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Declaration of interests

The authors have declared that no conflict of interest exists.
Abstract

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. BMP receptor ALK1 is well-known for its role in blood vessel formation; however, its role within the immune system has never been revealed. 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 displayed 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. have proposed that during monocyte differentiation to 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, due to 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, ACVRL1) is a type I receptor of the TGFβ receptor superfamily with two 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 Listeria monocytogenes and overwhelming disseminated infections. Taken together, our data revealed a previously unappreciated role of ALK1 signaling in maintaining KC homeostasis and function.
**Results**

Loss of Alk1, rather than Tgfb2, Alk2 and Alk3, leads to an altered phenotype of KCs

Recently, Clec4F was identified as a specific surface marker for Kupffer cells (5). To specifically target KCs, we first generated Clec4f\(^{Cre/DTR}\) mice (hereafter referred to as Clec4f\(^{Cre}\)), in which an expression cassette encoding an internal ribosomal entry site (IRES), the Cre enzyme, a self-cleaving 2A peptide and the human DTR was inserted into the 3’ untranslated region of the Clec4f gene. Clec4f\(^{Cre}\) mice were crossed 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\(^{Cre}\) strain efficiently recombined in CD64\(^+\)F4/80\(^+\) KCs (>90%), and almost all tdTomato-positive cells were KCs (Supplemental Figure 1, A and B, and Supplemental Figure 2A). Furthermore, tdTomato expression was not detected in other tissues (including CD45\(^+\) and CD45\(^-\) 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 h after diphtheria toxin (DT) administration, KCs were efficiently deleted in Clec4f\(^{Cre}\) mice, as determined by immunostaining and flow cytometry (Supplemental Figure 1, F and G). Thus, the Clec4f\(^{Cre}\) 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 to 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 to 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 $\text{Alk1}^{fl/fl}\text{Clec4f}^\text{Cre}$ mice to test this hypothesis. We also prepared $\text{Alk2}^{fl/fl}\text{Alk3}^{fl/fl}\text{Clec4f}^\text{Cre}$ 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 $\text{Tgfbr2}^{fl/fl}\text{Clec4f}^\text{Cre}$ mice. $\text{Alk2}$, $\text{Alk3}$ and $\text{Tgfbr2}$ were efficiently deleted in KCs from $\text{Alk2}^{fl/fl}\text{Alk3}^{fl/fl}\text{Clec4f}^\text{Cre}$ and $\text{Tgfbr2}^{fl/fl}\text{Clec4f}^\text{Cre}$ mice, respectively, but the deficiency of these genes did not affect the expression of $\text{Id1}$ and $\text{Id3}$ (Supplemental Figure 4, A and B). In contrast, ablation of ALK1 resulted in dramatic reduction of the expression of $\text{Id1}$ and $\text{Id3}$ in CD64$^+$F4/80$^+$ hepatic macrophages from $\text{Alk1}^{fl/fl}\text{Clec4f}^\text{Cre}$ mice, although the expression of $\text{Alk1}$ was reduced by approximately 60% in these macrophages (Figure 1A). We further analyzed the total hepatic macrophage population in $\text{Alk1}^{fl/fl}\text{Clec4f}^\text{Cre}$ mice. No difference was observed in the number of KCs between $\text{Alk1}^{fl/fl}\text{Clec4f}^\text{Cre}$ and $\text{Alk1}^{fl/fl}$ mice (Figure 1B), but the KC surface phenotype in $\text{Alk1}^{fl/fl}\text{Clec4f}^\text{Cre}$ mice was altered, with a reduced population of Clec4F$^+$Tim4$^+$ KCs and increased populations of Clec4F$^-$Tim4$^+$ KCs and Clec4F$^-$Tim4$^+$ KCs (Figure 1C). In addition, no difference was observed in the cell number and surface phenotype of KCs between $\text{Alk2}^{fl/fl}\text{Alk3}^{fl/fl}\text{Clec4f}^\text{Cre}$, $\text{Tgfbr2}^{fl/fl}\text{Clec4f}^\text{Cre}$ and their control mice (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 $\text{Id1}$ and $\text{Id3}$ 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$^+$Tim4$^+$ cells. However, upon KC loss, newly arrived monocyte-derived KCs (MoKCs) are initially Clec4F$^-$Tim4$^-$ cells and then differentiate into Clec4F$^+$Tim4$^-$ cells (5). Among these Clec4F$^+$Tim4$^-$ MoKCs, only some acquire the expression of Tim4 (5). Thus, if KCs are constantly replaced by monocytes, three hepatic macrophage subsets are usually detected, based on the expression of Clec4F and Tim4, including Clec4F$^+$Tim4$^+$, Clec4F$^+$Tim4$^-$ and Clec4F$^-$Tim4$^+$ macrophages (20, 21). Interestingly, in addition to these macrophage subpopulations, we identified a population of Clec4F$^+$Tim4$^+$ KCs in $\text{Alk1}^{fl/fl}\text{Clec4f}^\text{Cre}$ mice (Figure 1C) that has never been reported previously. Indeed, Clec4F$^+$Tim4$^+$ KCs were also detected in the livers of tamoxifen-treated $\text{Alk1}^{fl/fl}\text{UBC}^\text{CreERT2}$ mice (Figure
1D), in which Clec4F and Tim4 expression was examined 5 days after tamoxifen treatment. Thus, these results suggested that the presence of Clec4F-Tim4+ KCs may be caused by Alk1 loss.

We next performed a single-cell RNA sequencing analysis (scRNA-seq) on sorted CD64+F4/80+Tim4+ KCs from Alk1lox/loxClec4fCre 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, 13690 cells remained (6295 cells from Alk1lox/lox mice and 7395 cells from Alk1lox/loxClec4fCre mice). Five clusters were identified by generating a UMAP from the transcriptome data using the Seurat pipeline (Figure 2A). Cluster 0 was predominantly composed of cells originating from Alk1lox/lox mice, whereas cells in clusters 1 and 2 mainly originated from Alk1lox/loxClec4fCre mice (Figure 2A). Because we were unable to determine which cells expressed full-length or floxed mRNA using the 3’ Assay from 10X Genomics, we next analyzed the differentially expressed (DE) genes between these clusters to find markers that could distinguish the different populations by flow cytometry. Analysis of scRNA-seq 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 found that Clec4F was expressed in clusters 0, 2, 3 and 4 but not in cluster 1 (Figure 2A and Supplemental Figure 5A). Interestingly, Id1 and Id3 expression was also significantly reduced in cluster 1 (Figure 2A). Given that Id1 and Id3 are target genes of ALK1 signaling in KCs, these results suggested that Clec4F-Tim4+ KCs (cluster 1) may be deficient in Alk1. We performed qPCR and genomic PCR on sorted Clec4F-Tim4+ and Clec4F+Tim4+ KCs from Alk1lox/loxClec4fCre mice to verify this result and found that Clec4F-Tim4+ KCs efficiently deleted Alk1, whereas Clec4F+Tim4+ KCs were heterozygous for the Alk1 deletion (Supplemental Figure 5, B and C). KCs of Alk1lox/+Clec4fCre mice did not display a similar phenotype to that of Alk1lox/loxClec4fCre mice (Supplemental Figure 5D), suggesting no obvious effect of Alk1 haploinsufficiency on KCs.

In Alk1lox/lox Clec4fCre mice, Cre recombinase is expressed under the control of the Clec4F promoter, implying that Clec4F was once expressed in Clec4F-Tim4+ KCs. We prepared Alk1lox/loxClec4fCreR26yfp reporter mice to examine this possibility and observed high expression of the YFP reporter gene in Clec4F-Tim4+ 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 $\text{Alk1}^{0/0}\text{Clec4f}^{\text{Cre/Cre}}$ 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 (Figure 2C).

Based on the results described above, we identified cluster 1 as $\text{Alk1}^{-/-}$ KCs from $\text{Alk1}^{0/0}\text{Clec4f}^{\text{Cre}}$ mice and cluster 0 as $\text{Alk1}^{+/+}$ KCs from $\text{Alk1}^{0/0}$ mice. Cells in cluster 2 from $\text{Alk1}^{0/0}\text{Clec4f}^{\text{Cre}}$ mice were identified as $\text{Alk1}^{+/+}$ KCs. Clusters 3 and 4 were proliferating cells expressing DNA replication-associated genes such as $\text{Mcm2}$-7 and $\text{Mki67}$ (Supplemental Figure 5A). Then, we compared the transcriptional profiles between clusters and found that among the 25 top core genes of KCs described previously (20), the expression of 16 core genes was significantly reduced upon the loss of $\text{Alk1}$ in KCs (Figure 2D), suggesting that ALK1 plays a critical role in maintaining the identity of KCs.

The transcription factors $\text{Zeb2}$ and $\text{Nr1h3}$ are required for the identity of KCs (20). We found that the expression of $\text{Nr1h3}$ but not $\text{Zeb2}$ was significantly decreased in $\text{Alk1}^{-/-}$ KCs (Figure 2D and Supplemental Table1). 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 CD163 and CD91 (encoded by $\text{Lrp1}$) 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 $\text{Spic}$ expression in KCs.

The maintenance of KCs requires ALK1 signaling

Decreased expression of Tim4 in KCs from $\text{Alk1}^{0/0}\text{Clec4f}^{\text{Cre}}$ mice suggested that circulating monocytes might replenish liver macrophages. We generated shielded chimeras in which the livers of $\text{Alk1}^{0/0}\text{Clec4f}^{\text{Cre}}$ mice and $\text{Alk1}^{0/0}$ littermate controls were shielded during irradiation and these mice were reconstituted with congenic CD45.1 WT BM (bone marrow) to examine this possibility (Figure 3A). As expected, partial shielding resulted in mixed chimerism in blood Ly6C$^\text{hi}$ monocytes in all groups (Figure 3B). KCs in $\text{Alk1}^{0/0}$ mice were not chimeric, as KCs were self-maintained under steady-state conditions independent of circulating monocytes (Figure 3B). However,
hepatic macrophages from Alk1fl/fl/Clec4fCre mice displayed chimerism (Figure 3B), suggesting that the ALK1 deficiency may lead 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). EdU incorporation assays revealed that Clec4F-Tim4+ KCs had a reduced capability to proliferate compared to 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, 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 investigate whether ALK1 deficiency results in KC disappearance, we established BM chimeras in which CD45.1+ mice were lethally irradiated and were injected with congenic CD45.2+ Alk1fl/fl/UBC^CreERT2^ 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). As 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-negative at 5 and 10 days after the last treatment, whereas Clec4F-Tim4+ KCs were no longer detected in the liver at 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 investigate whether ALK1 signaling is required for the maintenance of other tissue-resident macrophages, we generated mixed BM chimeras. We took advantage of $\text{Alk1}^{fl/fl}\text{Vav1}^\text{Cre}$ mice, in which Cre recombinase is expressed at high levels in hematopoietic stem cells and its activity remains robust during reconstitution following transplantation (26). Recipient mice (CD45.1) were lethally irradiated and reconstituted with equal amounts of WT (CD45.1/CD45.2) and $\text{Alk1}^{fl/fl}\text{Vav1}^\text{Cre}$ (CD45.2) BM or WT (CD45.1/CD45.2) and $\text{Alk1}^{fl/fl}$ (CD45.2) BM (Figure 4A). After 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 $\text{Alk1}^{fl/fl}\text{Vav1}^\text{Cre}$ (CD45.2) chimeras compared to WT (CD45.1/CD45.2) and $\text{Alk1}^{fl/fl}$ (CD45.2) chimeras (Figure 4B), suggesting that liver macrophages deficient in $\text{Alk1}$ are outcompeted by their wild-type counterparts. In contrast, blood monocytes and other tissue macrophages, including lung macrophages, kidney macrophages, brain macrophages and spleen macrophages, were reconstituted at an equal ratio in WT (CD45.1/CD45.2) and $\text{Alk1}^{fl/fl}\text{Vav1}^\text{Cre}$ (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, is required for the expression of $\text{Id1}$, $\text{Id3}$ and $\text{Clec4f}$. Consistent with these results, BMP9 and BMP10 treatment maintained higher expression of $\text{Id1}$, $\text{Id3}$ and $\text{Clec4f}$ in cultured KCs than BMP2 (ALK3 ligand) and BMP6 (ALK2 ligand) (Figure 5A). However, Clec4F expression in KCs from $\text{Bmp9}$ KO mice was unaltered compared to 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 $\text{Bmp9}$ 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 $\text{Id1}$, $\text{Id3}$, $\text{Clec4f}$, $\text{Fabp7}$, $\text{Cd5l}$ and $\text{Cdh5}$, were most significantly downregulated in KCs from $\text{Bmp9}$ KO mice treated with the anti-BMP10 antibody compared to 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 Smad4^flo^Clec4f^Cre^ mice to assess whether ALK1 signaling functions in KCs via the Smad pathway. Similar to the effect of Alk1 deletion, KCs from Smad4^flo^Clec4f^Cre^ mice displayed altered expression of Clec4F and Tim4 (Figure 6A). Protected chimeras revealed that Smad4 deficiency led to the replenishment of KCs from monocytes (Figure 6B). Similar to Alk1^flo^UBC^CreERT2^ mice, Clec4F^Tim4^+ cells were also detected in KCs from tamoxifen-treated Smad4^flo^UBC^CreERT2^ 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, while Clec4F^Tim4^- KCs maintained the expression of Smad4 at levels comparable to KCs isolated from untreated Smad4^flo^UBC^CreERT2^ 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, ALK1 signaling regulates 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 need to prepare ALK1 conditional knockout mice, in which Alk1 was completely targeted in these cells. Alk1 is not efficiently deleted in KCs from Alk1^flo^Clec4f^Cre^ mice, so we analyzed Alk1^flo^Vav1^Cre^ 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 Alk1^flo^Vav1^Cre^ mice compared to those from Alk1^flo^ mice (Supplemental Figure 6A), and Clec4F expression was not detected in either Tim4^+ or Tim4^- KCs from Alk1^flo^Vav1^Cre^ mice (Supplemental Figure 6B). We also analyzed the number of hepatic myeloid cells, including KCs, neutrophils, monocytes, pDCs and cDCs, and found that these cell counts were normal in Alk1^flo^Vav1^Cre^ mice (Supplemental Figure 6, C-F). Similarly, Smad4 was also efficiently targeted in KCs from Smad4^flo^Vav1^Cre^ 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 Alk1^flo^Vav1^Cre^ and Smad4^flo^Vav1^Cre^ mice to investigate whether
the liver architecture was affected by ALK1 and Smad4 deficiency. E-cadherin and glutamine synthetase were expressed in the periportal and central vein regions of the liver lobules, respectively. The regional localization of E-cadherin and glutamine synthetase in the liver lobules of $\text{Alk}1^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ and $\text{Smad}4^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ mice was not affected (Supplemental Figure 7, A and B), and KCs from these mice still closely interacted with LSECs and HSCs (Supplemental Figure 7, C and D), suggesting that the liver architecture was intact.

VSIG4 (also known as CR1g), 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 $\text{Vsig4}$ in KCs was obviously upregulated after BMP9 and BMP10 treatment (Figure 5A). The transcriptomic analysis revealed that $\text{Vsig4}$ expression was significantly decreased following the deletion of $\text{Alk1}$ in KCs (Figure 2D). Confocal microscopy and flow cytometry also confirmed this significant reduction in KCs from $\text{Alk1}^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ and $\text{Smad}4^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ mice (Figure 7, A-D).

In addition to the cell surface phenotype, KC function is also governed by their three-dimensional morphology within the vasculature, as their 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, $\text{Alk1}^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ and $\text{Smad}4^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ mice lacked the periportal polarization of KCs observed in their controls (Figure 8, A and B). In addition, KCs from $\text{Alk1}^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ and $\text{Smad}4^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ mice were generally smaller with a decreased cell surface area and volume compared with KCs from $\text{Alk1}^{\text{fl/fl}}$ controls (Figure 9, A and B). Taken 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 *Listeria monocytogenes***

Next, we infected $\text{Alk1}^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ and $\text{Alk1}^{\text{fl/fl}}$ mice with *Listeria monocytogenes* (Lm) and found that approximately 80% of $\text{Alk1}^{\text{fl/fl}}\text{Vav1}^{\text{Cre}}$ mice died within 7 days of infection, whereas no $\text{Alk1}^{\text{fl/fl}}$ mice succumbed to the infection (Figure 10A). Similarly,
Smad4\textsuperscript{fl/fl} Vav1\textsuperscript{Cre} mice exhibited higher mortality to Lm infection than Smad4\textsuperscript{fl/fl} controls (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 Lm infection is KC intrinsic, we infected Alk1\textsuperscript{fl/fl}Clec4f\textsuperscript{Cre/Cre} and Alk1\textsuperscript{fl/fl} mice with Lm and found that Alk1\textsuperscript{fl/fl}Clec4f\textsuperscript{Cre/Cre} mice were more susceptible to Lm infection than their controls (Figure 10B), suggesting that ALK1 has a KC-intrinsic role in protecting the host from Lm infection. Moreover, we used intravital microscopy 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 Lm by KCs (Figure 10, C and D, Supplemental Figure 8B and Supplemental movies 1-3), despite having a similar number of KCs compared to their controls (Supplemental Figure 7, E and F). As a result, rapid systemic bacterial dissemination occurred in Alk1\textsuperscript{fl/fl}Vav1\textsuperscript{Cre} mice, with less Lm in the liver but significantly more bacteria in the lung and blood (Figure 10E). Based on these results, the ability of KCs to capture bacteria from the bloodstream was significantly impaired in Alk1\textsuperscript{fl/fl}Vav1\textsuperscript{Cre} mice, thereby resulting in systemic bacterial dissemination and increased mortality of the host.

KCs are essential for host survival in Lm infection, but most Lm are not killed by the KCs (30). Elimination of the pathogen in Lm infection requires the recruitment of neutrophils to the liver and the specific binding of these neutrophils to KCs (30). We found neutrophil recruitment to the liver occurred at 2h post-infection, and no difference was observed in their recruitment and interaction with KCs between Alk1\textsuperscript{fl/fl} Vav1\textsuperscript{Cre} and Alk1\textsuperscript{fl/fl} mice (Supplemental Figure 9). IFN-γ is essential for the innate defense against Lm infection (31). We observed increased expression of intracellular IFN-γ in NK, CD4\textsuperscript{+} T and CD8\textsuperscript{+} T cells from Alk1\textsuperscript{fl/fl}Vav1\textsuperscript{Cre} mice at 24h post-infection (Supplemental Figure 10A), which coincided with higher bacterial counts in the livers and spleens of Alk1\textsuperscript{fl/fl}Vav1\textsuperscript{Cre} mice than in Alk1\textsuperscript{fl/fl} mice (Supplemental Figure 10B). Recently, it has been shown that IL-17A plays a critical role in innate defense against Lm infection in the liver (32). Consistent with the previous report (32), IL-17A was mainly expressed by TCR γδ\textsuperscript{+} T rather than CD4\textsuperscript{+} T cells at 5 days post Lm infection (Supplemental Figure 10C). The proportion of IL-17A-producing cells in TCR γδ\textsuperscript{+} T cells in the liver of Alk1\textsuperscript{fl/fl}Vav1\textsuperscript{Cre} mice was
comparable to that in the liver of Alk1<sup>fl/fl</sup> mice (Supplemental Figure 10C), indicating that the ability of TCR γδ<sup>+</sup>T cells to produce IL-17A was not impaired in Alk1<sup>fl/fl</sup>Vav1<sup>Cre</sup> mice.

To investigate whether KC phagosome maturation and activation are altered in the absence of ALK1. We quantified phagocytosis and ROS production by KCs that engulfed Lm in vivo using pH- and ROS-sensitive probe labeled bacteria. No differences were observed in the acidification and ROS production of Lm-containing phagosomes in KCs between Alk1<sup>fl/fl</sup>Vav1<sup>Cre</sup> and Alk1<sup>fl/fl</sup> mice (Supplemental Figure 11, A and B). Furthermore, we also determined cytokines expression by KCs from Alk1<sup>fl/fl</sup>Vav1<sup>Cre</sup> and Alk1<sup>fl/fl</sup> mice in response to Lm infection and found that the expression of the TNF-α and MCP-1 mRNAs in KCs was slightly reduced in the absence of ALK1 (Supplemental Figure 11C), indicating decreased KC activation in Alk1<sup>fl/fl</sup>Vav1<sup>Cre</sup> mice during Lm infection. Overall, these results suggested that the ALK1 signaling pathway is critical for host defenses against Lm infection and that this effect appears to be KC-specific.

**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 the 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 mRNA and protein levels. Moreover, KCs no longer expressed Clec4F when Alk1 was completely deleted, as observed in KCs from Alk1fl/flVav1Cre and Alk1fl/flClec4fCre/Cre mice. Together, these results suggested that ALK1 signaling is essential for Clec4F expression in 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 hepatic stellate cells (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/flVav1Cre mice and Smad4fl/flVav1Cre 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/flVav1Cre mice was not affected (our unpublished observations), suggesting that the alteration of anatomical localization was not due to the lack of CXCR3 expression. In fact, monocytes constantly replenish the macrophage pool in the livers of Alk1fl/flVav1Cre mice and Smad4fl/flVav1Cre mice. These less-mature Mo-KCs may contribute to a uniform distribution of KCs. Nevertheless, our study is the first to provide insights into how the tissue-specific functions of KCs are affected by factors that imprint their identity.

Deletion of Alk1 and Smad4 resulted in disruption of KC homeostasis, exhibited by loss of KCs over time and replacement by monocyte-derived macrophages. These immature monocyte-derived macrophages displayed reduced expression of VSIG4 and altered function of KCs. Finally, Alk1/Smad4-deficient mice exhibited increased susceptibility
to infection with gram-positive *Listeria monocytogenes*. In fact, mutations in *Alk1* result in Hereditary Hemorrhagic Telangiectasia (HHT), which is a rare genetic disease characterized by recurrent epistaxis, cutaneous telangiectasia, and 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 *Alk1*+/−/Vav1Cre mice showed a significant reduction in the capture of circulating *Staphylococcus aureus* by KCs (Supplemental Figure 12). Thus, it is possible that the high incidence of infectious diseases observed in HHT patients may be due to 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 monocytes-derived macrophages (43-45). More importantly, the alteration of KC homeostasis also occurs in human liver diseases, as the number of liver macrophages was significantly reduced in patients with liver fibrosis (46). It has been reported that patients with acute liver failure and advanced cirrhosis displayed the high risk of bacterial infections, and dysfunction of liver macrophages may play a role (46, 47). This suggested that therapeutic interventions aimed to prevent the loss of KCs and (or) promote the maturation of newly arrived monocytes-derived macrophages might help these patients to reduce infection susceptibility.

Recently, Bonnardel et al. and Sakai et al. proposed a two-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 two papers provided the background for understanding how the 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 *Listeria monocytogenes* infection. Altogether this study revealed how the liver microenvironment regulates KCs to maintain their phenotypes and form an effective defense against bacterial infection.

**Methods**
Mice

Clec4f<sup>Cre</sup>/DTR mice were generated by Nanjing BioMedical Research Institute of Nanjing University (NBRI) using CRISPR/Cas9-mediated genome editing on a C57/BL/6J background. R26<sup>tdTomato</sup> mice (Stock No:007914), UBC<sup>CreERT2</sup> (Stock No:007001) mice were obtained from Jackson Laboratory. Tgfbr2<sup>2/−</sup> mice and Smad4<sup>2/−</sup> mice were kindly provided by Dr. Xiao Yang (Beijing Institute of Lifeomics). Bmp9 KO mice were kindly provided by Dr. Se-Jin Lee (Johns Hopkins University). Alk1<sup>2/−</sup> mice were kindly provided by Dr. Zhihong Xu (Fudan University, Shanghai). Alk2<sup>2/−</sup> mice were kindly provided by Dr. Vesa Kaartinen (University of Michigan). Alk3<sup>2/−</sup> mice were kindly provided by Dr. Yuji Mishina (University of Michigan). Vav1<sup>Cre</sup> mice were kindly provided by Dr. Bing Liu (Fifth Medical Center of Chinese PLA General Hospital). CD45.1/Ly5.1 mice were kindly provided by Dr. Mingzhao Zhu (Institute of Biophysics, Chinese Academy of Sciences). Mice and their littermates were used between six- to sixteen-week-old unless otherwise specifically indicated. All mice were maintained at the 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 (sigma) at 37°C for 30 min and vigorously pipette. 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 min. 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 min. Liver cell suspensions were further enriched by iodixanol gradient (OptiPrep) as previously described (49).

For surface marker analysis, cell pellets were stained with appropriate antibodies at 4°C for 20-30 min. 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 70μm cell strainer followed by being lysed by
RBC lysis buffer. The hepatic mononuclear cells were stimulated with cell stimulation cocktail (eBioscience) for 6 hours, followed by intracellular IL-17A staining. Flow cytometry was performed using an LSRII Fortessa (Becton Dickinson/BD Biosciences). The acquired data were analyzed with FlowJo software (Tree Star). For cell sorting, 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). After overnight, the culture medium was replaced by serum-free X-VIVO15 media (Lonza, Basel, Switzerland), supplemented with 20 ng/ml M-CSF in the presence or absence of 50 ng/ml rmBMP2 (Peprotech), rhBMP6 (Peprotech), rhBMP9 (Peprotech) or 50 ng/ml rmBMP10 (R&D). The half media was changed every other day. At day 7, KCs were acquired, and RNA was extracted by using RNeasy plus Mini Kit (Qiagen).

Bone marrow chimeras

For establishment of total body irradiation, C57BL/6 (CD45.1) mice were lethally irradiated (10Gy) by X-ray and were transplanted with 1×10⁷ BM cells. Mice were analyzed 8 weeks post transplantation.

For establishment of partially-shielded irradiation, livers of Alk1⁻/⁻Clec4f⁻/⁻ mice (CD45.2) and Smad4⁻/⁻Clec4f⁻/⁻ mice (CD45.2) or their WT controls (CD45.2) were protected by a lead cover and then irradiated (8Gy) by X-ray. The irradiated mice were transplanted with 1×10⁷ BM cells from gender-matched WT mice (CD45.1). 4 weeks after transplantation mice were analyzed.

EdU staining

Mice were administrated with 0.5 mg of 5-ethynyl-20-deoxyuridine (EdU, Thermo Fisher Scientific) via intraperitoneal injection. After 20 h, KCs were obtained and EdU incorporation was measured by flow cytometry using the Click-it EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific), according to manufacturer’s instructions.

Quantitative Real-Time PCR
Total RNA was isolated with RNeasy plus Mini Kit (Qiagen) and cDNA was synthesized with Prime Script RT Reagent Kit (Takara). Quantitative PCR was performed with a SYBR Green PCR kit (Toyobo, Japan) in 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 were first isolated by F4/80 magnetic beads (Miltenyi) and then sorted by FACS from Alk1<sup>fl/fl</sup> Clec4f<sup>Cre</sup> mice. Genomic DNA was extracted using QIAamp DNA Micro Kit (Qiagen). PCR was used as follows: 95℃ 3min, 35 cycles of 95℃ 30s, 62℃ 30s, 72C 45s, followed by a 2min incubation at 72C. PCR primers for Alk1 flox were: forward, 5’-GCTTGCAATGCTTGGCTCTAC-3’, reverse, 5’-GGGAGGAGCCATGTTCTCAG-3’; PCR primers for Alk1 deletion were: forward, 5’-GTGCGTGGAGGAGGACAGTAGTC-3’, reverse, 5’-TGGAGACCTGTCTGAATGTCTG-3’.

**Bacterial burden**

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

**Single-Cell RNA-Seq**

A total of single cell Tim4<sup>+</sup>CD64<sup>+</sup>F4/80<sup>+</sup>Ly6C<sup>-</sup>CD45<sup>+</sup> cells were sorted from livers of Alk1<sup>fl/fl</sup>Clec4f<sup>Cre</sup> and Alk1<sup>fl/fl</sup> littermate controls. FACS-purified cells were washed with 0.04% BSA DPBS for three times and were resuscitated to a concentration of 700-1200 cells/μl (viability≥85%). Individual cells were then loaded on 10X Genomics Chromium controller to generate single-cell Gel Bead-in-EMulsion (GEMs). Single-cell sequencing libraries were prepared following the 10x Genomics Chromium Single Cell 3’ Reagent Kits (V3 chemistry) and sequenced on an NovaSeq 6000 platform (Illumina). Raw reads were demultiplexed and aligned to the mouse transcriptome.
(mm10) using 10X Genomics Cell Ranger pipeline (version 3.1.0). All downstream single-cell analyses were performed using R package Seurat (version 3.2.2). Additional low quality (expressed gene < 500, UMI counts < 2000, % of mitochondrial genes < 10% or % of ribosome genes < 14%) and contaminating cells (lymphocytes) were removed from the analysis.

Data availability

The raw RNA-seq data generated from this study are available in NCBI SRA under accession code PRJNA705814.

Intravital imaging of liver and L. monocytogenes capture

Multichannel confocal microscope was used to image mouse liver as previously described (50). Briefly, mice were anaesthetized (2.5% Avertin, 20 ml/kg, i.p., Sigma). 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 covered on the liver to keep liver moist and stable. For the visualization of liver macrophages, platelets and neutrophils, 2 μg of anti-F4/80 (BM8) or 2.5 μg of anti-Ly6G (1A8) (Biolegend) was administered intravenously. Image acquisition was performed using an inverted Olympus FV3000 confocal microscope with a 20x/0.75 UPLANSAPO objective lens. Laser excitation wavelengths of 488 nm, 561 nm and 647 nm and HyD or PMT detectors were used for fluorescence detection. For bacteria catching experiments, bacteria were resuspended in PBS and labeled by incubating with CFSE (100 μM, Invitrogen) for 30min. Acquisition of images was initiated and labeled bacteria (4x10^7 CFU) was injected via tail vein immediately and images were acquired every 5 seconds for 25 minutes. Functions of Coloc and spot in the Imaris software were used to identify and quantify captured bacteria within Kupffer cells per field of view at every time point.
For the visualization of cell interaction, acquisition of images was initiated and 5x10^8 Lm was injected via tail vein and images were acquired every 10 seconds for 50min. A stitched image of 4x4 field of view was acquired 2h post-infection to visualize neutrophil recruitment. Functions of Coloc and surface in the Imaris software (Bitplane) were used to measure voxel overlap between two cell surface and to identify interactions between Kupffer cells and platelets or neutrophils.

**Immunofluorescence staining**

Livers were perfused with 0.5% Heparin (damas-beta) in 20 ml cold PBS, fixed in 4% PFA for 12 hours, followed by dehydration in 15% and 30% sucrose before embedding in OCT compound (Sakura Finetek). Sections (50 μm) were cut on a CM1950 cryostat (Leica) and adhered to Superfrost Plus slides. Frozen sections were blocked for 2h with 1% bovin serum albumin, 0.3 M Glycine and 10% donkey or goat serum after permeabilization in PBS with 0.2% Triton for 30 min at room temperature, followed by staining with antibodies diluted in PBS with 0.2% Tween for 12h at 4°C and secondary antibodies and Hoechst for 2h at room temperature. The following antibodies were used: anti-CD31 (390, BD Bioscience), anti-F4/80 (BM8, Biolegend), anti-E-Cadherin (DECMA-1, Biolegend), anti-Desmin (polyclone, Abcam), anti-Glutamine Synthetase (polyclone, Abcam), anti-VSIG4 (NLA14, Invitrogen) and anti-Clec4F (polyclone, R&D systems). Fluorescent-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Sections were mounted with Fluoromount G (YEASEN) and images were acquired on a FV3000 confocal microscope with a 20x/0.75 UPLANSAPO or 60x/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). Kupffer cells number, cell surface area and cell volume were quantified using surface function
in Imaris. Surface was created for F4/80 and threshold was determined by background subtraction algorithm, and the fluorescent intensity of Clec4F and VSIG4 for every individual Kupffer cells were calculated. Positions of Kupffer cells were obtained and the distance to the center of each CV/PV was calculated in Excel.

**Statistics**

Data are presented as mean ± SEM. All statistics analyses were performed in GraphPad Prism (GraphPad Software). Statistical significance was assessed by unpaired, two-tailed, Student’s t-test or one-way ANOVA with Tukey’s multiple comparisons test or two-way ANOVA with Sidak’s multiple comparisons test where appropriate. Survival analysis was assessed by Mantel-Cox test. *p < 0.05; **p < 0.01; ***p < 0.001; ****P < 0.0001; ns, not significant. Each symbol represents an individual mouse. Number of animals is indicated as “n”.

**Study approval**

All experimental procedures in mice were approved by the Institutional Animal Care and Use Committee at the Beijing Institute of Lifeomics.

**Author contributions**

Z.D., conceived the study, funding acquisition, designed and performed the experiments, analyzed and curated the majority of data, interpreted results and wrote the manuscript. Y.F., performed the experiments, analyzed the data, edited the manuscript and visualized images. W. Y., performed the experiments, analyzed the data and visualized images. L. S., performed the experiments and visualized images. L.Y., performed bioinformatics analysis. H.F., provided technical and material support. Y.W., L.D., T.Y., and L.Q., provided technical support. T. L., conceived the study, edited the manuscript, funding acquisition and supervised the study. W.J., edited the manuscript, funding acquisition and supervised the study. F.H., supervised the study. Z.D., Y.F., W. Y., and L. S., contributed equally to this work. The order of the co-first authors was determined on the basis of their contributions to this project.
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References


26. Perez-Cunningham J, Boyer SW, Landon M, and Forsberg EC. Hematopoietic stem cell-specific GFP-expressing transgenic mice generated by genetic


Figure 1. ALK1 controls KC surface phenotype. (A) qPCR analysis of \( \text{Alk1} \), \( \text{Id1} \) and \( \text{Id3} \) expression in sorted KCs (CD45\(^+\)Ly6C\(^-\)CD64\(^+\)F4/80\(^+\)) from \( \text{Alk1}^{\text{fl/fl}} \text{Clec4f} \text{Cre} \) mice and \( \text{Alk1}^{\text{fl/fl}} \) controls (n = 5-6 per group). Gating strategy for KCs was shown in Supplemental Figure 2A. (B) Representative flow cytometry data (left) and total cell number (right) of CD64\(^+\)F4/80\(^+\) KCs (pregated on CD45\(^+\)Ly6C\(^-\)) in \( \text{Alk1}^{\text{fl/fl}} \text{Clec4f} \text{Cre} \) mice and \( \text{Alk1}^{\text{fl/fl}} \) controls (n = 7-10 per group). (C) Expression (left) of Clec4F and Tim4 in KCs (pregated on CD45\(^+\)Ly6C\(^-\)CD64\(^+\)F4/80\(^+\)) and percentage (right) of Clec4F\(^+\)Tim4\(^+\), Clec4F\(^+\)Tim4\(^-\), Clec4F\(^-\)Tim4\(^+\) and Clec4F\(^-\)Tim4\(^-\) KCs in \( \text{Alk1}^{\text{fl/fl}} \text{Clec4f} \text{Cre} \) mice and \( \text{Alk1}^{\text{fl/fl}} \) controls at the age of 8 weeks (n = 6 per group). (D) \( \text{Alk1}^{\text{fl/fl}} \text{UBC}^{\text{CreERT2}} \) mice were treated with or without tamoxifen (10 mg) 2 times every other day via oral gavage and Clec4F and Tim4 expression in KCs were examined 5 days after the last treatment. Data are representative of at least three independent experiments. The results represent mean ± SEM. ***P < 0.001, ****P < 0.0001, calculated by two-tailed Student's t-test (A-C).
Figure 2. ALK1 controls KC identity. (A) UMAP plot of scRNA-seq data of KCs from Alk1\(^{fl/fl}\)Clec4f\(^{Cre}\) mice and Alk1\(^{fl/fl}\) controls, showing clusters and distribution of cells on different samples and expression of Id1, Id3, and Clec4f in KCs. (B) Flow cytometry of YFP in indicated hepatic macrophage populations from Alk1\(^{fl/fl}\)Clec4f\(^{Cre}\)R26\(^{yfp}\) mice and Alk1\(^{fl/fl}\)R26\(^{yfp}\) controls. Data are representative of three independent experiments. (C) Expression of Clec4F and Tim4 in KCs (pregated on CD45\(^{+}\)Ly6C\(^{-}\)CD64\(^{+}\)F4/80\(^{+}\)) from Alk1\(^{fl/fl}\)Clec4f\(^{Cre/Cre}\) mice and their controls at the age of 5 weeks. The experiment was repeated two times. (D, E) Heatmap showing indicated genes expressed differentially across KC cluster from scRNA-seq data. Genes in red and blue are significantly upregulated and downregulated, respectively.
Figure 3. The maintenance of KCs requires ALK1 signaling. (A) Schematic of experimental set-up. (B) Expression of CD45.1 (donor) and CD45.2 (recipient) in blood monocytes and total KCs from Alk1fl/fl and Alk1fl/fl Clec4fCre chimeras. Percentage (right) of total chimerism of KCs in Alk1fl/fl and Alk1fl/fl Clec4f Cre chimeras (n = 6 per group). Schematic of experimental set up is indicated in Figure 3A. (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 controls (n = 7-12 per group) at the age of 2, 5 and 12 weeks. (D) Representative flow cytometry 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 were examined 5, 10 and 25 days after the last treatment (n = 4-8 per group). The results represent mean ± SEM. **P < 0.01, ***P < 0.001, ****P < 0.0001, calculated by two-tailed Student’s t-test (B) and one-way ANOVA(C-E).
Figure 4. ALK1 is dispensable for the maintenance of macrophages located in the lung, kidney, brain and spleen. (A) Schematic of experimental set-up. (B-G) Representative flow cytometry data (left) and percentage (right) of CD45.1+CD45.2+ and CD45.2+ of KCs (B), blood monocytes (C), AMs (D), kidney macrophages (E), brain macrophages (F) and spleen macrophages (G, n = 5-8 per group). Data are pooled from two independent experiments. The results represent mean ± SEM. ****P <0.0001, calculated by two-way ANOVA. Gating strategies for the indicated macrophages (B-G) were shown in Supplemental Figure 2.
Figure 5. BMP9 and BMP10 control the expression of KC-specific signature gene. (A) BMP2, BMP6, BMP9 and BMP10 stimulated sorted KCs and indicated genes were determined by qPCR. Data are representative of at least three independent experiments. (B) Flow cytometry of Clec4F and Tim4 of KCs from Bmp9 KO and WT mice treated with PBS or anti-BMP10 antibody (15 mg/kg) 4 times every day via i.p. Data are representative of three independent experiments. (C) KCs were sorted from mice described as (B) and indicated genes were determined by qPCR (n = 3 per group). The results represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, calculated by one-way ANOVA (C).
Figure 6. Loss of Smad4 in KCs repeats the altered phenotype of loss ALK1. (A) Expression of Clec4F and Tim4 in KCs (pregated on CD45+Ly6C-CD64+F4/80+) in Smad4fl/flClec4fCre mice and their controls. Data are representative of at least three independent experiments. (B) Expression of CD45.1(donor) and CD45.2 (recipient) in blood monocytes and total KCs in Smad4fl/flClec4fCre and Smad4fl/fl chimeras. Percentage (right) of total chimerism of KCs in Smad4fl/flClec4fCre and Smad4fl/fl chimeras (n = 5 per group). (C) Smad4fl/flUBC<sup>CreERT2</sup> mice were treated with or without tamoxifen (10 mg) 2 times every other day via oral gavage and Clec4F and Tim4 expression in KCs (pregated on CD45+Ly6C CD64+F4/80) was examined 5 days after the last treatment. Data are representative of at least three independent experiments. (D) qPCR analysis of Smad4, Id1 and Id3 expression in sorted total KCs, Clec4F+Tim4<sup>+</sup> and Clec4F-Tim4<sup>+</sup> KCs from mice described as (C, n = 3 per group). The results represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, calculated by two-tailed Student’s t-test (B) and one-way ANOVA (D).
Figure 7. ALK1/Smad4 signaling is important for KCs to maintain the expression of VSIG4. (A, B) Immunofluorescence image of 20 μm-thick liver sections from Alk1<sup>fl/fl</sup>Vav1<sup>Cre</sup> mice (A), Smad4<sup>fl/fl</sup>Vav1<sup>Cre</sup> mice (B) and their littermate controls. Expression of Clec4F (green) and VSIG4 (red) in KCs (visualized with anti-F4/80, white) were assessed and blood vessels were visualized with anti-CD31 (blue). Data are representative of three independent experiments. Scale bar, 80μm. (C and D) Flow cytometry of VSIG4 and Tim4 of KCs in Alk1<sup>fl/fl</sup>Vav1<sup>Cre</sup> (C), Smad4<sup>fl/fl</sup>Vav1<sup>Cre</sup> (D) and their littermate controls. Data are representative of at least three independent experiments.
Figure 8. ALK1/Smad4 signaling is important for KCs to maintain their location. (A, B) Zoom-in representative immunofluorescence image (upper) of liver sections from Alk1^{fl/fl}Vav1^{Cre} mice (A), Smad4^{fl/fl}Vav1^{Cre} mice (B) and their littermate controls showing distribution of KCs (anti-F4/80, green) at the periphery of central veins (CV, visualized with anti-Glutamine Synthetase, red) and portal veins (PV), blood vessels were visualized with anti-CD31 (blue). KC number in each region of 200μm radius around CV or PV was measured (lower). Scale bar, 50μm. Data are pooled from n = 3 mice per group. The results represent mean ± SEM. ****p < 0.0001, calculated by two-way ANOVA (A, B).
Figure 9. ALK1/Smad4 signaling is important for KCs to maintain their morphology. (A, B) 117 rendered surfaces of KCs from Alk1\textsuperscript{fl/fl}Vav1\textsuperscript{Cre} mice (A), Smad4\textsuperscript{fl/fl}Vav1\textsuperscript{Cre} mice (B) and their littermate controls were displayed and arranged by surface area (upper), and 3D analysis of individual KC surface area (lower) and surface volume (lower). Data are pooled from n = 3 mice per group. The results represent mean ± SEM. ****p < 0.0001, calculated by two-tailed Student’s t-test (A, B).
Figure 10. Alk1-deficient mice exhibit increased susceptibility to Lm infection. (A, B) Survival of Alk1fl/flVav1Cre mice (A, n = 11), Alk1fl/flClec4fCre/Cre (B, n = 7) and their littermate controls (n = 8-14 per group) infected with 5×10⁵ CFU Lm was monitored every day. (C-D) Representative IVM images (left) and quantification (right) showing KCs (anti-F4/80, red) capturing circulating Lm (CFSE, green) within 25 minutes after infection in Alk1fl/flVav1Cre mice (C, n = 3), Alk1fl/flClec4fCre/Cre mice (D, n = 3) and their littermate controls (n = 3-4 per group). Bacteria captured by Kupffer cells are highlighted (arrows). Scale bar, 10 μm. (E) Colony-forming units (CFU) were assayed in livers, lungs and blood of Alk1fl/flVav1Cre mice and their littermate controls at 10 min following injection of Lm (4×10⁷ CFU, n = 4 per group). The experiment was repeated two times. The results represent mean ± SEM. **P<0.01, ***P <0.001, ****P <0.0001, calculated by Mantel-Cox test (A, B), two-way ANOVA (C, D), two-tailed Student’s t-test (E).