Megakaryocyte derived immune-stimulating cells regulate host-defense immunity against bacterial pathogens

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Abstract

Megakaryocytes (MKs) continuously produce platelets in bone marrow to support hemostasis. However, MKs also play roles beyond thrombopoiesis as they regulate hematopoietic stem cell quiescence and erythropoiesis, which suggests the functional heterogeneity of MKs. Here, using single-cell sequencing we identified an MK-derived immune-stimulating cell (MDIC) population, which plays an important role in host-protective response against bacteria. In contrast to platelet-generating MKs, MDICs highly express cell migration, immune-modulatory, and response genes. Upon *Listeria (L.) monocytogenes* infection, MDICs egress to circulation and infiltrate into the spleen, liver and lung. MDICs interact with myeloid cells to promote their migration and tissue infiltration. More importantly, MDICs stimulate phagocytosis of macrophages and neutrophils by producing TNF α and IL-6 and facilitating antigen-specific T cell activation via IL-6 to enhance anti-bacterial response. Ablation of MKs reduced innate immune response and compromised T cell activation in spleen and liver, impairs the anti-bacterial effects in mice under *L. monocytogenes* challenge. Finally, infection-induced emergency megakaryopoiesis efficiently stimulated MDICs generation upon bacterial infection. Overall, we identify MDICs as a novel MK subpopulation, which regulates host-defense immune response against bacterial infection.

1 Introduction

2 Megakaryocytes (MKs), a rare hematopoietic lineage in bone marrow, continually
3 produce blood platelets to support hemostasis and thrombosis ^{1,2}. MK progenitors
4 undergo multiple rounds of endomitosis during maturation to achieve polyploid ³⁻⁷ and
5 migrate between distinct microenvironments and organs for their proliferation,
6 maturation, and biological functions ⁸⁻¹⁴. Although platelet generation is the prominent
7 role of MKs, evidence suggests that MKs have other biological functions.

8 Mature MKs physically interact with hematopoietic stem cells (HSCs) and
9 constitute a unique niche for HSC regulation in bone marrow. MKs produce multiple
10 factors, including transforming growth factor-beta (TGFβ) ^{15,16} and platelet factor (PF4 or
11 CXCL4) ¹⁷ to maintain HSC quiescence. MKs also interact with other niche cells, such as
12 osteoblasts ^{18,19}, non-myelinating Schwann cells ^{20,21}, and blood vessels ^{8,22} to further
13 influence the attraction and retention of hematopoietic stem and progenitor cells during
14 homeostasis and stress. MKs express multiple immune receptors, including IgG Fc
15 receptors, toll-like receptors (TLRs), major histocompatibility complex (MHC) ^{12,23-25},
16 and produce numerous cytokines, such as PF4, TGFβ, IL-1, and IL-6, which regulate
17 immune cell differentiation, survival, and migration ²⁶⁻³⁰. These suggest that MKs might
18 play roles in inflammation and infection ³¹⁻³⁴; however, the precise mechanism remains
19 elusive.

20 Here, using single-cell RNA sequencing (scRNA-seq), we identified a novel
21 immune-stimulating MK subpopulation, which participates in host defense against
22 microbial pathogens.

23

1   **Methods**

2   Detailed descriptions of experimental methods are in Supplemental Methods.

3   **Mice**

4   C57BL/6-Tg(*Pf4-cre*)Q3Rsko/J (*Pf4-cre*), C57BL/6-Gt(ROSA)
5   26Sortm1(HBEGF)Awai/J (*iDTR*), C57BL/6-Gt(ROSA)26Sortm4(ACTB-tdTomato,-
6   EGFP)Luo/J (*mTmG*), Gt(ROSA)26Sortm9(CAG-tdTomato)Hze (*tdTomato*) mice were
7   obtained from the Jackson Laboratory. *Scl-CreER* mice were provided by J.R. Goethert.
8   All mice were maintained in the C57BL/6 background. Animals were blindly included in
9   the experiments according to genotyping results as a mix of male and female. All animal
10   experiments were performed according to protocols approved by the institutional animal
11   care and use committee.

12   **Single cell RNA sequencing**

13   Sorted CD41⁺FSC^{high} single cells from control mice and mice at three days after *L.*
14   *monocytogenes* infection were processed through the Chromium Single Cell. Platform
15   using the Chromium Single Cell 3' Library and Gel Bead Kit v3 (10X Genomics, PN-
16   1000075) and the Chromium Single Cell B Chip Kit (10X Genomics, PN-1000074) as
17   the manufacturer's protocol. In brief, over 7,000 cells of each sample were loaded onto
18   the Chromium instrument to generate single-cell barcoded droplets. Cells were lysed and
19   barcoded reverse transcription of RNA was occurred. Libraries were prepared by
20   following amplification, fragmentation, adaptor, and index attachment, then sequenced on
21   an Illumina NovaSeq platform.

22   **scRNA-seq preprocessing**

23   The scRNA-seq reads were aligned to the mm10 reference genomes, and unique

1 molecular identifier (UMI) counts were obtained by CellRanger 3.0.2. Normalization,
 2 dimensionality reduction and clustering were performed with the Seurat 3.0 R package ³⁵
 3 on RStudio. For control and *Listeria (L.) monocytogenes* infection group, we loaded one
 4 10x Genomics well each and detected 5,663 and 5,948 cells which passed the cellranger
 5 pipeline, respectively. To ruled out potentially contamination, cells with >12%
 6 mitochondrial content or have <200 detected genes were excluded, with Seurat function
 7 subset (percent.mt<12 & nFeature_RNA>200). We ruled out doublets with default
 8 parameters of DoubletDecon R package, and 54 of control cells and 939 of *L.*
 9 *monocytogenes* infected cells were excluded. Following standard procedure in Seurat's
 10 pipeline, in combination with spearman correlation test and MetaNeighbor method, we
 11 obtain 3,272 MKs from control mice (1,712 MKs) and mice with *L. monocytogenes*
 12 infection (1,560 MKs) (3,897 and 3,449 immune cells were discarded respectively).

13 **Data sharing statement**

14 The scRNA-seq data generated in this study are deposited in GEO (GSE168224,
 15 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE168224>).

17 **Results**

18 **Single-cell atlas identifies an MK-derived immunoregulatory cell population.**

19 To explore the heterogeneity of MKs, we applied droplet-based single-cell RNA
 20 sequencing (scRNA-seq) with CD41⁺ forward scatter (FSC)^{high} MKs in bone marrow. To
 21 adequately enrich MKs at various stages, we gated over the top 1% of CD41⁺ cells as
 22 described and combined transcriptomic analysis to capture sufficient and accurate MKs ³⁶

(Figure 1A; Supplemental Figure 1A-C). We successfully detected a total of 5,368 high-quality cells and excluded 3,656 immune cells according to their transcriptional profiles to obtain 1,712 MKs. MKs, with an average of ~4200 genes per cell, expressed MK-specific marker genes including *Pf4* and *Itga2b* (CD41) and exhibited high similarity to published megakaryocyte single-cell transcriptome rather than immune cells³⁶⁻³⁸ (Figure 1B-D, Supplemental Figure 1D-M). MKs expressed higher mRNA and more genes than immune cells, presumably due to the polyploid of MKs³⁹ (Supplemental Figure 1N). Re-clustering 1,712 MKs revealed five clusters termed MK1 to MK5, each expressed MK marker genes (*Pf4*, *Itga2b*, *Vwf*, *Mpl*, *Itgb3*) and transcription factors required for MK differentiation (*Fli1*, *Nfe2*, *Gata1*, *Runx1*) and exhibited megakaryocyte transcriptional profiles compared to published data^{36-38,40,41} (Figure 1B-D, Supplemental Figure 1O). Then we annotated these five clusters based on the feature genes of each cluster through enriched gene ontology (GO). MK1 highly expressed nuclear division genes, including *Mki67*, *Ube2c*, *Nuf2*, *Cdk1*^{5,42,43} (Figure 1E-H), indicating endomitosis during MK polarization². MK2 showed enrichment of genes related to DNA replication and repair genes such as *Mcm2*, *Ung*, *Cdca7*, *Mcm3*^{44,45} (Figure 1E-H). MK3 contained feature genes associated with blood coagulation such as *Alox12*, *Gp6*, *Myl9*, *Thbs1*⁴⁶⁻⁴⁹ (Figure 1E-H), indicating the role of platelet generation and thrombosis. MK4 exhibited B cell differentiation and antigen processing and presentation pathways, including feature genes *Cd74*, *H2-Aa*, *Ly6c2*, *H2-Eb1*, *S100a4*, *Ifi2712a* and *Ctss*⁵⁰⁻⁵³ (Figure 1E-H). MK5 enriched cell migration, immune-modulatory and inflammatory response genes, including cell migration genes *Ccl3*, *Ccl4*, *Ccl9*, *Cyp11a1*, *Cdh1*, *Cxcr4*, cytokine genes *Il6*, *Il15*, *Il18rap*, IgG Fc receptor gene *Fcer1a*, *Fcer1g*, and inflammatory response genes *Ier3*,

1 *Cd200r3*, *Junb*⁵⁴⁻⁶³ (Figure 1E-H, Supplemental Figure 1P), indicating that this
 2 subpopulation might directly sense the immune signals to participate in immune
 3 responses³². Therefore, we referred this novel MK subpopulation as MK-derived
 4 immune-stimulating cells (MDICs). Interestingly, MDICs (MK5) exhibit a slightly lower
 5 expression of some canonical MK markers (*Vwf*, *Mpl*, *Itgb3*), transcription factors
 6 (*Gata1*), and a little higher expression of *Spi* (encoding PU.1), which is involved in
 7 immune cell differentiation, than other populations⁶⁴ (Figure 1C, Supplemental Figure
 8 1P).

9 Overall, using scRNA-seq, we identified a new immune-stimulating MK
 10 subpopulation termed MDICs that might participate in cell migration, immune-
 11 modulatory functions, and inflammatory response.

13 **MDICs migrate to peripheral organs upon *L. monocytogenes* infection.**

14 To obtain purer MKs by CD41 markers through flow cytometry, we performed a tighter
 15 gating strategy than cell sorting for scRNA-seq to avoid CD41⁻ cells containing immune
 16 cells. We further performed CD41 immunostaining to confirm the CD41 expression and
 17 polyploid in sorted MKs (Supplemental Figure 2A-B). Moreover, we seldom observed
 18 CD41⁺ hematopoietic stem and progenitor cells (HSPCs) with a tighter gating strategy
 19 because of lower CD41 levels in HSCs, consistent with single-cell analysis showing
 20 rarely expressed HSC marker genes in MKs⁶⁵⁻⁶⁸ (Supplemental Figure 1P, 2C). To assign
 21 the immune response and modulatory functionality to potential clusters, we identified
 22 surface protein and cytokinin encoded marker genes: *Cdh1* (encoding CD324/E-cadherin)
 23 and *Il6* enriched in MDICs (MK5), *Ccr2* enriched in MK4 and MDICs (MK5), and high

1 *Cxcr4* expression in MDICs, compared to low *Cxcr4* level in classic MKs ^{69,70} (Figure 1G
2 and I). Single cells expressed a high level of *Cxcr4* also enriched cell migration, immune-
3 modulatory, and inflammatory response pathways (Supplemental Figure 1Q). Meanwhile,
4 high levels of CXCR4 were associated with IL-6 labeled MDICs (MK5) according to
5 flow cytometry (Supplemental Figure 2D), which indicated that high CXCR4 expression
6 could be a potential marker to enrich MDICs. Indeed, MDICs (marked with CXCR4^{high},
7 hereafter) had smaller cell size and produced less platelets under Thrombopoietin (TPO)
8 stimulation ⁷¹ (Supplemental Figure 2E). This showed that MDICs had distinct responses
9 to TPO stimulation compared to classic MKs.

10 We further co-stained CD324, CCR2, or CXCR4 with CD41 in bone marrow cells
11 from control mice and mice at three days after *Listeria (L.) monocytogenes* infection
12 (Figure 2A), we found CXCR4^{high} and CD324⁺ MKs, but not CXCR4^{low} and CD324⁻
13 MKs, were reduced after infection without cell proliferation and apoptosis (28.8% and
14 30.5% decreased respectively, Figure 2B-F, Supplemental Figure 2F-H). However,
15 CCR2⁺ MK numbers did not show a significant change after infection in bone marrow
16 (Supplemental Figure 2I-J). Note transcriptomic profiles showed that MDICs (MK5)
17 enriched cell migration genes, including *Cxcr4* (Figure 1E, H, 2G). These results
18 indicated that MDICs (MK5) might migrate in the bone marrow microenvironment or
19 egress to circulation in response to infection. First, we explored the spatial distribution of
20 MKs in bone marrow at three days after *L. monocytogenes* infection. Although HSCs are
21 dramatically activated upon infection ⁷², we did not observe a significant influence of the
22 association between MKs and HSCs upon *L. monocytogenes* infection (Figure 2H-I),
23 albeit the critical role of perivascular MKs in maintaining HSC quiescence ^{15,17,73}.

1 However, ~ 80% MKs directly contacted blood vessels three days after *L. monocytogenes*
2 infection, which was 1.61-fold higher than control MKs (~ 40%) (Figure 2H, J, and
3 Supplemental Figure 2K). Furthermore, significantly more CXCR4^{high} MKs contacted
4 with blood vessels compared to CXCR4^{low} MKs after *L. monocytogenes* infection (1.23-
5 fold increase; Figure 2K-L). In addition, we developed a live imaging method to trace
6 MK migration in bone marrow (see Methods). Using *Pf4-cre; tdTomato* (tdT) reporter
7 labeled MKs, we observed small MKs which did not rupture or release platelet rapidly
8 migrated into sinusoid upon *L. monocytogenes* infection (Figure 2M, movie 1).
9 Consistently, we found that CXCR4^{high} MKs increased in peripheral blood (3.04-fold
10 increase, Figure 2N). However, CXCR4^{low} MKs did not differ at three days after *L.*
11 *monocytogenes* infection (Supplemental Figure 2L). These results indicated MDICs
12 might egress into circulation from bone marrow upon infection.

13 Accordingly, we observed a significant increase of CXCR4^{high} MKs in the liver
14 (2.51-fold) and spleen (2.18-fold) at three days after *L. monocytogenes* infection (Figure
15 2O-P, Supplemental Figure 2M-N). To further investigate the migration of MKs into
16 peripheral organs upon infection, we employed *Pf4-cre; Rosa26-cell membrane-localized*
17 *tdTomato (mT)/ cell membrane-localized EGFP (mG) (R26R^{mT/mG})* reporter mice, in
18 which cell membrane-localized EGFP (mGFP) is expressed exclusively in MK lineage ⁷⁴.
19 Using this reporter line, we found that mGFP⁺ MKs were dramatically increased in the
20 liver (10.4-fold) and slightly increased in the spleen (1.29-fold, no significant difference)
21 at three days after *L. monocytogenes* infection (Figure 2Q-R). To further confirm the
22 infiltration of MDICs, we transplanted tdT⁺ bone marrow cells into recipients, which
23 were infected by *L. monocytogenes* for one day (Supplemental Figure 2O). Interestingly,

we found that the majority of tdT⁺CXCR4^{high} MKs migrated into the liver (92.1%) and spleen (92.5%); however, much fewer tdT⁺CXCR4^{high} MKs migrated to bone marrow (33.3%) when tdT⁺ bone marrow cells were injected to recipients for two days (Supplemental Figure 2P). On the contrary, tdT⁺CXCR4^{low} MKs preferred migrating to bone marrow (66.7%) rather than the liver (7.93%) or spleen (7.53%) (Supplemental Figure 2P).

Overall, our data showed that bacterial infection increased the egressing MDICs in circulation, which might infiltrate into the liver and spleen, suggesting a role of MDICs in host-protective response in tissues.

MDICs promote myeloid cell migration upon *L. monocytogenes* infection.

Our scRNA-seq also revealed that MDICs (MK5) population highly expressed cell adhesion cell chemotaxis and myeloid cell activation genes compared to classic MK populations (Figure 1H, 3A). This indicated that MDICs might regulate immune cell chemotaxis, which is essential for host-defense response^{75,76}. To test this, we performed an *ex vivo* transwell assay and found that CXCR4^{high} MKs effectively promoted the mobilization of macrophages (2.50-fold), dendritic cells (1.99-fold), and neutrophils (1.87-fold), which was much more efficient compared to simultaneously purified CXCR4^{low} MKs (Figure 3B-C). Consistently, MKs from the liver and spleen also efficiently promoted immune cell mobilization (Supplemental Figure 3A). However, CCR2 and CD324 could not enrich MKs that promote myeloid cell mobilization (Figure 3D, Supplemental Figure 3B). We noticed that MKs and myeloid cells were preferably trapped in the sinusoid (MK 2.33-fold, myeloid cell 2.95-fold increase) three days after *L.*

1 *monocytogenes* infection compared to control mice (Figure 3E-F). Moreover, the direct
2 association between MKs, blood vessels, and myeloid cells increased (1.93-fold) in the
3 bone marrow of mice three days after *L. monocytogenes* infection (Figure 3E, G).
4 Moreover, we found that more than 2-fold myeloid cells were associated with CXCR4^{high}
5 MKs in bone marrow compared to CXCR4^{low} MKs in mice at three days after *L.*
6 *monocytogenes* infection (Figure 3H-I). Consistently, the myeloid cell-CXCR4^{high} MK
7 association (mean distance 15.36 μm) was significantly closer than the myeloid cell-
8 CXCR4^{low} MKs association (mean distance 25.62 μm, $p = 7.03 \times 10^{-4}$ by KS test, Figure
9 3J). Furthermore, bone marrow myeloid cells were found preferably adjacent to
10 CXCR4^{high} MK-blood vessel intersection rather than CXCR4^{low} MK-blood vessel
11 intersection in mice at three days after *L. monocytogenes* infection (Figure 3K). These
12 observations indicated that CXCR4^{high} MKs might promote the tissue infiltration of
13 myeloid cells to promote host-defense response.

14 To investigate the role of MKs in immune cell infiltration upon *L. monocytogenes*
15 infection, we employed *Pf4-Cre; iDTR* mice, in which MKs were rendered sensitive to
16 diphtheria toxin (DT) ¹⁵. As four-time DT injections efficiently ablated MKs in bone
17 marrow, we gave the *L. monocytogenes* challenge at the second DT injection and
18 analyzed the immune response three days after infection (Figure 3L-M). MK ablation
19 markedly increased the numbers of hematopoietic stem and progenitor cells in the bone
20 marrow and peripheral blood as previously reported ^{15,77} (Supplemental Figure 3C-D).
21 We also noticed an increase of innate immune cells (macrophages 2.04-fold, neutrophils
22 5.32-fold) in the bone marrow of MK ablated mice upon *L. monocytogenes* challenge
23 (Figure 3N). However, MK ablated mice had dramatically reduced numbers of innate

immune cells in the liver (macrophage 44.4% decrease, DC 67.2% decrease) and spleen (monocyte 22.5% decrease, macrophage 40.0% decrease, DC 32.9% decrease, neutrophil 55.8% decrease) three days after *L. monocytogenes* infection (Figure 3O-P).

Overall, our observations indicated that upon *L. monocytogenes* infection, MDICs facilitated the infiltration of innate immune cells against bacterial infection.

MDICs enhance host-defense response against *L. monocytogenes* infection.

To characterize the role of MDICs in the bacterial clearance capability of myeloid cells, we co-cultured MKs with neutrophils or macrophages before phagocytosis assays. We found that CXCR4^{high} MKs efficiently stimulated the phagocytosis ability of neutrophils and macrophages against bacteria. CCR2⁺ MKs and CD324⁺ MKs also showed stimulation effects on macrophage phagocytosis (Figure 4A-D). Our scRNA-seq revealed that MDICs (MK5) population enriched cytokine production compared to classic MK populations (Figure 4E). We further explored the molecular insights of MDICs and found that CXCR4^{high} MKs highly expressed *Ccl6* (CCL6), *Tnf* (TNF α), and *Il6* (IL-6), which regulate inflammatory responses and immune cell proliferation^{78,79 80} (Figure 4F). We further confirmed that more CXCR4^{high} MKs producing TNF α (4.08-fold) and IL-6 (6.96-fold) compared to CXCR4^{low} MK in mice with *L. monocytogenes* infection (Figure 4G, I). Consequently, higher protein levels of TNF α (3.80-fold) and IL-6 (6.11-fold) were observed in CXCR4^{high} MKs than CXCR4^{low} MKs, which is consistent with their phagocytosis promotion in mice with *L. monocytogenes* infection (Figure 4H, J). These suggested that MDICs might directly facilitate the host-defense response by producing TNF α and IL-6 to stimulate myeloid cell phagocytosis for bacterial clearance^{78,79}. To test

this hypothesis, we investigated the role TNF α and IL-6 in the MDIC-stimulated phagocytosis ability of macrophages and neutrophils. We found that the CXCR4^{high} MK-stimulated bacterial phagocytosis abilities in macrophages and neutrophils were significantly compromised when TNF α or IL-6 were blocked by neutralizing antibody (anti-TNF α antibody: neutrophils 63.8% decrease, macrophages 51.6% decrease; anti-IL-6 antibody: neutrophils 46.7% decrease, macrophages 44.6% decrease; Figure 4K-N). This showed that MDICs promoted the phagocytosis function of neutrophils and macrophages by producing proinflammatory cytokine TNF α and IL-6. Consequently, *in vivo* the bacterial burdens were dramatically increased in the liver (81.2-fold), and spleen (1.93-fold) in MK ablated mice at three days after *L. monocytogenes* infection (Figure 4O-Q).

Overall, our data demonstrate that MDICs enhance innate immunity against *L. monocytogenes* infection.

Bacterial infection promotes coagulation and immune response in distinct MK subpopulations.

To explore the role of MDICs during pathogen infection, we further elaborated the single-cell transcriptional profile of MKs in mice at three days after *L. monocytogenes* infection (Figure 5A-D; Supplemental Figure 4A-B). We performed the low-quality cell filtration and immune cell removing using the same strategy as scRNA-seq in control MKs. *L. monocytogenes* infection stimulated the immune response and cytokine production genes in multiple immune cells (Figure 5C). We obtained 1,560 MKs from mice upon infection, which also presented five populations as in control mice and exhibited high correlation

with published MK transcriptome rather than immune cells (Figure 5D and Supplemental Figure 4C). MK1 and MK2 showed no significant changes in cell fractions, transcriptional profiles (Figure 5E, Supplemental Figure 4D), and the polyploid after infection (Supplemental Figure 4E). Platelet-generating MKs (MK3) and MK4 increased, whereas the MDICs (MK5) decreased (Figure 5E, Figure 2D) in infected mice compared to control mice. Platelet-generating MKs (MK3) enriched blood coagulation genes with a slight increase of immune response pathways upon *L. monocytogenes* infection (Figure 5F). MDICs (MK5) exhibited stimulation of immune response, especially interferon (IFN) signaling pathway and antigen processing and presentation, including elevated gene expression of *Ifitm3*, *Stat1*, *Bst2*, *Irgm1*, *H2-Q7*, *H2-Q6*, *H2-K1*⁸¹⁻⁸⁴ (Figure 5F-G), consistent with lung MKs^{36,37}. As lung is an important site of platelet generation¹², we compared MK1 to MK5 with these published lung MK scRNA-seq datasets^{36,37}. We found that MDICs (MK5) and platelet-generating MKs (MK3), which are involved in immunoregulatory and coagulation, showed greater similarity to lung MKs. (Figure 5H). MK5 also enriched genes involved in antigen processing and presentation pathway upon *L. monocytogenes* infection, in line with published lung MKs³⁶ (Figure 5I). Thus, bacterial infection stimulated platelet-generating MKs and activated immune-response MDICs.

Bacterial infection stimulates thrombopoiesis and T cell proliferation via MKs.

scRNA-seq analysis showed an increased platelet-generating MKs (MK3) ratio, indicating active thrombopoiesis upon *L. monocytogenes* infection to meet the demand of increased platelet consumption⁸⁵ (Figure 5E). In supporting this, more ruptured MKs

1 were observed in bone marrow three days after *L. monocytogenes* infection (Figure 6A-
2 B). Consequently, platelets were quickly recovered during day 3 to day 7 post-*L.*
3 *monocytogenes* infection (Figure 6C), although platelets were initially damaged upon
4 acute infection^{31,86}.

5 Antigen processing and presentation pathway in MDICs was activated upon *L.*
6 *monocytogenes* infection in scRNA-seq. We next asked whether MDICs regulate T cell
7 response. Interestingly, we observed that CXCR4^{high} MKs efficiently promoted T cell
8 proliferation (7.06-fold increase), which was more efficient than CXCR4^{low} MKs (4.70-
9 fold increase; Figure 6D-E). Moreover, the CXCR4^{high} MKs, but not CXCR4^{low} MKs,
10 induced T cell proliferation was significantly compromised (27.8% decrease) when IL-6
11 was blocked by neutralizing antibody (Figure 6E). This indicated that MDICs promoted
12 T-cell proliferation by producing IL-6.

13 We further investigated the *in vivo* function of MKs in promoting adaptive immunity
14 against bacterial infection. We used ovalbumin (OVA)-expressing recombinant microbe
15 (*L. monocytogenes*-OVA₂₅₇₋₂₆₄) to infect control, or MK ablated mice (Figure 6F). At
16 seven days after *L. monocytogenes*-OVA₂₅₇₋₂₆₄ infection, we found that MK ablation
17 dramatically reduced OVA-specific T cells activation, in which the number of CD4⁺
18 IFN γ ⁺ Th1 cells, CD4⁺IL4⁺ Th2, and CD8⁺ cytotoxic T lymphocytes were markedly
19 reduced (42.9%, 37.9%, and 57.4% decrease, respectively) in MK ablated mice, although
20 the total numbers of CD4⁺ T cell and CD8⁺ T cells were not affected (Figure 6G and
21 Supplemental Figure 5A, D). These observations of MDICs exhibited a similar ability to
22 stimulate T cell activation through antigen processing and presentation to depend on
23 MHC II after bacteria engulfment as previously reported in lung MKs³⁷. Interestingly,

1 through tdTomato labeled MK transplantation, we found MKs more rapidly infiltrated
2 into the lung upon *L. monocytogenes* (Figure 6H-J). These results indicated that immune-
3 modulatory lung MKs shared similar functionality with MDICs.

4 MK-committed HSCs drive emergency megakaryopoiesis to replenish platelet
5 consumption during acute inflammation⁸⁷. We hypothesized that immune-modulatory
6 MDICs might be derived from committed HSCs during *L. monocytogenes* infection.
7 Using *Scl-creER*; *tdTomato*⁺ reporter mice, in which HSPC promoted hematopoiesis can
8 be traced through tamoxifen induction²¹, we analyzed tdTomato labeled MKs and
9 progenitors at 18 hours after infection (Figure 6K). We found that tdTomato⁺ MKs,
10 including MDICs (CXCR4^{high}) and classic MKs (CXCR4^{low}), were increased upon
11 infection (Figure 6L). However, progenitors were not significantly increased, consistent
12 with previous report⁸⁷ (Figure 6M). This indicated that emergency megakaryopoiesis
13 might participate in the defense against pathogens through producing MDICs.

14 Overall, our observation indicated that acute pathogen infection enhances
15 thrombopoiesis and adaptive immune responses of MKs through inflammation-induced
16 emergency megakaryopoiesis.

17

18 Discussion

19 MKs express multiple immune receptors, which participate in megakaryocyte maturation,
20 platelet activation, and potentially influence neutrophils and the adaptive immune cells
21^{32,88-90}. The expression of immune receptors might confer MKs with immune sensing
22 capacity in inflammation and infection³². Accordingly, MKs prevent the spread of
23 dengue virus infection by enhancing the type 1 interferons pathway in murine and clinical

1 biospecimens⁹¹. Recent scRNA-seq studies also suggested the existence of a potential
2 immunoregulation subpopulation in MKs^{37,92}. However, how MKs regulate host-defense
3 response remains elusive. In our work, using scRNA-seq, we identified an immune-
4 stimulating MK subpopulation, MIDCs, which highly expressed chemokine receptor
5 CXCR4 and enhanced both native and adaptive immunity against bacterial infection.

6 Upon acute inflammation, platelet-generating MKs were consumed for platelet
7 production³¹. Furthermore, CXCR4^{high} MDICs egressed into circulation and infiltrated
8 into peripheral organs, such as the liver, spleen, and lung. This is consistent with previous
9 reports that MKs egress and migrate to the pulmonary capillary^{12,37,93}, and CXCR4
10 navigates cell migration upon chemotactic stimuli^{8,70}. HSCs like MK progenitors induce
11 emergency megakaryopoiesis to generate mature MKs for platelet production⁸⁷.
12 However, our data showed that inflammation-induced emergency megakaryopoiesis more
13 efficiently induced CXCR4^{high} MDICs than classic MKs, which further suggested the role
14 of MKs in host-defense response.

15 MDICs showed phagocytosis ability and displayed efficient ability to stimulate
16 bacterial phagocytosis ability of macrophages and neutrophils through producing TNF α
17 and IL-6, and promoted T cell proliferation by producing IL-6. Our scRNA-seq data also
18 showed that MDICs expressed various cytokines, such as CCL3, CCL4, CCL6 and
19 CCL9, which regulates antimicrobial immunity⁹⁴, and IL-6, IL-15, which increases T
20 cell proliferation and activation^{80,95}. Consistently, lung MKs internalize and process both
21 antigenic proteins and bacterial pathogens to induced CD4⁺ T cell activation in an MHC
22 II-dependent manner³⁷, and exhibit transcriptomic similarity to bone marrow MDICs.
23 Moreover, inflammation stimulates MK infiltration into the lungs. Therefore, further

1 studies are warranted to determine more elaborate cell surface makers and mechanisms
2 that MDICs regulate host-defense response in various organs.

3 Overall, we first identified a novel immune-stimulating subpopulation, MDICs,
4 within MKs and provided functional evidence that MDICs regulate host-defense response
5 by enhancing the innate and adaptive immunoresponses.

6

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15

16 **Authorship**

17 J.W., J.X., X.H. designed and performed most of the experiments, analyzed the data and
18 generated figures. D.W. contributed to histology and live imaging. M.C. contributed to
19 MK ablation experiments. L.J. contributed for scientific discussion and manuscript
20 preparation. M.Z. supervised the project and wrote the manuscript.

21

22 **Conflict-of-interest disclosure**

23 The authors declare no competing financial interests.

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1 **Figure legends**

2 **Figure 1. Single-cell atlas identifies an MK derived inflammatory cell population.**

3 (A) Schematic depicting the strategy of cell isolation, MK sorting, scRNA-seq and data
4 analysis. (B) UMAP of 1,712 MKs from bone marrow, colored by clusters. (C) Violin
5 plots showing the expression of MK selected marker genes. (D) Feature plots of MK
6 marker genes. (E) Heat map showing the row-scaled signature genes of MKs (fold-
7 change > 1.5 , p value < 0.05) with exemplar genes listed on the right (top, color-coded by
8 clusters). Columns denote cells; rows denote genes. (F) Dot plot showing the scaled
9 expression of exemplar signature genes for each cluster, colored by the average
10 expression of each gene in each cluster scaled across all clusters. Dot size represents the
11 percentage of expressed cells in each cluster. (G) Feature plots of selected signature genes
12 in each cluster. (H) Gene Ontology (GO) analysis of signature genes for each cluster. GO
13 terms are selected with Benjamini–Hochberg-corrected p values < 0.05 and colored by –
14 $\log_{10}(p$ value). (I) Violin plots and the corresponding feature plots of selected signature
15 genes of MK5.

16 **Figure 2. MDICs migrate to peripheral organs upon *L. monocytogenes* infection. (A)**

17 Schematic depicting the strategy of *L. monocytogenes* infection and analysis. (B-D) Bone
18 marrow MK (B), CXCR4^{low} MK (C) and CXCR4^{high} MK (D) numbers of control mice
19 and mice at three days after *L. monocytogenes* infection ($n = 8$ mice). (E-F) Fraction of
20 CXCR4^{high} BrdU⁺ MKs (E) and CXCR4^{high} Annexin V⁺ MKs (F) in bone marrow from
21 control mice or mice at three days after *L. monocytogenes* infection ($n = 5$ mice). (G)
22 GSEA evaluated enrichment of chemotaxis and cell migration genes in MK5 cells
23 compared to classic MK populations (MK1 to 4). Normalized enrichment scores (NESs)

1 and adjusted p values were calculated by permutation tests. (H) Representative images of
2 CD41 (blue), CD150 (red), EMCN (green) and lineage cells (white) in bone marrow from
3 control mice or mice at three days after *L. monocytogenes* infection. d_{HSC} and d_{EC} indicate
4 the distance between the MK (blue, marked with an asterisk) and the closest HSC (red),
5 endothelia cell (green), respectively. Yellow boxes indicate the locations of the magnified
6 images. Arrowheads indicate HSCs. EMCN, endomucin; EC, endothelial cell. (I-J)
7 Comparison of distance between HSCs to MKs ($n = 96$ control and 127 infected HSCs, p
8 $= 0.21$ by two-sample KS test) (I), and MKs to ECs ($n = 119$ control and 103 infected
9 MKs) (J) in bone marrow of control mice or mice at three days after *L. monocytogenes*
10 infection. (K) Representative images of CD41 (blue), CXCR4 (red) and EMCN (green) in
11 bone marrow of mice at three days after *L. monocytogenes* infection. Yellow arrows
12 indicate CXCR4^{high} MKs, while white arrowheads indicate CXCR4^{low} MKs. (L)
13 Comparison of distance between CXCR4^{low} or CXCR4^{high} MKs and ECs ($n = 68$
14 CXCR4^{low} and 78 CXCR4^{high} MKs). (M) Visualization of MK migration (red, arrowhead)
15 into the BM sinusoids (green) by live imaging in *Pf4-cre; tdTomato*^{+/-} mice at three days
16 after *L. monocytogenes* infection. S, indicates sinusoid and dashed lines demarcate the
17 border of sinusoids. (N-P) CXCR4^{high} MKs in peripheral blood (N), livers (O) and
18 spleens (P) from control mice or mice at three days after *L. monocytogenes* infection ($n =$
19 3 mice). (Q-R) Representative images and cell numbers of *mGFP*⁺ cells (green) in livers
20 (Q) and spleens (R) from control mice or mice at three days after *L. monocytogenes*
21 infection ($n = 4$ mice). Scale bars without indicated, 20 μ m. Data represent mean $\pm$ s.e.m.
22 Two-tailed Student's t -test was performed to assess statistical significance in (B-F, J, L,

1 N-R). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s., no significant difference. Two-sample
2 KS test was performed to assess statistically significant in (I).

3 **Figure 3. MDICs promote myeloid cell migration upon *L. monocytogenes* infection.**

4 (A) GSEA evaluated enrichment of cell-cell adhesion and myeloid cell activation genes
5 in MK5 cells compared to classic MK populations (MK1 to 4). NESs and adjusted p
6 values were calculated by permutation tests. (B) Schematic depicting transwell assay for
7 myeloid cells in absence or presence of MKs as indicated. (C-D) Numbers of indicated
8 transmigrated cells, normalized to Ctrl (only media was added to the lower chambers).

9 Mon, monocytes; MΦ, macrophages; DC, dendritic cells; Neu, neutrophils. (E)

10 Representative images of MKs (blue), vascular endothelial cells (green) and myeloid
11 cells (white) in mouse bone marrow. Arrowheads indicate the myeloid cells in the

12 sinusoids. d_{MK} and d_{EC} indicate the distance between the myeloid cell marked with an
13 asterisk to the closest MK and endothelial cell (EC), respectively. (F) Number of MKs

14 and myeloid cells in the sinusoids of control mice or mice at three days after *L.*

15 *monocytogenes* infection ($n = 3$ mice). (G) Two-dimensional probability distribution of

16 distances from myeloid cells to MKs (d_{MK}) and EC cells (d_{EC}) in bone marrow of control
17 mice or mice at three days after *L. monocytogenes* infection (control, $n = 119$ CD11b⁺

18 cells; infection, $n = 144$ CD11b⁺ cells; $p = 3 \times 10^{-5}$ by 2D KS test). (H) Representative

19 images of MKs (blue), CXCR4 (red), vascular endothelial cells (green) and myeloid cells
20 (white) in mouse bone marrow showing the distribution of myeloid cells to CXCR4^{low} or

21 CXCR4^{high} MKs and vascular cells at three days after *L. monocytogenes* infection. Yellow

22 arrows indicate CXCR4^{high} MKs and white arrowheads indicate CXCR4^{low} MKs. (I-J)

23 Distance (I) and mean distance ($p = 7 \times 10^{-4}$ by KS test, J) of myeloid cells to the closest

1 CXCR4^{low} and CXCR4^{high} MKs. (K) Two-dimensional probability distribution of distances
2 from myeloid cells to CXCR4^{low} or CXCR4^{high} MKs and vascular cells ($n = 104$ CD11b⁺
3 cells; $p = 0.04$ by 2D KS test). (L) Schema for DT treatment and *L. monocytogenes*
4 infection design. *L.m.*, *L. monocytogenes*. (M) Representative images of MKs (blue,
5 arrows) and vascular endothelial cells (green) in bone marrow of mice after 4 days
6 diphtheria toxin (DT) treatment. (N-P) Percentage or cell number of innate immune cells
7 in bone marrow (N), livers (O) or spleens (P) of *Pf4-cre; iDTR^{+/-}* mice after four-time DT
8 injections ($n = 6$ mice). Scale bars, 20 μ m. Data represent mean $\pm$ s.e.m. Repeated-
9 measures one-way ANOVA followed by Dunnett's test for multiple comparisons in (C-
10 D), ‡ $p < 0.05$, # $p < 0.01$, ## $p < 0.001$, n.s., no significant difference. Two-dimensional-
11 two-sample KS test was performed to assess statistically significant in (G, K). Two-
12 sample KS test was performed to assess statistically significant in (J). Two-tailed
13 Student's *t*-test was performed to assess statistical significance in (F, N-P). * $p < 0.05$, **
14 $p < 0.01$, *** $p < 0.001$, n.s., no significant difference.

15 **Figure 4. MDICs enhance host-defense response against *L. monocytogenes* infection.**

16 (A) Representative images of neutrophil phagocytosis capacity with or without
17 CXCR4^{low} MKs or CXCR4^{high} MKs co-cultured. CD11b, red; *E. coli*, green; DAPI, blue.
18 (B) Quantification of neutrophil phagocytosis capacity with indicated MKs co-cultured.
19 Blank, neutrophil phagocytosed without co-cultured cells (Blank, $n = 20$; CXCR4^{low} MKs,
20 $n = 19$; CXCR4^{high} MKs, $n = 20$; CD324⁺ MKs, $n = 23$; CCR2⁺ MKs, $n = 19$;
21 CCR2⁺CXCR4^{high} MKs, $n = 18$). (C) Representative images of macrophage phagocytosis
22 capacity with or without CXCR4^{low} MKs or CXCR4^{high} MKs co-cultured. F4/80, red; *E*
23 *coli*, green; DAPI, blue. (D) Quantification of macrophage phagocytosis capacity with

1 indicated MKs co-cultured. Blank, macrophage phagocytosed without co-cultured cells
2 (Blank, $n = 110$; CXCR4^{low} MKs, $n = 69$; CXCR4^{high} MKs, $n = 94$; CD324⁺ MKs, $n = 40$;
3 CCR2⁺ MKs, $n = 184$; CCR2⁺CXCR4^{high} MKs, $n = 66$). (E) GSEA evaluated enrichment
4 of cytokine production involved in immune response genes in MK5 cells compared to
5 classic MK populations (MK1 to 4). NES and adjusted p value were calculated by
6 permutation tests. (F) Correlation analysis between expression profiles of *Cxcr4* and
7 cytokines, feature genes of MK subpopulations. (G-J) TNF α and IL-6 protein levels in
8 CXCR4^{low} and CXCR4^{high} MKs were shown by FACS (G, I) and immunostaining using
9 sorted MKs comparing to their functional levels of macrophages (H, J). (K-L)
10 Quantification of neutrophil (K) and macrophage (L) phagocytosis capacity with or
11 without CXCR4^{high} MKs co-cultured in absence or presence of TNF α neutralizing
12 antibody (neutrophil: $n = 30$ for each group; macrophage: Blank, $n = 36$; CXCR4^{high}
13 MKs, $n = 34$; CXCR4^{high} MKs + TNF α neutralizing antibody, $n = 34$). Blank, neutrophil
14 phagocytosed without co-cultured cells. (M-N) Quantification of neutrophil (M) and
15 macrophage (N) phagocytosis capacity with or without CXCR4^{high} MKs co-cultured in
16 absence or presence of IL-6 neutralizing antibody (neutrophil: Blank, $n = 20$; CXCR4^{high}
17 MKs, $n = 20$; CXCR4^{high} MKs + IL-6 neutralizing antibody, $n = 19$; macrophage: Blank,
18 $n = 44$; CXCR4^{high} MKs, $n = 36$; CXCR4^{high} MKs + IL-6 neutralizing antibody, $n = 39$).
19 (O) Schema for DT treatment and *L. monocytogenes* infection. (P-Q) Numbers of *L.*
20 *monocytogenes* CFUs of livers (P) and spleens (Q) of *Pf4-cre; iDTR*⁺ mice at three days
21 after *L. monocytogenes* infection ($n = 6$ mice). Scale bars in (A, C), 20 μ m; scale bars in
22 (H, J), 5 μ m. Data represent means $\pm$ first and third quartiles (B, D, H, J-N) or mean $\pm$
23 s.e.m (G, I, P, Q). Two-sample KS test was performed to assess statistically significant in

(B, D, H, J-N). Two-tailed Student's *t*-test was performed to assess statistical significance in (G, I, P, Q), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s., no significant difference.

Figure 5. Bacterial infection promotes coagulation and immune response in distinct

MK subpopulations. (A) Schematic depicting the strategy of scRNA-seq using bone

marrow MKs from mice at three days after *L. monocytogenes* infection. (B) UMAP of

5,368 cells from control bone marrow and 4,276 cells from *L. monocytogenes* infection

bone marrow, colored by cell types. Neu, neutrophil; MP, myeloid progenitor; Mon,

monocytes; B, B cells; T, T cells. (C) GO analysis of up-regulated genes after *L.*

monocytogenes infection (fold-change > 1.5 , $p < 0.05$) for each cell types. GO terms are

selected with Benjamini–Hochberg-corrected p values < 0.05 and colored by $-\log_{10}(p$

value), dot size represents the gene ratio. (D) UMAP of 3,272 MKs from control (1,712

MKs) and *L. monocytogenes* infection (1,560 MKs) bone marrow, colored by clusters.

(E) Fraction of each MK cluster from control MKs or *L. monocytogenes* infection MKs.

(F) Gene set variation analysis (GSVA) of each MK cluster under control or *L.*

monocytogenes infection, colored by row-scaled GSVA enrichment scores. (G) Heat map

showing the row-scaled differential genes of control and infection MK5 cells (fold-

change > 1.4 , p value < 0.05), with exemplar genes listed on the right (top, color-coded

by conditions). Columns denote cells; rows denote genes. (H) Similarities by

MetaNeighbor R package of scRNA-seq defined MK populations in control and *L.*

monocytogenes infection BM with the BM MK and lung MK reported by Pariser et al ³⁷

and Yeung et al ³⁶. (I) Heat map showing the row-scaled antigen processing genes of

control and infection MK5 cells, comparing with BM MK and lung MK reported by

Yeung et al ³⁶.

Figure 6. Bacterial infection stimulates thrombopoiesis and T cell proliferation via MKs. (A-B) Representative images (A) and statistical analysis (B) of CD41 (green) and DAPI (blue) in bone marrow from control mice or mice at three days after *L. monocytogenes* infection. Arrows indicate ruptured MKs, yellow boxes indicate the locations of the magnified images. (C) Platelets in peripheral blood in control mice or mice after *L. monocytogenes* infection on indicated days. (D) Representative flow cytometry gating and the cell numbers of proliferating T cells with CXCR4^{high} MK co-cultured for 24 and 72 hours. (E) Reduced CFSE intensity of proliferating T cells with CXCR4^{low} or CXCR4^{high} MK co-cultured for 72 hours in absence or presence of IL-6 neutralizing antibody. Blank indicates T cells proliferated without co-cultured cells. (F) Schema for DT treatment, *L. monocytogenes*-OVA₂₅₇₋₂₆₄ infection and *in vitro* re-activation assay. *L.m.*-OVA, *L. monocytogenes*-OVA₂₅₇₋₂₆₄; OVA, OVA₂₅₇₋₂₆₄. (G) Activated T cell numbers in spleens of *Pf4-cre*; *iDTR*⁺ mice after *in vitro* re-activation at seven days after *L. monocytogenes*-OVA₂₅₇₋₂₆₄ infection (*n* = 4 mice). (H) Schema for *L. monocytogenes* infection and *Pf4-cre*⁺; *tdTomato*⁺ cells transplantation. (I-J) Representative images and statistical analysis of lungs in control recipients or upon *L. monocytogenes* infection (*n* = 3 mice). Arrows indicate *tdTomato*⁺ cells in lungs. (K) Schema for HSC lineage tracing upon *L. monocytogenes* infection using *Scl-cre*⁺; *tdTomato*^{+/-} mice. (L) *tdTomato*⁺ CXCR4^{low} and *tdTomato*⁺ CXCR4^{high} MK numbers in control and *L. monocytogenes* infected *Scl-cre*⁺; *tdTomato*^{+/-} mice. (M) *tdTomato*⁺ progenitors in control and *L. monocytogenes* infected *Scl-cre*⁺; *tdTomato*^{+/-} mice, CMP, common myeloid progenitors; GMP, granulocyte-monocyte progenitors; MEP, megakaryocyte-erythroid progenitors. Scale bars, 20 μ m. Data represent mean $\pm$ s.e.m (B,

- 1 C, E, G, J, L, M). Two-tailed Student's *t*-test was performed to assess statistical
- 2 significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s., no significant difference.

Figure 1

Figure 2

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Figure 3

Figure 4

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Figure 6