REVIEW ARTICLE

New insights into the generation and function of megakaryocytes in health and disease

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Abstract

Megakaryocytes are canonically viewed as specialized hematopoietic cells that merely produce platelets and that are generated through a series of hematopoietic stem and progenitor cells in the bone marrow. Despite their essential physiological functions, the generation and function of megakaryocytes remain incompletely understood. Recent studies, mostly in mice, have begun to redefine the cellular hierarchy of megakaryopoiesis, and shed new light on the alternative routes and mechanisms of megakaryopoiesis. Moreover, the conception of megakaryocytes as a homogeneous cell population with the sole purpose of platelet production is being challenged. Indeed, megakaryocytes are being shown to be a heterogeneous population of cells with distinct routes of differentiation and versatile functions. In particular, megakaryocytes show abilities reminiscent of immune cells, and are increasingly considered as the root dictating the hemostatic and immune functions of platelets in various physiopathological conditions. Furthermore, although megakaryocytes are well known as a component of the bone marrow niche, maintaining hematopoietic stem cells during homeostasis, the newly identified properties of megakaryocytes further indicate that they may be key supporters of stressed or diseased hematopoietic stem cells during myeloablative injury, aging and hematopoietic malignancy. Therefore, the generation and function of megakaryocytes are more diverse than we previously thought. Here, we review the recent literature that expands our views of megakaryocyte differentiation and heterogeneity, as well as the functions of megakaryocytes, with special focuses on their immune functions and supporting roles for stressed or diseased hematopoietic stem cells.

Introduction

Megakaryocytes are large, polyploid hematopoietic cells primarily responsible for platelet production. Their diameter and ploidy range from 20-100 μm and 2-128N, respectively.[1] Megakaryocyte generation, which is also termed megakaryopoiesis, is highly specialized and traditionally viewed as a process whereby hematopoietic stem cells (HSC) differentiate into megakaryocytes through a series of increasingly restricted progenitors, along the myeloid branch of hematopoiesis. Once mature, megakaryocytes undergo a process of terminal differentiation involving polyploidization, to increase in size, and the formation of the demarcation membrane system to provide extra membrane necessary for platelet biogenesis.[1] Despite having been observed in multiple tissues, it is only recently that the bone marrow (BM) has been definitively established as the primary site of megakaryocyte and platelet production,[2] whereas most evidence for platelet production outside the BM comes from mouse studies and there is a lack of definitive evidence for this in humans.[3-5] Megakaryopoiesis is regulated at multiple levels, primarily by thrombopoietin (TPO), a cytokine mainly sourced from the liver. Recently, we and others have also identified a wide array of nutrients, hormones and other cytokines,[6-8] as well as megakaryocyte/platelet-derived extracellular vesicles[9,10] and extracellular matrix,[11-14] as critical regulators of megakaryopoiesis during homeostasis or stress, indicating a sophisticated regulation and an extensive association with a variety of pathophysiological processes.

Recent studies, mostly in mice, have focused on alternative routes by which megakaryocytes are directly differentiated from hematopoietic stem and progenitor cells.[15] Besides platelet production, megakaryocytes are being increasingly

appreciated as having diverse functions such as serving as immune cells[16] and hematopoietic stem cell (HSC) supporters.[17] In the face of stress of physical, chemical or biological origin, such as myeloablative injury and infection, there are also fundamental changes in the route and functional output of megakaryopoiesis as well as functional remodeling of megakaryocytes, which will significantly affect not only hemostasis but also immunity and overall hematopoiesis.[18-20] These exciting new findings not only expand our prior limited view of megakaryocyte generation and function, but also profoundly affect our understanding of the pathogenesis of many diseases such as hematopoietic disorders and immune-related diseases. Herein, we review these novel emerging concepts and highlight how they reshape our view of megakaryocyte generation and function in both health and disease.

Revised differentiation route and functional output of megakaryopoiesis

Alternative routes of megakaryopoiesis and megakaryocyte-biased hematopoietic stem cells and multipotent progenitors

Canonically, megakaryocytes are thought to be generated from HSC in a stepwise manner sequentially through multipotent progenitors (MPP) and a series of lineage-restricted/committed progenitors including common myeloid progenitors, megakaryocyte-erythrocyte progenitors and megakaryocyte progenitors (MkP) (Figure 1). Using single-cell techniques, increasing studies are revealing an alternative route in mice whereby HSC unilaterally and directly differentiate into MkP.[21] Moreover, the relative contribution of these two routes of differentiation can be modulated by pathophysiological demands, with chemotherapy-induced myeloablative injury and lipopolysaccharide-induced acute inflammation enhancing, respectively, the direct and stepwise routes of megakaryopoiesis (Figure 1).[18] Meanwhile, the different routes of megakaryopoiesis give rise to distinct subsets of MkP. MkP generated through the direct route show upregulation of CD24a and von Willebrand factor (VWF), while the expression of CD48 is low or absent; MkP generated through the stepwise route show high expression of CD48 and CD49b.[22] Furthermore, single-cell techniques have also shown that multiple megakaryocyte-biased/restricted HSC (Mk-HSC) with skewed production of megakaryocytes while retaining their multi-lineage potential are present in the mouse HSC compartment and are thought to initiate the direct route of megakaryopoiesis.[23,24] Besides, downstream MPP have recently been identified as a set of lineage-biased progenitors (designated MPP1-4), with MPP2 being biased towards the megakaryocyte/erythroid lineage.[25] MPP2 comprises a large number of megakaryocyte-primed cells (21.9%) and presents a preference for MkP production, bypassing common myeloid progenitors and megakaryocyte-erythrocyte progenitors. At steady state, it is estimated that the direct sources of megakaryocytes from HSC and MPP2 account for approximately 31%.[25]

Phenotypically, VWF$^+$HSC have been defined most commonly as Mk-HSC.[22-24] Although VWF$^+$HSC were suggested to be positioned at the apex of the hematopoietic hierarchy a decade ago,[23] a recent study from the same group demonstrated a non-hierarchical relationship between VWF$^+$HSC and VWF$^-$ multipotent HSC.[22] VWF$^+$HSC are primed to differentiate into MkP, and, in a few instances, also into MPP2 and megakaryocyte-erythrocyte progenitors with exclusive megakaryocyte potential. As a consequence, VWF$^+$HSC replenish platelets in a fast-track manner, in contrast to VWF$^-$HSC which give rise to all blood cell lineages through a slower stepwise pathway. It was suggested that the slower but more potent platelet replenishment by VWF$^-$HSC is the default pathway at steady state, while the shorter and faster pathway initiated by VWF$^+$HSC becomes more prominent after myeloablative injury that reduces MkP.[22] CD41$^+$HSC exhibiting a bias towards both megakaryocyte and myeloid lineages are also considered as Mk-HSC.[26-28] Gekas *et al.*[26] showed that CD41$^+$HSC are more quiescent than CD41$^-$HSC and may be hierarchically equal to or above CD41$^-$HSC. Shin *et al.*[29] revealed that c-Kit^{hi}HSC are an intrinsically megakaryocyte-biased population as well. Compared to c-Kit^{lo}HSC, c-Kit^{hi}HSC have diminished self-renewal potential, differentiate more rapidly, and are less quiescent and arose from c-Kit^{lo}HSC. Excitingly, a subset of Mk-HSC has also been identified recently in humans, although the markers remain to be defined.[30] It should be noted that these Mk-HSC are not equivalent to each other. For example, Sanjuan-Pla *et al.*[23] found co-expression of VWF and CD41 on only 67.9% of HSC. Collectively, although a variety of Mk-HSC can be identifiable through distinct markers, their differences should be borne in mind when defining Mk-HSC using these markers. Furthermore, although MkP generation through the direct route is intuitively thought to be faster than generation through the stepwise route, it does not necessarily mean that the direct route is truly the faster pathway, as MkP can be produced with even faster kinetics through the stepwise route, derived downstream of megakaryocyte-erythrocyte progenitors rather than from HSC/MPP.[18] This should be kept in mind when evaluating MkP and megakaryocyte generation kinetics by different routes of differentiation in different contexts.

Megakaryocyte-committed progenitors with phenotypes and functions distinct from HSC and MkP are also being increasingly identified in the mouse HSC compartment. Haas *et al.*[31] reported that the BM HSC compartment contains a population of stem-like megakaryocyte-committed progenitors (SL-MkP). Molecularly, megakaryocyte-specific transcripts such as CD41 are expressed in SL-MkP at steady state, but their protein synthesis is suppressed. Function-

ally, although SL-MkP are dormant and contribute little to steady-state megakaryopoiesis, they become activated and undergo functional and cellular maturation including megakaryocyte protein synthesis and polyploidization upon acute inflammation, resulting in a rapid replenishment of the megakaryocyte/platelet pool. Recently, Liu *et al.*[32] validated the enrichment of an early megakaryocyte-committed progenitor (MgP) in $CD201^{-}CD48^{-}$ and $CD48^{+}$ subpopulations of BM HSC, and demonstrated that HSC give rise to MkP through MgP. MgP express megakaryocyte-related genes, which are transcriptionally heterogeneous. Besides, MgP have little repopulating potential in comparison to Mk-HSC, but have greater platelet reconstitution potential than MPP2 and MkP. In a mouse model of mutant calreticulin-driven essential thrombocythemia, Prins *et al.*[33] identified an over-representation of "proliferative MkP (pMkP)" that exhibit an intermediate phenotype between HSC and MkP. They showed that pMkP represent a group of cells with megakaryocyte bias and increased proliferative potential relative to MkP, and are generated from HSC in an MPP2-independent manner. Similar to MgP, SL-MkP and MkP, pMkP have short-term platelet reconstitution potential as well. More interestingly, HSC can differentiate into restricted progenitors including MkP without undergoing cell division and even before entering the S phase of cell cycle. Compared to HSC, these undivided but differentiated progenitors lose multipotency and express lineage-specific genes.[34] These findings raise the questions of whether these phenotypically and functionally different megakaryocyte-committed progenitors are intermediate cells between HSC and MkP and are generated in different contexts. Further investigations are warranted to accurately mark and distinguish these megakaryocyte-committed progenitors from each other and from HSC, especially Mk-HSC. Furthermore, it should be noted that $Lin^{-}c\text{-}Kit^{+}Sca1^{-}CD41^{+}CD150^{+}$ cells, which are canonically defined as MkP in mice, also exhibit myeloid and erythroid potential upon transplantation,[35,36] highlighting the need for further optimization of markers for *bona fide* MkP as well.

Megakaryocyte heterogeneity

Emerging evidence from single-cell RNA-sequencing studies is also revealing the existence of molecularly and functionally different subpopulations in the mouse mature megakaryocyte compartment, including at least three subtypes: platelet-producing megakaryocytes, niche-supporting megakaryocytes and immune megakaryocytes (Figure 1). These megakaryocyte subpopulations are heterogeneous in terms of frequency, morphology, function and spatial distribution in the BM, despite sharing megakaryocyte markers. Platelet-producing megakaryocytes are the largest, characterized by high expression of genes involved in platelet production and hemostasis, and account for the largest proportion (~25%) in total BM megakaryocytes. Niche-supporting megakaryocytes account for ~18% of total BM megakaryocytes, are enriched in 8-32N megakaryocytes and are characterized by high expression of growth- and survival-promoting cytokines such as insulin-like growth factor 1 (IGF-1), platelet factor 4 (PF4), fibroblast growth factors (FGF) and transforming growth factor β (TGF-β). Immune megakaryocytes represent ~5% of total BM megakaryocytes, are small and enriched in megakaryocytes of low ploidy (≤8N), and highly express genes related to the inflammatory response and myeloid leukocyte activation. Platelet-producing and niche-supporting megakaryocytes are in close proximity to sinusoids and HSC, respectively.[37] Similar transcriptional heterogeneity is also established within human embryonic and BM mature megakaryocytes *in vivo*.[37-39] Although the ploidy distribution is similar between human and mouse BM megakaryocyte subpopulations, there are clearly different transcriptional signatures between human and mouse megakaryocytes as well as between embryonic and BM megakaryocytes. For example, enriched terms in human embryonic niche-supporting megakaryocytes are mainly related to extracellular matrix organization, while those in human and mouse BM niche-supporting megakaryocytes are related to cytokine-mediated cell-cell signaling. The immune programs in both human and mouse BM immune megakaryocytes seem to be more mature and diverse compared with human embryonic immune megakaryocytes. Molecularly, LSP1 and CD53 have been identified as markers for mouse BM immune megakaryocytes, while CD14 and CD148/CD48 have been identified as markers for human embryonic and BM immune megakaryocytes, respectively.[37-39]

Megakaryocytes are also present in various extramedullary tissues, including the lungs, spleen, and even the liver, which also operate, albeit minimally, to meet the enormous daily needs for platelet replenishment during homeostasis. Although extramedullary megakaryocytes seem to have the same megakaryocyte origin and share many megakaryocyte-specific characteristics, such as common megakaryocyte markers and the ability to produce platelets,[1,2] they can still be distinguished from BM megakaryocytes. Compared to BM megakaryocytes, lung megakaryocytes are smaller and characterized by an enrichment of 2N cells.[3,37] The ploidy of splenic megakaryocytes ranges from 8N to 64N, with a larger proportion of 64N cells compared with BM megakaryocytes.[5] With regard to lifespan, lung megakaryocytes are long-lived and present for up to 4 months, while BM megakaryocytes have a lifespan of <1 week.[4] Functionally, single-cell RNA-sequencing has demonstrated that lung megakaryocytes exhibit a cellular heterogeneity resembling that of BM megakaryocytes, with immune megakaryocytes being particularly abundant.[37] Compared to BM megakaryocytes, splenic megakaryocytes also display an immune-like transcriptional signature at steady state.[5] Taken together, it appears that extramedullary megakaryocytes are positioned to allow them to function more efficiently as immune cells.

Mechanisms underpinning the generation of megakaryocyte-biased hematopoietic stem cells/multipotent progenitors

Aging,[30,35,40] as well as aging-associated diseases such as myeloproliferative neoplasm (MPN)[28,41] and metabolic disease,[42] are always associated with Mk-HSC expansion in both humans and mice. Garyn *et al.*[43] reported that DNA damage, such as that elicited by replication stress, induces megakaryocyte priming in HSC through provoking G2 arrest. This may partially explain the expansion of Mk-HSC during aging, which is closely associated with DNA damage accumulation.[44] Inflammatory cytokines such as type I interferon (IFN-I)[28,45] and interleukin 1β (IL-1β)[40] can induce megakaryocyte priming of HSC directly, or indirectly through upregulating TPO which is crucial for Mk-HSC maintenance.[46] Upon acute inflammation, IFN-I also mediates the activation and maturation of SL-MkP through a post-transcriptional mechanism involving signal transducer and activator of transcription 1/mechanistic target of rapamycin (STAT1/mTOR) and/or Toll-like receptor 4/myeloid differentiation factor 88 (TLR4/MYD88) signaling.[31] Recently, we have also identified that renal Klotho and inorganic phosphate sup-

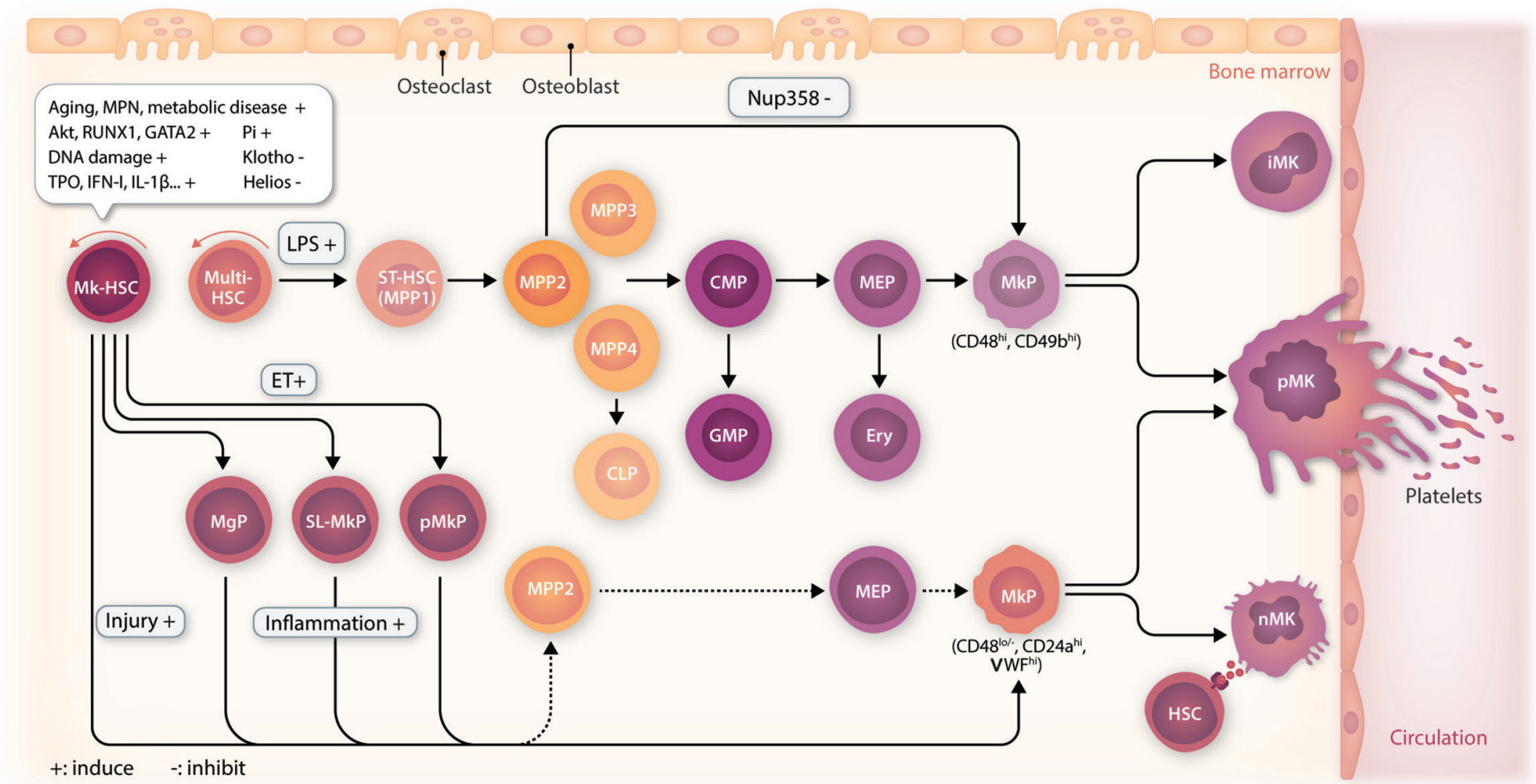

Figure 1. Overview of the differentiation routes and functional outputs of megakaryopoiesis. Megakaryocytes are generated through stepwise and direct routes of differentiation. The stepwise route of megakaryopoiesis involves differentiation through a multipotent hematopoietic stem cell, short-term hematopoietic stem cell (or multipotent progenitor 1), multipotent progenitors (primarily multipotent progenitor 2 [MMP2]), a common myeloid progenitor and megakaryocyte-erythrocyte progenitor, and ultimately gives rise to a megakaryocyte progenitor (MkP) highly expressing CD48 and CD49b. Besides, MPP2 can differentiate directly into MkP, bypassing common myeloid progenitors and megakaryocyte-erythrocyte progenitors. Megakaryocyte-biased/restricted hematopoietic stem cells (Mk-HSC) can differentiate directly into MkP with high expression of CD24a and von Willebrand factor and low/absent expression of CD48, forming the direct route of megakaryopoiesis. In a few instances, Mk-HSC also differentiate into MPP2 and megakaryocyte-erythrocyte progenitors which have exclusive MkP potential. In homeostasis and disease, early megakaryocyte-committed progenitors, stem-like megakaryocyte-committed progenitors and proliferative MkP may act as intermediate phenotypes between Mk-HSC and MkP. Although the stepwise route is the default manner for megakaryopoiesis at steady state, the direct route becomes prominent after myeloablative injury. The functional outputs of these two routes of megakaryopoiesis are different, with the direct and stepwise routes contributing, respectively, to niche-supporting megakaryocytes and immune megakaryocytes, while contributing to platelet-producing megakaryocytes together. MPN: myeloproliferative neoplasm; Akt: protein kinase B; RUNX1: runt-related transcription factor 1; GATA2: GATA binding protein 2; TPO: thrombopoietin; IFN-I: type I interferon; IL-1β: interleukin 1β; Pi: inorganic phosphate; Mk-HSC: megakaryocyte-biased/restricted hematopoietic stem cell; multi-HSC: multipotent hematopoietic stem cell; LPS: lipopolysaccharide; ST-HSC: short-term hematopoietic stem cell; MMP: multipotent progenitor; Nup358: nucleoporin 358kD; CLP: common lymphoid progenitor; CMP: common myeloid progenitor; GMP: granulocyte-monocyte progenitor; MEP: megakaryocyte-erythrocyte progenitor; Ery: erythrocyte; MkP: megakaryocyte progenitor; iMK: immune megakaryocyte; MgP: early megakaryocyte-committed progenitor; SL-MkP: stem-like megakaryocyte-committed progenitor; pMkP: proliferative megakaryocyte progenitor; VWF: von Willebrand factor; HSC: hematopoietic stem cell; pMK: platelet-producing megakaryocyte; nMK: niche-supporting megakaryocyte.

press and potentiate, respectively, megakaryocyte priming of HSC through antagonistically regulating Akt-mediated GATA binding protein 2 (GATA2) activation.[7] In patients with familial platelet disorder with a predisposition to myeloid malignancy (FPDMM), germline monoallelic mutations in runt-related transcription factor 1 (RUNX1) result in a deficiency of Mk-HSC potentially due to augmented TGF-β receptor type 1 (TGFBR1) signaling.[47] Besides, Wang *et al.*[27] revealed that RUNX1 governs the direct differentiation potential of Mk-HSC into megakaryocytes through transcriptionally regulating the expression of multiple essential megakaryocyte developmental genes including *Spi1*, *Selp*, and *Itga2b*. Cova *et al.*[48] reported that transcription factor Helios, which is highly expressed in hematopoietic stem and progenitors cells, represses megakaryocyte priming of HSC potentially through inducing chromatin compaction at the regulatory regions of megakaryocyte-specific genes recognized by GATA2 and RUNX1. These lines of evidence highlight the central roles of transcription factors RUNX1 and GATA2 in Mk-HSC generation. With regard to the generation of megakaryocyte-biased MPP2, it has been reported that nucleoporin Nup358, a component of nuclear pore complexes, dictates the lineage priming of MPP2 into the megakaryocyte/erythroid or myeloid lineage through regulating nuclear translocation of histone deacetylase 3 in a SUMOylation-independent manner.[49]

Mechanisms underpinning megakaryocyte heterogeneity

Although the mechanisms dictating functional specialization of megakaryocyte subpopulations remain largely undefined, a recent study highlighted a close association with the routes of megakaryopoiesis.[18] Using several fate-mapping systems, Li *et al.*[18] were able to distinguish the two routes of megakaryopoiesis and showed that the direct and stepwise routes contribute, respectively, to niche-supporting megakaryocytes and immune megakaryocytes, while they contribute to platelet-producing megakaryocytes together. It is tempting to speculate that stepwise megakaryopoiesis endows the megakaryocytes generated with a phenotype resembling that of myeloid immune cells by going through the myeloid branch of hematopoiesis. At the molecular level, in response to immune stimuli, rapid expansion of immune megakaryocytes accompanies strong upregulation of a pre-existing PU.1- and interferon regulatory factor 8 (IRF8)-associated monocytic-like transcriptional program, which regulates the expression of immune-related genes.[37] Protein arginine methyltransferase 1 (PRMT1) expression positively correlates with the expression of immune-related genes and thus is high in immune megakaryocytes. It has been suggested that PRMT1 may be essential for immune megakaryocyte generation via its substrates RUNX1, RNA binding motif protein 15 (RBM15), and dual specificity phosphatase 4 (DUSP4).[50]

The tissue environment also significantly influences the phenotypic and functional identities of megakaryocytes. For example, the immune phenotype of lung megakaryocytes is plastic and likely acquired from the immune environment of the lungs such as airway epithelial cell secretion of IL-33 as well as serving as an interface with the microbiome and environmental stress.[3] During sepsis, elevated levels of IL-3 specifically induce the differentiation of splenic MkP into immune-skewed megakaryocytes, while BM megakaryocytes are nearly unaffected, except for increased ploidy.[5] Notably, the molecular phenotypes of BM megakaryocytes are plastic as well. The immune phenotypes and functions of all BM megakaryocyte subpopulations will strengthen in response to pathogen challenge, resulting in an increase in immune megakaryocytes.[18,39] Thus, despite distinguishable features, whether these megakaryocyte subpopulations are truly functionally distinct and fixed or generated through environment/context-dependent mechanisms remains to be determined.

Multifaceted functions of megakaryocytes in immunity

Although megakaryocytes express a large repertoire of immune molecules, their roles as immune cells are relatively poorly understood in general. Actually, although BM megakaryocytes reside in a relatively immunosuppressive environment and seem to be inactive from an immune point of view at steady state, they have potent immune plasticity, similar to immune cells.[18,39] Besides, immune pathways are implicated in the process of production of platelets from megakaryocytes and the platelets inherit functional immune molecules from megakaryocytes.[16,51] These findings highlight the multifaceted roles of megakaryocytes in immunity (Figure 2).

Immune functions of megakaryocytes

Increasing studies show that megakaryocytes, particularly immune megakaryocytes or megakaryocytes that express C-X-C motif chemokine receptor 4 (CXCR4) strongly,[52] express high levels of a diverse array of immune-related receptors including TLR1-6,[53] NLR receptors,[54] C-type lectin receptors,[55] cyclic GMP–AMP synthase/stimulator of interferon genes (cGAS/STING),[19] melanoma differentiation-associated gene 5/mitochondrial antiviral-signaling protein (MDA5/MAVS),[56] and IFN-I receptor,[19] enabling them to recognize a wide range of pathogen- or damage-associated molecular patterns and respond to immune cytokines (Figure 2). Megakaryocytes also express major histocompatibility complex class I/II and co-stimulatory molecules CD80, CD80L and CD40L, enabling them to phagocytize pathogens and process and present antigens to trigger T-cell activation.[57,58] Besides, megakaryocytes express immune effector genes such as interferon-induced transmembrane protein 3 (IFITM3), whose activation provokes IFN-I secretion.[59] In response to bacterial infection, megakaryocytes

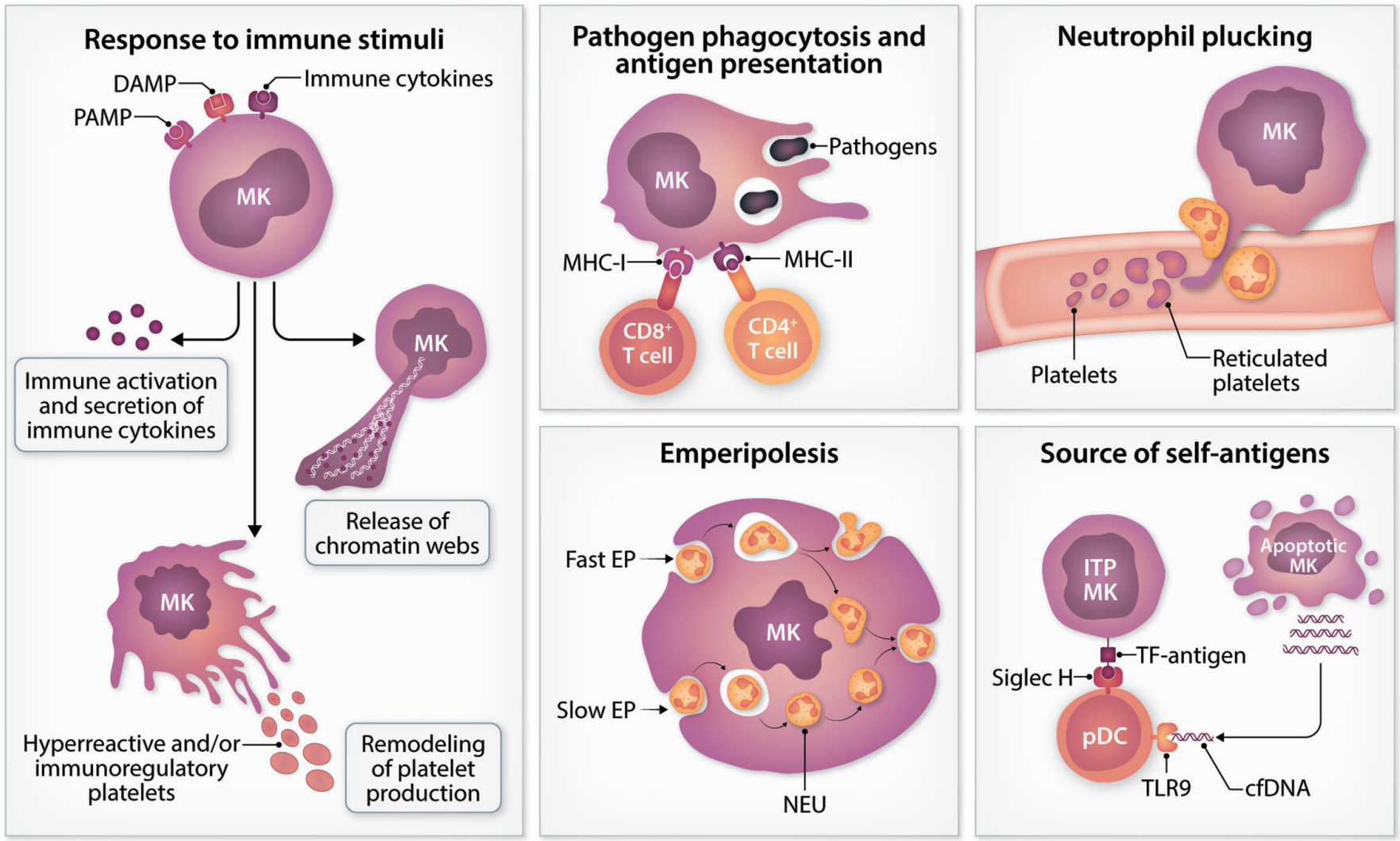

Figure 2. The multifaceted functions of megakaryocytes in immunity. Megakaryocytes can phagocytize pathogens and express major histocompatibility complex class I/II molecules to present antigens to CD4⁺ and CD8⁺ T cells. Megakaryocytes also express immune-related receptors and effectors to respond to immune stimuli including damage-associated molecular patterns, pathogen-associated molecular patterns and immune cytokines. Immune stimulation can provoke the secretion of immune cytokines and release of chromatin webs to regulate immune responses, as well as the remodeling of platelet production to produce hyperreactive and/or immunoregulatory platelets, which may be involved in the amplification and propagation of megakaryocyte immune functions. Megakaryocytes actively interact with neutrophils through processes including emperipolesis and neutrophil plucking. Megakaryocytes also serve as sources of self-antigens such as Thomsen-Friedenreich antigen, exposed by megakaryocytes during immune thrombocytopenia, and cell-free DNA, released by apoptotic megakaryocytes. DAMP: damage-associated molecular pattern; PAMP: pathogen-associated molecular pattern; MK: megakaryocyte; MHC-I/II: major histocompatibility complex class I/II; EP: emperipolesis; NEU: neutrophil; ITP: immune thrombocytopenia; TF antigen: Thomsen-Fruedenreich antigen; pDC: plasmacytoid dendritic cell; TLR9: Toll-like receptor 9; cfDNA: cell-free DNA.

also undergo chemotaxis and are capable of releasing chromatin webs resembling neutrophil extracellular traps.[60] All these endow megakaryocytes with abilities reminiscent of myeloid immune cells. Of note, *Mpl*$^{-/-}$ mice deficient in megakaryocytes and platelets have only limited CD4⁺ T-cell activation,[3] implying that megakaryocytes/platelets may also support T-cell responses via indirect mechanisms. Indeed, megakaryocytic secretion of tumor necrosis factor α (TNF-α) and IL-6 can stimulate migration and bacterial phagocytosis of macrophages and neutrophils.[52]

Besides phagocytizing pathogens, megakaryocytes also internalize cells of other hematopoietic lineages, especially neutrophils, in an intriguing cell-in-cell interaction termed emperipolesis (Figure 2). Megakaryocyte emperipolesis reflects at least two distinct processes as observed *in vitro*: approximately half of neutrophils exit megakaryocytes rapidly (≤10 minutes, fast emperipolesis) and display ameboid morphology when passing through megakaryocytes, while the remaining neutrophils reside within megakaryocytes for at least 60 minutes (slow emperipolesis) and are morphologically intact, with most of them residing within emperisomes. Furthermore, some neutrophils are able to exit the emperisome to enter the cytoplasm and be in contact with the demarcation membrane system.[61] Although the pathophysiological roles of emperipolesis are poorly defined, Cunin *et al.*[62] showed that emperipolesis permits membrane exchange and enhances platelet production, resulting in the production of platelets bearing membrane from neutrophils. Although rare at steady state, emperipolesis is always augmented during inflammation and myelofibrosis, which is potentially driven by the C-X-C-motif ligand 1 (CXCL1)-CXCR1/2 signaling that is crucial for neutrophil chemotaxis.[62,63] These findings imply that emperipolesis may represent an immune behavior of

megakaryocytes. In addition, Petzold *et al.*[64] reported that neutrophils migrate towards perisinusoidal megakaryocytes at steady state in a CXCR4–CXCL12-dependent manner and pluck intravascular megakaryocyte extensions to accelerate proplatelet growth and platelet release, although the immunological significance of this process remains to be determined (Figure 2).[64]

On the other hand, megakaryocytes also serve as an essential source of self-antigens. During platelet production, megakaryocytes show signs of apoptosis. Apoptotic megakaryocytes are a rich source of cell-free DNA, which is a potent activator of plasmacytoid dendritic cells via the TLR9–MYD88 pathway. The subsequent production of IFN-I by plasmacytoid dendritic cells then triggers local on-demand proliferation and maturation of MkP.[65] Besides, megakaryocytes are major contributors to circulating cell-free DNA (~26%) in healthy individuals, and the methylation pattern of megakaryocyte-specific cell-free DNA is suggested to serve as a biomarker for thrombopoietic disorders.[66] Plasmacytoid dendritic cells also surveil sialylated O-glycans on megakaryocytes via Siglec H receptors. Aberrantly sialylated O-glycans increase the exposure of Thomsen-Friedenreich antigen on megakaryocytes, which can be recognized by plasmacytoid dendritic cells and stimulates the secretion of IFN-I by plasmacytoid dendritic cells to inhibit proplatelet formation (Figure 2), thus contributing to the pathogenesis of immune thrombocytopenia.[67]

Immune-stimulated megakaryocytes produce phenotypically and functionally distinct platelets

Accumulating studies are establishing a close association between megakaryocyte immune stimulation and platelet production. For instance, we recently reported that both human and mouse megakaryocytes are intrinsically programmed to be apoptosis-resistant through reprogramming the BCL-xL–BAX/BAK axis during megakaryocyte maturation. Thus, in the face of irradiation-induced myeloablative injury, mouse megakaryocytes undergo minority mitochondrial outer membrane permeabilization rather than apoptosis, resulting in the activation of the cGAS–STING pathway via release of mitochondrial DNA.[19] The subsequent IFN-I secretion by megakaryocytes *in vivo* upregulates an IFN-stimulated gene guanylate-binding protein 2 (GBP2) which, as a large GTPase, may power cytoskeleton reorganization in megakaryocytes to generate large, hyperreactive platelets.[19] Consistently, during viral infection, IFN-I also stimulates megakaryocyte maturation and production of hyperreactive platelets *in vivo*.[31,45] Of note, azacitidine-induced hypomethylation and rapid accumulation of double-stranded RNA species stimulates activation of RNA sensors including TLR3 and MDA5/MAVS complex to secret IFN-I in human MkP *in vitro*. At this moment, however, IFN-I constrains TPO-induced MkP growth and differentiation through STAT1/suppressor of cytokine signaling 1 (SOCS1) signaling to induce thrombocytopenia.[56] Besides, as described above, secretion of IFN-I by plasmacytoid dendritic cells appears to have opposite effects on platelet production during homeostasis and immune thrombocytopenia.[65,67] These lines of evidence point to pivotal regulatory roles of IFN-I in platelet production as well as complex effects of IFN-I on the megakaryocyte lineage potentially depending on differentiation stages, contexts and species.

Apart from IFN-I, stimulation by other immune cytokines, including interleukins and TNF-α, has been well demonstrated to promote platelet production independently of TPO signaling.[51] Recently, increasing studies have shown that immune cytokines also remodel platelet functions through acting on megakaryocytes. For example, elevated levels of TNF-α play an important role in the development of platelet hyperreactivity during physiological aging through the reprogramming of inflammatory, metabolic and mitochondrial pathways in BM megakaryocytes, which is then passed on to platelets and results in increased platelet mitochondrial mass and reactivity.[68] During sepsis, IL-3 induces splenic megakaryocytes to produce a unique CD40L^{hi} platelet population that shows potent CD40L-dependent immunomodulatory functions such as inducing NETosis.[5] Furthermore, a wide array of other immune cytokines can induce platelet hyperreactivity.[69] The redundancy in signaling among immune cytokines suggests that megakaryocyte production of hyperreactive and/or immunomodulatory platelets may be a common consequence of immune cytokine stimulation. Overall, given the fundamental immune functions of platelets,[16] these findings infer that remodeling of the phenotype and function of produced platelets may act as a part of the megakaryocyte immune responses. Moreover, since platelets circulate throughout the body, it seems that the immune functions of megakaryocytes could be propagated and amplified by their progeny, platelets.

Megakaryocyte functions in supporting stressed or diseased hematopoietic stem cells

Megakaryocytes serve as hematopoietic stem cell-supporting cells

HSC homeostasis is tightly regulated by both intrinsic and extrinsic mechanisms, the latter of which are implemented by the specialized microenvironment termed niche. The HSC niche is composed of various types of cells, cytokines, extracellular matrix and nutrients.[17,70,71] Among these, megakaryocytes have attracted particular attention because a population of niche-supporting megakaryocytes, described above, localize, in particular, adjacent to HSC[72,73] and directly regulate the maintenance of HSC by acting as a biomechanical restrainer,[74] through release of extracellular vesicles[75] and synthesis of extracellular matrix such as fibronectin, collagen and laminin,[76] as well as being a paracrine source

of cytokines including PF4,[77] IGF-1/IGF-1 binding protein-3 (IGFBP-3),[78] FGF1 and TGF-β.[79] More interestingly, although being enriched in megakaryocytes of 8-32N, niche-supporting megakaryocytes with different ploidy may have different HSC-supporting functions. For example, only 32N, not 8/16N megakaryocytes, express IGFBP-3 transcript, while 8N megakaryocytes express more IGF-1 transcript than 16/32N megakaryocytes.[78] Indirectly, megakaryocytes also interact with mesenchymal stromal cells such as osteomacs to support HSC maintenance through upregulation of Embigin and CD166.[80] Molecularly, it has been reported that serglycin plays a key role in balancing the distribution of HSC-supporting factors particularly PF4 between megakaryocyte α-granules and their adjacent extracellular spaces.[81] Simultaneously, membrane CLEC2 mediates megakaryocyte production of HSC-supporting factors, particularly TPO, through downstream signaling molecules including Syk, Lcp2, and Plcg2.[82] The regulatory roles of megakaryocytes in HSC biology during homeostasis have been extensively described.[15] Below we focus on new findings that expand the supporting functions of megakaryocytes for stressed or diseased HSC (Figure 3).

Megakaryocyte regulation of hematopoietic stem cell injury and regeneration

The crosstalk between megakaryocytes and HSC becomes more prominent in the context of hematopoietic injury. Usually, hematopoietic injury induces multiple modalities of HSC death, whereas megakaryocytes are injury-resistant and remain functional in rodents for even 7~10 days after injury.[19,20] Particularly, in the face of sublethal irradiation-induced myeloablative injury, megakaryocytes become a predominant component of the HSC niche.[19] These surviving megakaryocytes undergo phenotypic and functional shifts towards niche-supporting megakaryocytes and become much closer to HSC after injury, accompanied by augmented IGF-1 secretion in comparison to other niche cells, thus becoming a predominant source of IGF-1 in the HSC niche. Functionally, megakaryocytic IGF-1 hypersecretion promotes self-renewal and expansion of HSC through delicately coordinating proliferation, mitochondrial oxidative metabolism and ferroptosis to safeguard efficient HSC regeneration after injury.[20] Even after lethal irradiation-induced myeloablative conditioning, megakaryocytes also survive, migrate to the endosteal niche and drive endosteal niche expansion through the paracrine effect of platelet-derived growth factor-BB to facilitate the engraftment of transplanted HSC.[83] Moreover, after chemotherapy-induced myeloablative injury, megakaryocytic FGF1 signaling dominates over TGF-β signaling, which restricts HSC proliferation, to trigger HSC regeneration.[79] These studies suggest that megakaryocytes act as a last line of defense against myeloablative injury. Of note, stress of biological origin, such as viral infections, can stimulate megakaryocytic IFITM3 to provoke IFN-I secretion, which protects neighboring HSC from the viral infection.[59] More interestingly, megakaryocytes are reported to reside particularly adjacent to Mk-HSC and be essential for their megakaryocyte priming.[72] It is tempting to speculate that the recently reported ferroptosis-resistant property of Mk-HSC in homeostasis and injury[70,71] may be related to their close interaction with megakaryocytes, such as megakaryocytic paracrine effects of IGF-1. Megakaryocytes can indirectly regulate HSC regeneration as well. As reported by Luis *et al.*,[84] antibodies against platelets can activate megakaryocytes and promote their secretion of IL-1, which distinctively activates Mk-HSC through stimulating BM leptin receptor-positive perivascular niche cells expressing IL-1 receptor, thereby promoting rapid platelet replenishment.

Megakaryocyte regulation of hematopoietic stem cell aging

HSC aging, which is characterized by Mk-HSC expansion, reduced reconstitution capacity, increased replication stress, accumulated DNA mutations, increased apoptosis resistance, loss of autophagy capacity, and epigenetic, transcriptional and translational alterations,[44] has long been a well-described paradigm for the investigation of stem cell aging. Although cell-intrinsic defects seem to play dominant roles in driving HSC aging, emerging evidence argues for a contribution of the aged niche.[17] Quantitative and qualitative alterations of megakaryocytes occur with age. Despite the fact that the megakaryocyte lineage expands with age,[36] aged HSC are unexpectedly farther away from megakaryocytes than are young HSC, in which reduced β_3-adrenergic receptor–NOS1 activity may be implicated.[85,86] Whether megakaryocyte expansion is a consequence or a cause of HSC aging remains elusive. In support of the promotive role of megakaryocytes in HSC aging, in a mouse model in which *Jak2*^{V617F} is expressed solely in the megakaryocyte lineage, *Jak2*^{V617F}-bearing megakaryocytes promote HSC aging potentially through decreased direct interaction and increased secretion of pro-inflammatory and anti-angiogenic factors.[87] Besides, platelet expression and circulating levels of PF4 decline with age and are closely associated with the development of age-related diseases,[88] suggesting an involvement in HSC aging given the fundamental role of megakaryocytic PF4 in HSC homeostasis.[77] Consistently, work by Zhang *et al.*[89] suggests that age-related attrition of the megakaryocyte niche and the resultant PF4 deficiency is a central mechanism in HSC aging, potentially through downregulating low density lipoprotein receptor signaling and CXCR3 signaling.

Megakaryocyte regulation of malignant hematopoietic stem cells

MPN, which are prevalent in the eldery, are clonal diseases originating from somatically-mutated HSC, characterized by megakaryocyte/myeloid bias of HSC and a tendency to progress to acute myeloid leukemia.[90] *Jak2*^{V617F}, a gain-of-function *Jak2* mutation, is present in most MPN. Quantitative

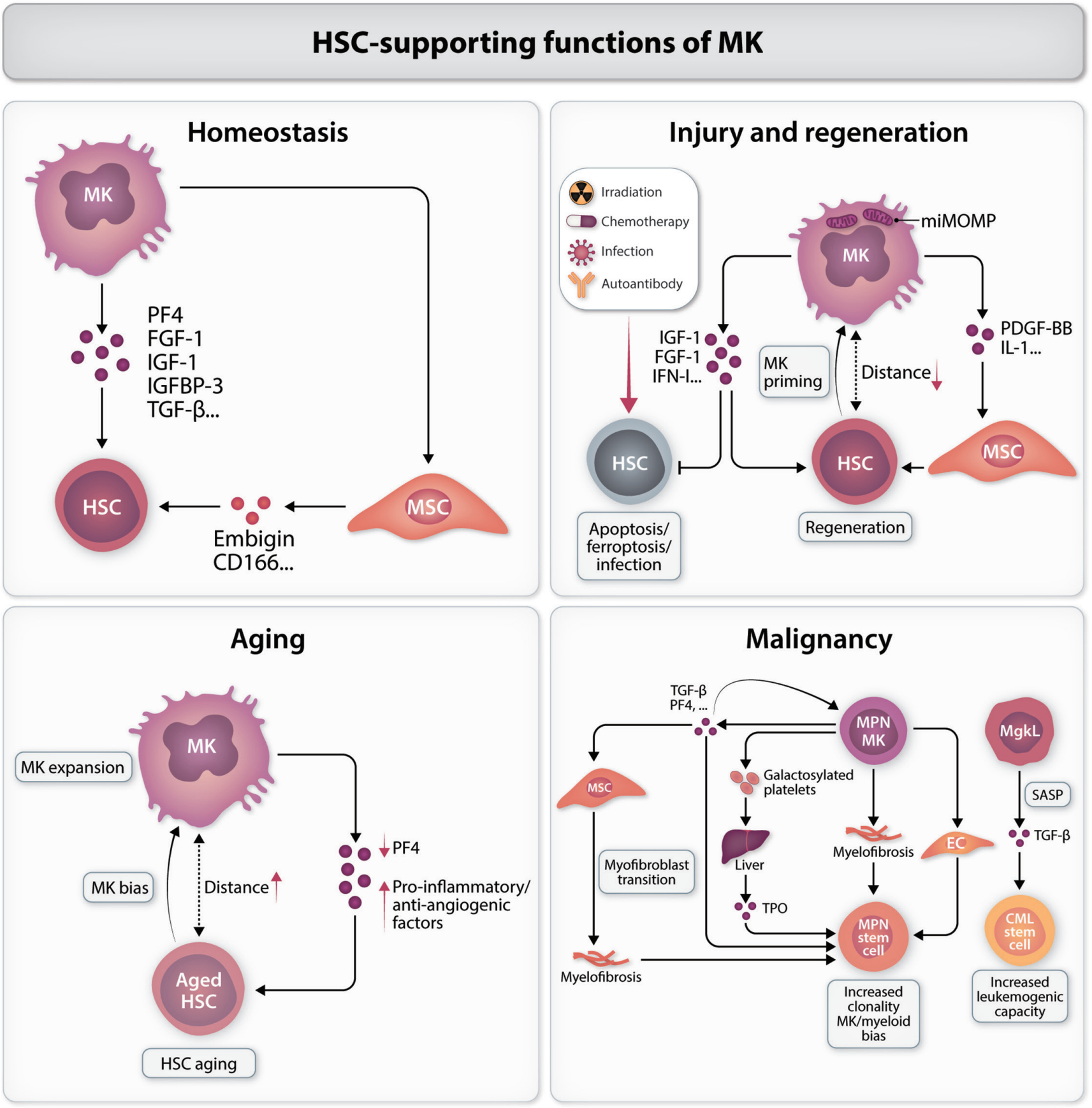

Figure 3. Megakaryocyte functions in supporting stressed or diseased hematopoietic stem cells. At homeostasis, megakaryocytes support hematopoietic stem cell (HSC) maintenance directly through secretion of platelet factor 4, fibroblast growth factor 1, insulin-like growth factor 1, IGF binding protein 3, transforming growth factor β and others, or indirectly through interacting with mesenchymal stromal cells, such as osteomacs, to upregulate Embigin and CD166. In the face of myeloablative injury, megakaryocytes undergo minority mitochondrial outer membrane permeabilization but not apoptosis, becoming closer to HSC, and secrete cytokines such as insulin-like growth factor 1 and fibroblast growth factor 1 to concurrently stimulate HSC activation and prevent HSC demise, thus safeguarding efficient HSC regeneration. Following myeloablative conditioning and HSC transplantation, megakaryocytes also survive and secrete platelet-derived growth factor-BB to expand the endosteal niche to facilitate HSC engraftment. Upon viral infection, megakaryocytes secrete type I interferon to protect neighboring HSC from viral infection. Besides, megakaryocytic secretion of insulin-like growth factor 1 and type I interferon may underlie the megakaryocyte priming of HSC that is frequently observed after hematopoietic injury. Indirectly, megakaryocytic secretion of interleukin 1 acts on leptin receptor-positive perivascular cells to activate megakaryocyte-biased HSC after autoantibody-induced platelet depletion. With age, although megakaryocytes expand, they get farther away from HSC. The decreased secretion of platelet factor 4 and increased secretion of pro-inflammatory and anti-angiogenic factors from megakaryocytes may contribute to HSC aging, which is characterized by megakaryocyte bias. In cases of myeloproliferative neoplasm (MPN), an altered megakaryocyte secretome, production of highly galactosylated platelets by the MPN megakaryocytes and the resultant stimulation of hepatic thrombopoietin secretion,

Continued on following page.

as well as remodeling of the bone marrow niche by the MPN megakaryocytes through altered interactions with endothelial cells/mesenchymal stromal cells and/or increased extracellular matrix synthesis, collectively increase the clonality and megakaryocyte/myeloid bias of MPN stem cells. In cases of chronic myeloid leukemia, increased secretion of transforming growth factor-β by senescent BCR-ABL^{+}CD41^{+}CD150^{+} leukemic megakaryocyte-lineage cells increases the leukemogenic capacity of the leukemic stem cells. MK: megakaryocyte; PF4: platelet factor 4, FGF-1: fibroblast growth factor 1; IGF-1: insulin-like growth factor 1; IGFBP3: IGF binding protein 3; TGF-β: transforming growth factor β; MSC: mesenchymal stromal cells; miMOMP: minority mitochondrial outer membrane permeabilization; IFN-I: type I interferon; PDGF-BB: platelet-derived growth factor-BB; IL-1: interleukin 1; TPO: thrombopoietin; EC: endothelial cell; MgkL: leukemic megakaryocyte-lineage cell; SASP: senescence-associated secretory phenotype; CML: chronic myeloid leukemia.

and qualitative alterations of megakaryocytes occur during MPN and are closely associated with MPN progression. As reported previously, *Jak2*^{V617F} megakaryocytes can promote MPN stem cell function by increasing the quiescence and repopulating capacity of these latter, potentially through altered megakaryocyte-endothelial interactions.[91,92] Increased expression and activity of β-1,4-galactosyltransferase 1 is observed in MPN megakaryocytes, which is associated with high platelet surface galactosylation. In turn, the highly galactosylated platelets promote hepatic TPO synthesis regardless of platelet mass,[93] which contributes to the maintenance and megakaryocyte/myeloid bias of MPN stem cells.[92] In primary myelofibrosis, TGF-β and PF4 released by MPN megakaryocytes are master drivers of mesenchymal stromal cell reprogramming into myofibroblasts, which drive myelofibrosis to promote the predominance of MPN stem cells.[94,95] In patients with polycythemia vera, downregulated megakaryocytic PF4 expression may contribute to the increased clonality of polycythemia vera HSC.[96] MPN also elicit other megakaryocyte-derived alterations. For example, increased TPO levels in MPN induce the release of TGF-β and consequent increase of extracellular matrix component synthesis by megakaryocytes,[97] and increased IL-13 levels promote MPN megakaryocyte growth and induce surface expression of TGF-β and collagen biosynthesis,[98] both of which may contribute to the deterioration of the BM niche. MPN megakaryocytes also show increased PIEZO1 expression, which may impair the maturation of megakaryocytes and thus contribute to aggravating some hallmarks of MPN.[99] In chronic myeloid leukemia, the oncogenic fusion gene *BCR-ABL* accelerates megakaryocyte-lineage differentiation and induces senescence and a senescence-associated secretory phenotype (SASP), leading to an increase in senescent *BCR-ABL*$^{+}$ CD41^{+}CD150^{+} leukemic megakaryocyte-lineage cells. In turn, TGF-β, as a representative SASP cytokine secreted from senescent leukemic megakaryocyte-lineage cells, maintains the leukemogenic capacity of chronic myeloid leukemia stem cells.[100]

Conclusions and future directions

Research into homeostasis as well as infectious, inflammatory and myeloablative situations has found distinct differentiation routes and functional outputs of megakaryopoiesis. Such new insights extend our understanding of the plasticity, heterogeneity and particular importance of megakaryocytes. It is now appreciated that megakaryopoiesis does not only occur as a stepwise process, but is dynamic and adapts to pathophysiological demands at multiple levels including HSC, MPP and MkP. Nevertheless, despite the distinguishable transcriptional features among megakaryocyte subpopulations, it is not sufficient to define different functional subtypes of megakaryocytes based solely on a set of markers or transcripts. This is of particular importance for immune megakaryocytes that are proposed to be small, low ploidy cells that may not even have the potential to produce platelets, all of which are contrary to the typical features of megakaryocytes. Critical questions remain open regarding whether megakaryocyte subpopulations are truly functionally distinct beyond their transcriptional profiles, whether megakaryocyte phenotypes are adaptive responses to different environmental cues or acquired through specialized routes of differentiation, whether immune megakaryocytes are a myeloid lineage cell that has gene and protein markers in common with megakaryocytes, to what degree the two differentiation routes replenish different megakaryocyte subpopulations during homeostasis and stress, and whether megakaryocytes of different origins and/or different subpopulations produce platelets with phenotypic and functional differences. Meanwhile, the newly identified properties of megakaryocytes also provide intriguing future prospects for fully understanding the pathophysiological functions of megakaryocytes in health and disease. There is still a notable lack of evidence regarding the sites, differentiation routes and functional outputs of human megakaryopoiesis as well as human megakaryocyte functions in immunity and HSC support. Although the megakaryocyte lineage shares much homology between humans and mice, caution should be employed in generalizing findings from mice to humans. We envision that future investigations in which fate tracing is coupled with multi-omics methods will help to provide a more comprehensive understanding of the origin, generation, function and heterogeneity of human and murine megakaryocytes in various contexts.

Overall, the changing dogma of megakaryopoiesis, as well as the emerging functions of megakaryocytes, particularly the notions that megakaryocytes serve as immune cells

and a last line of defense against hematopoietic injury, are not only expected to provide an updated framework for interpreting the pathophysiological roles of megakaryocytes, but also open new opportunities to harness the megakaryocyte/platelet system in health and disease.

Disclosures

No conflicts of interest to disclose.

Contributions

CD and JC carried out the literature searches, prepared figures, and wrote and edited the manuscript. JW and CD designed and revised the manuscript. All authors read and approved the final manuscript.

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