafted circulating monocytes differentiated into KCs. In the present study, we precluded the roles of TGF-β, BMP2, and BMP6 and showed that, in addition to BMP9, BMP10 also has a critical role in maintaining KC identity. In addition, we also demonstrated that this signaling pathway is important for KCs to accomplish their strategic role in protecting the host from L. monocytogenes infection. Altogether, this study revealed how the liver microenvironment regulates visceral arteriovenous malformations (AVMs) (40). It has been reported that patients with HHT are more susceptible to bacterial infection, especially gram-positive Staphylococcus aureus (41, 42). We also found that Alk1fl/fl Smad4fl/fl mice had a significant reduction in the capture of circulating S. aureus by KCs (Supplemental Figure S12). Thus, it is possible that the high incidence of infectious diseases observed in patients with HHT may be due to the impaired innate immune function of KCs seen in mice lacking ALK1. In addition, during liver injury or infection in mice, KC homeostasis was also disrupted, with loss of resident KCs and replenishment of monocyte-derived macrophages (43–45). More important, the alteration of KC homeostasis also occurs in human liver diseases, as the number of liver macrophages is significantly reduced in patients with liver fibrosis (46). It has been reported that patients with acute liver failure and advanced cirrhosis have a high risk of bacterial infections, and dysfunction of liver macrophages may play a role (46, 47). This suggests that therapeutic interventions aimed to prevent the loss of KCs and/or promote the maturation of newly arrived monocyte-derived macrophages might help reduce susceptibility to infection in these patients.

Figure 9. ALK1/Smad4 signaling is important for KCs to maintain their morphology. (A and B) Rendered surfaces (n = 117) of KCs from Alk1fl/fl Vav1Cre mice (A), Smad4fl/fl Vav1Cre mice (B), and their littermate controls were displayed and arranged by surface area (top) and 3D analysis of the surface area and surface volume of individual KCs (bottom). Data were pooled from 3 mice per group. Results represent the mean ± SEM. ****p < 0.0001, by 2-tailed Student’s t test (A and B).
KCs to maintain their phenotypes and form an effective defense against bacterial infection.

Methods

Mice. *Clec4fCre/DTR* mice were generated at the Nanjing BioMedical Research Institute of Nanjing University (NBRI) using CRISPR/Cas9-mediated genome editing on a C57/B16/J background. *R26tdTomato* mice (stock no. 007914) and *UBC-CreERT2* mice (stock no. 007001) were obtained from The Jackson Laboratory. *Tgfbr2 fl/fl* mice and *Smad4 fl/fl* mice were provided by Xiao Yang (Beijing Institute of Lifeomics). *Bmp9*-KO mice were provided by Se-Jin Lee (Johns Hopkins University, Baltimore, Maryland, USA). *Alk1fl/fl* mice were provided by Zhihong.
Xu (Fudan University, Shanghai, China). Alk2fl/fl mice were provided by Vesa Kaartinen (University of Michigan, Ann Arbor, Michigan, USA). Alk1fl/fl mice were provided by Bing Liu (Fifth Medical Center of Chinese PLA General Hospital, Beijing, China). CD45.1/Ly5.1 mice were provided by Mingzhao Zhu (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China). Mice and their littermates were used between 6 and 16 weeks of age unless otherwise specifically indicated. All mice were maintained at the specific pathogen-free (SPF) facilities of the Beijing Institute of Lifeomics.

Cell suspension preparations, flow cytometry, and antibodies. Cell suspensions were prepared as previously described (48). Briefly, CNS, spleen, lung, and kidney were cut into small pieces, incubated in collagenase type IV (MiliporeSigma) at 37°C for 30 minutes, and vigorously pipetted. The cell suspensions were filtered through a 70μm cell strainer to obtain a homogeneous cell suspension. CNS cell suspensions were further enriched by a Percoll gradient. The liver was perfused via the portal vein with approximately 20 mL HBSS, followed by perfusion with digestion buffer containing 0.05% collagenase type IV for 5 minutes. The digested livers were then excised and disrupted, and the cell suspension was passed through a 70μm cell strainer. Parenchymal cells were separated from nonparenchymal cells by centrifugation at 50g for 5 minutes. Liver cell suspensions were further enriched by iodixanol gradient (OptiPrep) as previously described (49).

For surface marker analysis, cell pellets were stained with the appropriate antibodies at 4°C for 20–30 minutes. For intracellular cytokine analysis, cells were stained with the Cytofix/Cytoperm kit according to the manufacturer’s instructions (eBioscience). To obtain hepatic mononuclear cells, mice were perfused via the portal vein with HBSS, and the livers were minced through a 70 μm cell strainer followed by lysis with RBC lysis buffer. The hepatic mononuclear cells were stimulated with a cell stimulation cocktail (eBioscience) for 6 hours, followed by intracellular IL-17A staining.

Flow cytometry was performed using an LSR II Fortessa (BD Biosciences). The acquired data were analyzed with FlowJo software (Tree Star). For cell sorting, a FACS Aria III (BD Biosciences) was used. The antibodies used are listed in Supplemental Table 2.

In vitro culture of KCs. Sorted KCs by FACS were seeded in 12-well plated in DMEM (HyClone) containing 10% FBS (Gibco, Thermo Fisher Scientific). After overnight, the culture medium was replaced by complete unedited blots int the supplemental material.)

Bacterial burden. Mice were injected with L. monocytogenes (strain 10403s) via the tail vein. The livers and spleens at the indicated post-infection time points were removed and homogenized. Serial dilutions of cell suspensions in PBS containing 1% Triton X-100 were seeded on brain heart infusion (BHI) agar plates. After overnight incubation at 37°C, CFU were counted.

PCR analysis for Alk1 deletion. Liver cells from Alk1fl/fl Clec4fcre mice were first isolated by F4/80 magnetic beads (Miltenyi Biotec) and then sorted by FACS. Genomic DNA was extracted using the QIAamp DNA Micro Kit (QIAGEN). PCR was performed as follows: 95°C for 3 minutes, 35 cycles at 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 45 seconds, followed by a 2-minute incubation at 72°C. PCR primers for floxed Alk1 were as follows: forward, 5’-GCTTGGAGCTGTGCTCTAC-3’; reverse, 5’-GGGAGGAGCCATGTTCTCAG-3’. PCR primers for Alk1 deletion were as follows: forward, 5’-GGACCTGAGAGGACAGAGTAGTCC-3’; reverse, 5’-TGGAGACCTGTCTGAGTCTG-3’. (See complete unedited blots in the supplemental material.)

IVM of liver and L. monocytogenes capture. A multichannel confocal microscope was used to image mouse liver as previously described (50). Briefly, mice were anesthetized (2.5% avertin, 20 mL/kg, i.p., MilliporeSigma). The tail vein was cannulated to administer fluorescent dyes and labeled bacteria. The abdominal cavity was exposed by removing the skin and muscles. The mouse was placed on a heated stage (37°C), and the largest lobe of the liver was positioned onto a coverslip; a small piece of sterile laboratory wipes was moisturized with saline and placed over the liver to keep it moist and stable. For the visualization of liver macrophages, platelets, and neutrophils, 2 μg anti-F4/80 (BM8) or 2.5 μg anti-Ly6G (IA8) (BioLegend) was administered i.v. Images were acquired using an inverted Olympus FY3000 confocal microscope with a 20×/0.75 UPLANSAPO objective lens. Laser excitation wavelengths of 488 nm, 561 nm, and 647 nm and a high-sensitivity spectral detector with a GaAsP incorporation was measured by flow cytometry using the Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions.

qPCR. Total RNA was isolated with RNeasy Plus Mini Kit (QIAGEN), and cDNA was synthesized with Prime Script RT Reagent Kit (Takara). qPCR was performed with a SYBR Green PCR kit in a CFX Connect Real-time PCR detection system (Bio-Rad). The specific qPCR primers used are listed in Supplemental Table 3.

PCR analysis for Alk1 deletion. Liver cells from Alk1fl/fl Clec4fcre mice were first isolated by F4/80 magnetic beads (Miltenyi Biotec) and then sorted by FACS. Genomic DNA was extracted using the QIAamp DNA Micro Kit (QIAGEN). PCR was performed as follows: 95°C for 3 minutes, 35 cycles at 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 45 seconds, followed by a 2-minute incubation at 72°C. PCR primers for floxed Alk1 were as follows: forward, 5’-GCTTGGAGCTGTGCTCTAC-3’; reverse, 5’-GGGAGGAGCCATGTTCTCAG-3’. PCR primers for Alk1 deletion were as follows: forward, 5’-GGACCTGAGAGGACAGAGTAGTCC-3’; reverse, 5’-TGGAGACCTGTCTGAGTCTG-3’. (See complete unedited blots in the supplemental material.)
photomultiplier tube (PMT) or a spectral detector with multialkali PMTs were used for fluorescence detection. For bacteria-catching experiments, bacteria were resuspended in PBS and labeled by incubation with CFSE (100 μM, Invitrogen, Thermo Fisher Scientific) for 30 minutes. Acquisition of images was initiated, labeled bacteria (4 × 10^7 CFU) were injected via the tail vein immediately, and images were acquired every 5 seconds for 25 minutes. Functions of colocalization and spot in the Imaris software were used to identify and quantify captured bacteria within KCs per field of view at every time point.

For the visualization of cell interaction, acquisition of images was initiated, 5 × 10^8 L. monocytogenes was injected via the tail vein, and images were acquired every 10 seconds for 50 minutes. A stitched image of 4 × 4 fields of view was acquired 2 hours after infection to visualize neutrophil recruitment. Functions of colocalization and surface in the Imaris software (Bitplane) were used to measure voxel overlap between 2 cell surfaces and to identify interactions between KCs and platelets or neutrophils.

Immunofluorescence staining. Livers were perfused with 0.5% heparin (ADAMAS-BETA) in 20 mL cold PBS and fixed in 4% paraformaldehyde (PFA) for 12 hours, followed by dehydration in 15% and 30% sucrose before embedment in OCT compound (Sakura Finetek). Sections (50 μm thick) were cut on a CM1950 cryostat (Leica) and adhered to Superfrost Plus slides. Frozen sections were blocked for 2 hours with 1% BSA, 0.3 M glycine, and 10% donkey or goat serum after permeabilization in PBS with 0.2% Triton X-100 for 30 minutes at room temperature, followed by staining with antibodies diluted in PBS with 0.2% Tween-20 for 12 hours at 4°C and with secondary antibodies and Hoechst for 2 hours at room temperature. The following antibodies were used: anti-CD31 (390, BD Biosience), anti-F4/80 (BM8, BioLegend), anti-E-cadherin (DECA M-A1, BioLegend), anti-desmin (polyclone, Abcam), anti-glutamine synthetase (polyclone, Abcam), anti-VSIG4 (NLA14, Invitrogen, Thermo Fisher Scientific), and anti-Clec4F (polyclone, R&D Systems). Fluorescence-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Sections were mounted with Fluoromount G (Yeasen Biotechnology), and images were acquired on a PV3000 confocal microscope with a 20×/0.75 UPLANSAPo or 60×/1.30 Sil UPLANSAPo objective lens. Laser excitation wavelengths of 405 nm, 488 nm, 561 nm, and 647 nm and HyD or PMT detectors were used for fluorescence detection.

Image processing and analysis. All videos and images were processed using Fiji (NIH) or Imaris (Bitplane) software. KC numbers, cell surface area, and cell volume were quantified using the surface function in Fiji. KC numbers, cell surface area, and cell volume were determined on the basis of their contributions to this project.

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