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# Novel Evidence That the Mannan-Binding Lectin Pathway of Complement Activation Plays a Pivotal Role in Triggering Mobilization of Hematopoietic Stem/Progenitor Cells by Activation of Both the Complement and Coagulation Cascades

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**OPEN**

# Novel evidence that the mannan-binding lectin pathway of complement activation plays a pivotal role in triggering mobilization of hematopoietic stem/progenitor cells by activation of both the complement and coagulation cascades

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Hematopoietic stem progenitor cells (HSPCs) circulate at low levels in peripheral blood (PB) and follow changes in circadian rhythm.[1] Evidence has accumulated that their egress from stem cell niches is significantly augmented in a complement cascade (ComC)-dependent manner.[2] The number of HSPCs circulating in PB increases during infection, tissue or organ injuries and particularly after administration of pharmacological drugs, such as granulocyte-colony stimulating factor (G-CSF) or the CXCR4 receptor antagonist AMD3100, and pharmacological mobilization is a means to obtaining HSPCs for hematopoietic transplants.[3] However, the mobilization process is still not well understood.

The ComC is activated by the classical, mannan-binding lectin (MBL) and alternative pathways.[4] Activation of the ComC and generation of cleavage fragments of the fifth component of the ComC (C5), such as C5a, $_{desArg}$C5a and C5b, by classical C5 convertase initiates events that are required for egress of HSPCs from bone marrow (BM) into PB.[5] Recent results indicate that the coagulation cascade (CoaC) is activated in parallel with activation of the ComC during the mobilization process and plays a supportive role, because thrombin has 'C5-like' convertase activity.[6] Although a requirement for ComC activation and the pivotal roles of the distal part of complement activation and the generation of C5 cleavage fragments in executing mobilization have been previously demonstrated,[5] mice with mutations in components that initiate the classical pathway ($C1q^{-/-}$ mice) do not show impairment in mobilization of HSPCs.[7]

Therefore, we became interested in the potential role of the MBL pathway of ComC activation in triggering the mobilization of HSPCs after administration of G-CSF or AMD3100. MBL is a soluble pattern-recognition receptor circulating in PB that is involved in the first line of defense of innate immunity and, as mentioned above, activates the ComC by engaging the so-called MBL-associated serine proteases (MASP-1 and -2). The MBL–MASP pathway also activates the CoaC, which, as also recently demonstrated, plays a role in the mobilization process.[6,8] On the basis of these findings, we hypothesized that the MBL-initiated ComC and CoaC activation pathways are involved in triggering mobilization of HSPCs and that MBL–MASP deficiency results in poor mobilization efficiency.

In our experiments, we employed 2-month-old, MBL-deficient ($MBL^{-/-}$) and MASP-1-deficient ($MASP\text{-}1^{-/-}$) mice as well as their normal wild type (WT) littermates, and animals were mobilized with G-CSF (100 μg/kg daily for 3 or 6 days) or AMD3100 (5 mg/kg). Following mobilization, we measured (i) the total number of white blood cells, (ii) the number of circulating clonogenic colony-forming unit granulocyte/macrophage (CFU-GM) progenitors and (iii) the number of Sca-$1^+$c-$kit^+$$lineage^-$ (SKL) cells in PB. In parallel, we evaluated activation of the ComC after administration of G-CSF or AMD3100 in experimental animals by employing C5a ELISA. Furthermore, to address the role of the CoaC in MBL–MASP-1- and MBL–MASP-2-induced mobilization, $MBL^{-/-}$ mice were treated

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in some of the experiments with an inhibitor of the CoaC (refludan).

We found that MBL-KO (Figure 1a) and MASP-1-KO (Figure 1b) mice are poor mobilizers in response to mobilizing agents compared with WT littermates. Moreover, to exclude defects in hematopoiesis in animals employed in this study that could be responsible for the observed mobilization defects, we found that under steady-state conditions MBL-deficient (Supplementary Figure 1) and MASP-1-deficient (Supplementary Figure 2) mice have normal PB cell counts (Panels A), red blood cell parameters (Panels B), numbers of bone marrow-residing HSPCs (Panels C) and numbers of clonogenic progenitors (Panels D) compared with WT animals.

Since, as mentioned above, the MBL–MASP-1 complex has been reported to also activate the CoaC, and thrombin provides C5-like convertase activity to activate/cleave C5,[6] which is pivotal for egress of HSPCs from BM into PB, we performed mobilization studies in MBL$^{-/-}$ and WT mice in the presence or absence of the CoaC inhibitor refludan. Figure 1c shows that, as expected, control mice exposed to refludan have impaired G-CSF-induced mobilization. However, administration of refludan did not augment the mobilization defect in MBL$^{-/-}$ mice, which indicates that the MBL–MASP pathway is most likely the crucial pathway in activation of the CoaC following G-CSF administration.

Overall, the salient observation of our work is that MBL and its downstream effector MASP-1 play a pivotal role in activation of the ComC during G-CSF- and AMD3100-mediated mobilization of HSPCs. For example, Figure 2a demonstrates defective generation of C5a in MBL$^{-/-}$ and MASP-1$^{-/-}$ animals, which explains our previous results in which mice that have a defect in activation of the classical pathway (C1q$^{-/-}$) mobilize HSPCs into PB normally,[7] because distal ComC pathway and C5 in C1q$^{-/-}$ mice is properly activated in MBM–MASP-dependent manner. We also demonstrate that, in addition to the ComC, the CoaC, which augments mobilization of HSPCs by providing thrombin-mediated C5-like convertase activity is also activated during mobilization in an MBL–MASP-dependent manner. On the basis of these and other published results, we propose the mechanistic scenario depicted in Figure 2b, which portrays mobilization of HSPCs in response to pharmacological agents (G-CSF or AMD3100).

Specifically, the first step during mobilization is activation of Gr-1$^+$ granulocytes and monocytes in the BM microenvironment, which are a source of several proteolytic[9,10] and, as recently demonstrated, also lipolytic enzymes[11] that together cooperate to

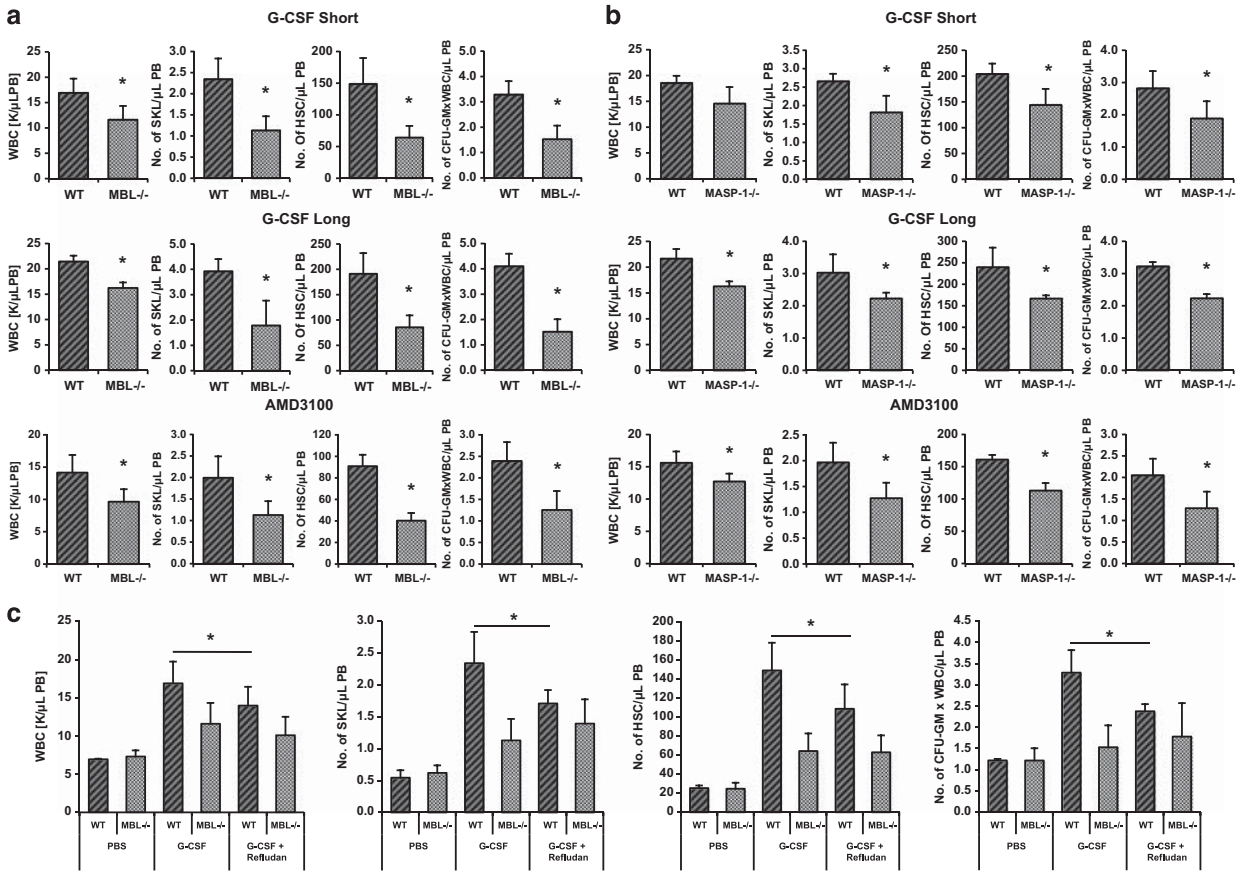


**Figure 1.** MBL$^{-/-}$ and MASP-1$^{-/-}$ mice are poor mobilizers in response to G-CSF and AMD3100. (**a**) MNCs were isolated from WT and MBL$^{-/-}$ mice after a short G-CSF mobilization (3 days, upper panel), long G-CSF mobilization (6 days, middle panel) or AMD3100 mobilization (lower panel). Mice were killed 6 h after the last G-CSF injection or 1 h after AMD3100 mobilization, and the numbers of white blood cells, SKL (Sca-1$^+$ c-kit$^+$ Lin$^-$) cells, HSCs (Sca-1$^+$ CD45$^+$ Lin$^-$) and CFU-GM clonogenic progenitors in PB were evaluated. Results from two separate experiments with five mice per group are pooled together, $^*P \leqslant 0.05$. (**b**) WT and MASP-1$^{-/-}$ mice were mobilized for 3 days with G-CSF (short mobilization, upper panel), 6 days with G-CSF (long mobilization, middle panel) or AMD3100 mobilization (lower panel). Mice were killed 6 h after the last G-CSF or 1 h after AMD3100 injection, the mononuclear cells were isolated, and the numbers of white blood cells, SKL (Sca-1$^+$ c-kit$^+$ Lin$^-$) cells, HSCs (Sca-1$^+$ CD45$^+$ Lin$^-$) and CFU-GM clonogenic progenitors in PB were evaluated. Results from two separate experiments with five mice per group are pooled together, $^*P \leqslant 0.05$. (**c**) The effect of inhibition of the CoaC on mobilization of HSPCs in MBL-deficient mice. MBL$^{-/-}$ mice were mobilized for 3 days with G-CSF (100 μg/kg per day, s.c.) in the presence or absence of refludan (administered daily for 3 days, 5 mg/kg, i.p.). The numbers of circulating leukocytes, SKL cells, HSCs and CFU-GM progenitors per microliter of PB are shown. Control mice were injected with PBS. Results from two separate experiments with five mice per group are pooled together, $^*P \leqslant 0.05$.

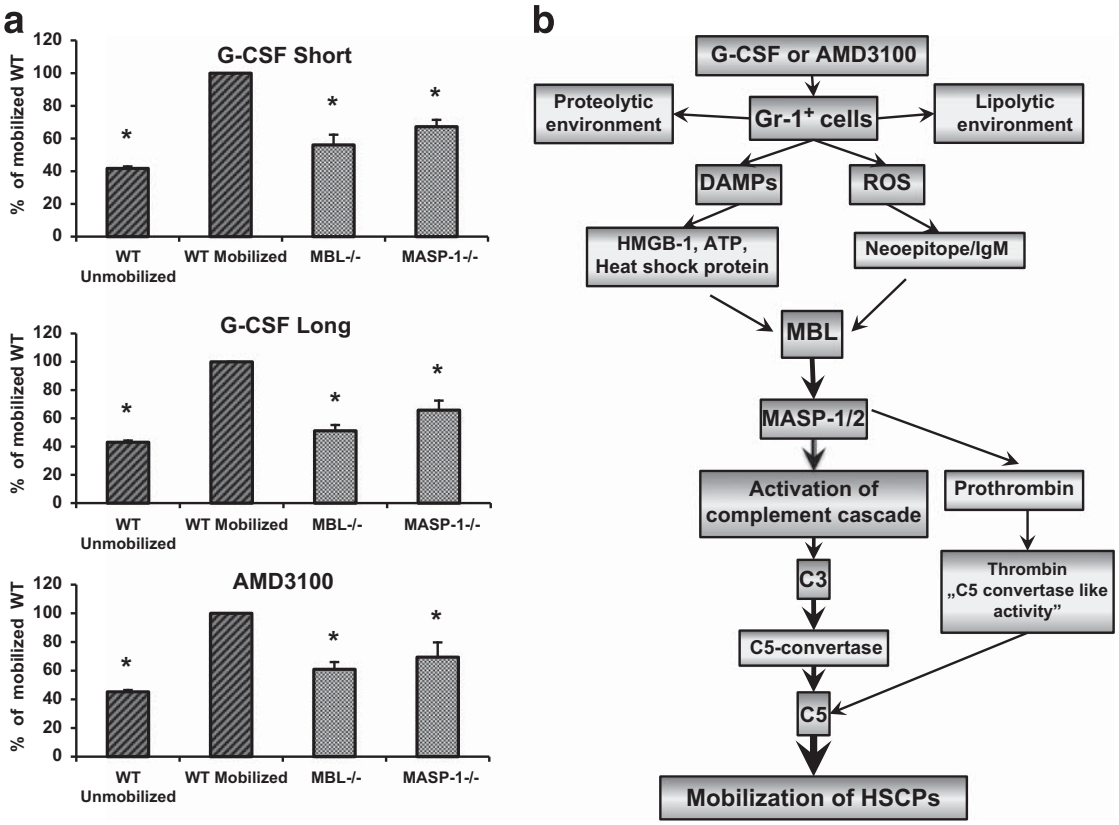


**Figure 2.** (**a**) Defective cleavage of C5 in MBL- and MASP-1-deficient mice during mobilization**.** Plasma collected from PB of mice mobilized with G-CSF (short or long) or AMD3100 show lower levels of C5a compared with WT mice. Results shown as a percentage of mobilized WT mice, $*P \leqslant 0.05$. (**b**) Interplay of components of innate immunity (the ComC, Gr-1$^+$ cells, naturally occurring IgM antibodies), and the CoaC in the mobilization of HSPCs. Mobilizing agents, G-CSF or AMD3100, activate Gr-1$^+$ neutrophils and monocytes and enhance secretion of ROS by these cells. In the BM microenvironment, ROS expose neoepitopes. Moreover, during mobilization, several types of DAMP molecules are released. Neoepitope–IgM complexes as well as DAMPs are recognized by MBL, which activates the ComC and the CoaC in a MASP-dependent manner. C5 convertases (classical and 'C5-like') generated in the next step cleave C5 to release cleavage fragments crucial to executing egress of HSPCs from BM.

impair retention signals for HSPCs in BM niches as well as disturb membrane lipid raft integrity. The fact that experiments with mouse mutants for several proteolytic enzymes that are released from activated Gr-1$^+$ cells in BM have failed so far to identify a crucial enzyme[3,12] suggests redundancy among enzymes and the involvement of several other proteases, such as cathepsin K. Moreover, it is widely acknowledged that proteolytic enzymes digest proteins involved in retention of HSPCs in BM niches, such as stromal-derived factor 1 and vascular cell adhesion molecule 1, expressed in the BM microenvironment, with the corresponding receptors, chemokine receptor CXCR4 and $\alpha_4\beta_1$ integrin receptor VLA-4, expressed on the surface of HSPCs.[3,14] Interestingly, in contrast to proteolytic enzymes, the lipolytic enzyme PLC-β2 has already been demonstrated to play an important role in mobilization, as it perturbs lipid raft integrity, which is necessary for proper signaling from CXCR4 and VLA-4.[11] At the same time, we cannot exclude the involvement of other lipolytic enzymes that could directly affect sphingosine-1-phosphate or ceramide-1-phosphate gradients.

Besides releasing proteolytic and lipolytic enzymes that create a proteolytic and lipolytic BM microenvironment, Gr-1$^+$ cells also secrete several other mediators that promote mobilization, such as (i) reactive oxygen species (ROS), which induce expression of neoepitopes in the BM microenvironment that bind naturally occurring IgM antibodies,[13] and (ii) danger-associated molecular pattern molecules (DAMPs), such as heat shock proteins, ATP and high-mobility group box 1. The role of these pathways in the mobilization of HSPCs is supported by the fact that Gr-1$^+$ cell-deficient mice,[3,12] ROS deficiency[14] or a lack of naturally occurring IgM antibodies[13] results in poor mobilizer status. What is important for this report, both neoepitope–IgM complexes and DAMPs, which are exposed or released at the initial phase of mobilization, are recognized by circulating MBL, which, in cooperation with MASP, triggers activation of both the ComC and the CoaC.[15]

Taking into consideration the pivotal role of the MBL pathway in the mobilization process, our studies have potential clinical implications for identifying so-called poor mobilizers. It is known that ~10% of normal healthy donors respond poorly to currently available mobilizing drugs. On the other hand, human MBL (*MBL2*) deficiency is the most common form of complement deficiency and is seen in ~10% of humans.[15] These numbers of poor mobilizers and MBL-deficient patients appear to match up, and it would be useful to evaluate activation of the MBL pathway in poor and good mobilizers. In human, MBL is produced in liver, and structural mutations in exon 1 of the human *MBL2* gene at codon 52 (Arg → Cys, allele D), codon 54 (Gly → Asp, allele B) and codon 57 (Gly → Glu, allele C) also independently reduce the level of functional serum MBL by disrupting the collagenous structure of the protein. Furthermore, several nucleotide substitutions in the promoter region of the human *MBL2* gene at positions − 550 (H/L polymorphism), − 221 (X/Y polymorphism), − 427, − 349, − 336, del (−324 to − 329), − 70 and +4 (P/Q polymorphisms) affect the MBL2 serum concentration.[15] Thus, further clinical studies are justified to see whether it is possible that the *MBL2* state can predict poor mobilizers.

In conclusion, we have identified a previously unrecognized role for the MBL–MASP-1 pathway in triggering both ComC and CoaC activation during the HSPC mobilization process. This finding explains the pivotal role of the MBL pathway in triggering activation of the proximal part of the ComC and explains why, with a deficiency in activation of classical pathway components (C1q), mobilization of HSPCs proceeds normally as long as the MBL pathway remains intact.[7] Taking into consideration that ~10% of normal people are poor activators of the MBL pathway[15] and that this percentage may correspond with the ~10% of the normal healthy population that are poor mobilizers, we are currently investigating whether MBL deficiency correlates with poor mobilization status in patients. If our hypothesis is correct, the MBL level could become an important predictive parameter for identifying poor mobilizers. Finally, our results again confirm a pivotal role of the ComC and other elements of innate immunity as well as involvement of the CoaC in the mobilization process.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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