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# IMPAIRED MOBILIZATION OF HEMATOPOITEIC STEM/ PROGENITOR CELLS IN C5-DEFCIENT MICE SUPPORTS THE PIVOTAL INVOLVEMENT OF INNATE IMMUNITY IN THIS PROCESS AND REVEALS NOVEL PROMOBILIZATION EFFECTS OF GRANULOCYTES

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## Abstract

We reported that complement cascade (CC) becomes activated in bone marrow (BM) during granulocyte colony stimulating factor (G-CSF) mobilization of hematopoietic stem/progenitor cells (HSPCs) and demonstrated that while the third CC component (C3)-deficient mice are easy mobilizers, the fifth CC component (C5)-deficient mice mobilize very poorly. To explain this, we postulated that activation/cleavage of CC releases C3a and C5a anaphylatoxins that differently regulate mobilization. Accordingly, C3a, by enhancing responsiveness of HSPCs to decreasing concentrations of stromal-derived growth factor-1 (SDF-1) in BM, prevents mobilization and promotes their BM-retention. As such, we focused on the mobilization-enhancing role of C5a herein. We found that C5a receptor (C5aR) is not expressed on the surface of HSPCs, and C5a-mediated pro-mobilization effects are mediated by stimulation of granulocytes. Overall, our data support a following model. First $C5aR^+$ granulocytes are chemoattracted by plasma C5 cleavage fragments, being the first wave of cells leaving BM. This facilitates subsequent egress of HSPCs. In the next step, after leaving the BM, granulocytes undergo degranulation in response to plasma C5a and secrete some cationic peptides (cathelicidin, β-defensin) that as demonstrated here for a first time highly enhance responsiveness of HSPCs to plasma SDF-1 gradient. In conclusion, our data reveal the underappreciated central role of innate immunity in mobilization where C5 cleavage fragments via granulocytes orchestrate this process.

### Keywords

Complement; C5; CXCR4; innate immunity; stem cell mobilization



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## Introduction

Hematopoietic stem/progenitor cells (HSPCs) circulate in peripheral blood (PB) under steady state conditions at very low levels to keep a pool of stem cells in balance in the bone marrow (BM) microenvironment, which is located in distant bones. Therefore, PB could be envisioned as a highway by which HSPCs relocate in the organism between hematopoietic BM endosteal and endothelial niches. Furthermore, after they enter tissues, these circulating HSPCs may return back to the PB via the lymphatic system and thoracic duct.1 This route of "circulation/tissue patrolling" is enforced during infection2, wherein mobilized HSPCs could be recruited to the affected peripheral tissues and give rise to tissue-resident myeloid and dendritic cells. In addition to infection, HSPCs are mobilized from BM into PB during tissue injury3,4 and after administration of some pharmacological agents [e.g., granulocyte colony stimulating factor (G-CSF)].5-8

The molecular mechanisms controlling mobilization of HSPCs are still not well understood. However, the attenuation of the stromal-derived growth factor-1 (SDF-1)-CXCR4 interaction between BM-secreted SDF-1 and HSPC-expressed CXCR49 and the adhesive interaction between Very Late Antigen-4 (VLA-4; $\alpha_1\beta_4$ integrin) expressed on HSPCs and its ligand Vascular Adhesion Molecule-1 (VCAM-1; CD106), which is expressed in the BM microenvironment, play a crucial role here.9

Augmenting evidence demonstrates that HSPC mobilization is regulated by elements of innate immunity, in particular by complement cascade (CC) protein cleavage fragments10-12, neutrophils13,14, and Toll receptors (TRs)15 that all play a pivotal and, until recently, underappreciated role in this process. To support this, work from our laboratory demonstrated that CC is in fact activated during mobilization16,17 and that several CC cleavage fragments (e.g., C3a, $_{desArg}$C3a, C5a, $_{desArg}$C5a anaphylatoxins) may modulate this process. We noticed that while C3 cleavage fragments (C3a, $_{desArg}$C3a) increase retention of HSPCs in BM11,18, C5 cleavage fragments (C5a, $_{desArg}$C5a) enhance egress of HSPCs into PB.12,19 This was evident in mobilization studies performed in C3- and C5-deficient animals, which revealed that C3-deficient ($C3^{-/-}$) mice are easy mobilizers10,17 and C5-deficient ($C5^{-/-}$) mice are poor mobilizers by contrast.12,19 Furthermore, it is also well known that granulocytes in order to mobilize HSPCs have to release several proteloytic enzymes that perturb the SDF-1-CXCR4 and VLA-4-VCAM-1 retention axes.20,21 Granulocytes are also the first cells that egress from BM during mobilization and the mobilization process is severely defective in leukocyte-deficient animals.22,23 Finally, mobilization studies in a murine model revealed that mobilization is somehow facilitated by activation of TRs on BM myeloid cells via lipopolysaccharide released by intestinal aneorobic bacteria24 or TRs binding polysaccharides.25,26

In this paper, we focus on the role played in mobilization of HSPCs by CC and granulocytes, two crucial components of innate immunity. Overall, our data support the central role of granulocytes in orchestration of mobilization as well as shed more light on novel granulocyte and C5 cleavage fragment-directed mechanisms that regulate egress of HSPCs.

## Materials and Methods

### Animals

Pathogen-free, 4- to 6-week-old $C5^{-/-}$, $C5^{+/+}$, and C5a receptor deficient ($C5aR^{-/-}$) mice were purchased from Jackson Laboratory (Bar Harbor, ME, http://www.jax.org). C57BL/6 and Balb/c mice were purchased from the National Cancer Institute (Frederick, MD, http://www.cancer.gov). All mice were adapted over 2 weeks and used for experiments at age 6 to 8 weeks. Animal studies were approved by the Animal Care and Use Committee of the University of Louisville (Louisville, KY).

### Mobilization

Mice were injected subcutaneously with 250 μg/kg of human G-CSF (Amgen, Thousand Oaks, CA, http://www.amgen.com) daily for 6 days. For zymosan mobilization, mice were injected intravenously with 0.5 mg of zymosan. At 6 h after the last G-CSF administration or at 1 h after zymosan injection, mice were bled from the retro-orbital plexus for complete blood count (CBC) and 450 μl of PB was obtained from the vena cava with a 25-gauge needle and 1 ml syringe containing 50 μl of 100 mM ethylenediaminetetraacetic acid (EDTA).

### CBC counts

Fifty microliters of PB was taken from the retro-orbital plexus of the mice and collected into microvette EDTA-coated tubes (Sarstedt Inc., Newton, NC, http://www.sarstedt.com/php/main.php). Samples were run within 2 h of collection on a HemaVet 950 (Drew Scientific Inc., Oxford, CT, http://www.drew-scientific.com).

### Colony forming unit-granulocytes/macrophage (CFU-GM) assay

Red blood cells (RBCs) were lysed with BD Pharm Lyse buffer (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com). Nucleated cells were subsequently washed twice and used for CFU-GM colonies as described elsewhere.10 Briefly, cells were resuspended in human methylcellulose base media provided by the manufacturer (R&D Systems, Inc., Minneapolis, MN, http://www.rndsystems.com) supplemented with 25 ng/ml recombinant murine granulocyte macrophage colony-stimulating factor (mGM-CSF) and 10 ng/ml recombinant murine interleukin-3 (mIL-3) (Millipore, Billerica, MA, http://www.millipore.com). Cultures were incubated for 7 days, at which time they were scored for the number of CFU-GM colonies under an inverted microscope.

### Evaluation of HSPC mobilization

The formula used for evaluation of circulating CFU-GM and $Sca\text{-}1^{+}/c\text{-}Kit^{+}/Lin^{-}$ (SKL) cells is: {[number of white blood cells (WBCs) x number of CFU-GM colonies]/number of WBCs plated = number of CFU-GM per microliter of PB} and {(number of WBCs x number of SKL cells)/number of gated WBCs = number of SKL cells per microliter of PB}, respectively.

### Transmission electron microscopy (TEM)

TEM analysis was performed as described elsewhere.27 Briefly, mice were bled and perfused with 50 ml Krebs-Ringer bicarbonate buffer and then fixed by continuous infusing of 50 ml carcodylate buffer (0.1 M) via the left ventricle at 6 h after final G-CSF administration. The BM tissue was harvested carefully and additionally fixed in 0.25 m glutaraldehyde in cacodylate buffer (pH 7.4) for 2 h at 4 °C, post-fixed in 0.04 M $OsO_4$, and dehydrated in ethyl alcohol (30-96%) and 100% acetone. Fixed tissues were subsequently embedded in LX112 and sectioned at 800 Å, stained with uranyl acetate and lead citrate, and viewed on a Philips CM10 TEM operating at 60 kV.

### Bone marrow nucleated cells (BMNCs)

BMNCs were prepared by flushing femurs and tibias of pathogen-free, 6- to 8-week-old mice without enzymatic digestion. They were lysed with BD Pharm Lyse buffer (BD Biosciences, San Jose, CA) to remove RBCs, washed, and resuspended in appropriate media for further analysis.

### Fluorescence-activated cell sorting (FACS) analysis

BMNC staining was performed in medium containing 2% fetal bovine serum (FBS). All monoclonal antibodies (mAbs) were added at saturating concentrations and the cells were incubated for 30 min on ice, washed twice, resuspended in staining solution at a concentration of $5\times10^6$ cells/ml, and subjected to analysis using an LSR II (Becton Dickinson, Mountainview, CA). The following anti-mouse antibodies were used to detect fluorescein isothiocyanate (FITC)-anti-CD117 (c-Kit) (clone 2B8, BioLegend, San Diego, CA) and Phycoerythrin (PE)-Cy5 anti-mouse Ly-6A/E (Sca-1) (clone D7, eBioscience™, San Diego, CA). All anti-mouse lineage markers (Lin) were conjugated by PE and purchased from the same company (BD Biosciences): anti-CD45R/B220 (clone RA3-6B2); anti-Gr-1 (clone RB6-8C5); anti-t-cell receptor β (TCRβ; clone H57-597); anti-TCRγδ (clone GL3); anti-CD11b (clone M1/70); and anti-Ter-119 (clone TER-119).

### Sorting of BMNCs

SKL cells and granulocytes ($Gr\text{-}1^+$) were purified as described elsewhere.27 Briefly, BMNCs ($1\times10^8$ cells/ml) were resuspended in Roswell Park Memorial Institute medium (RPMI) containing 2% heat-inactivated FBS (GIBCO, Grand Island, NY), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (GIBCO), and antibiotics (Mediatech Inc., Manassas, VA, http://www.cellgro.com) and incubated with saturating concentrations of directly labeled mAbs for 30 min on ice, washed twice, and sorted by multiparameter, live sterile cell sorting (MoFlo, Dako A/S, Fort Collins, CO).

### Transwell migration assay

Transwell migration assay was performed as described elsewhere.10 Briefly, unless otherwise indicated, RBC-lysed BMNCs from C57BL/6 mice were resuspended in assay media [RPMI containing 0.5% bovine serum albumin (BSA)] and equilibrated for 10 min at 37°C. Six hundred-fifty microliters of assay media containing test reagents in the presence or absence of 50 ng/ml SDF-1 (PeproTech, Rocky Hill, NJ) was added to the lower

chambers of a Costar Transwell 24-well plate (Costar Corning, Cambridge, MA). C3a, C5a, and $_{desArg}$C5a were purchased from Calbiochem (La Jolla, CA). Other test reagents LL-37 (AnaSpec, Inc. San Jose, CA) and β-defensin-2 (Chemicon, Themcula, CA) were also purchased. Aliquots of cell suspension ($1\times10^6$ cells/100 μl) were loaded onto the upper chambers with 5 μm-pore filters, which were incubated for 3 h (37°C, 95% humidity, 5% $CO_2$). Cells from the lower chambers were scored using FACS analysis for migration of BMNCs to test regents. The results are presented as a migration index, i.e., the ratio of the number of cells that migrated toward the medium containing test reagents to the number of cells that migrated toward the medium alone. In some experiments, migrated cells harvested from the lower chambers were plated in semisolid clonogenic assays supplemented with cytokines and were stimulated to grow CFU-GMs as described elsewhere.12 To evaluate the influence of lipid raft formation on cell migration, cells were preincubated before the transwell migration assay with molecules [1.0 or 2.5 mM methyl-β-cyclodextrin (MβCD, Sigma, St. Louis, MO)] that disturb lipid raft formation.10

### C5aR expression analysis

Expression of C5aR was evaluated by FACS as described below. C5aR was detected on BM and SKL cells with biotinylated rat anti-murine C5aR (clone 10/92, Abcam, Cambridge, MA) and a Streptavidin-Allophycocyanin/Cy7 (APC/Cy7) (Biolegend), washed, and subjected to analysis using a LSR II (Becton Dickinson, Mountainview, CA).

### Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total mRNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) from each 20,000 sorted cell populations. Messenger RNA was reverse-transcribed with 500U of Moloney murine leukemia virus reverse transcriptase (MoMLV-RT). The resulting cDNA fragments were amplified using 5U of Thermus aquaticus (Taq) polymerase. Primer sequences for β-2 microglobulin (β2M) were forward primer: 5′-CAT ACG CCT GCA GAG TTA AGC-3′ and reverse primer: 5′-GAT CAC ATG TCT CGA TCC VAG TAG-3′. For C5aR, primer sequences were forward primer: 5′-TCC TGC TGC TGG CTA CCA TT-3′ and reverse primer: 5′-AGT CCC GCG GAC CTT CTG-3′. For C5L2, primer sequences were forward primer: 5′-ATG GCC GAC TTG CTT TGT TG-3′ and reverse primer: 5′-TGG CCA GTG GCC CTT TT-3′. PCR products were run in 2% agarose gels containing ethidium bromide and photographed under UV light. The sizes of PCR products were verified against 100-base pair (bp) DNA ladders.

### Isolation of lipid rafts

Human pre-B cell line, Nalm-6 ($5\times10^7$), was lysed in 300 μL MEB buffer (150 mM NaCl, 20 mM MES, pH 6.5) containing 1% Triton X-100 and protease inhibitors [500 μM phenylmethylsulfonyl fluoride (PMSF) and 5 mM iodoacetamide] for 1 h on ice. The cells were mixed with an equal volume of 80% sucrose in MEB and were placed at the bottom of a centrifuge tube. Samples were overlain with 30% and 5% sucrose in MEB and were centrifuged at 100,000*g* for 17 h. Fractions were gently removed from the top of the gradient and n-octylglucoside was added to each fraction (60 μM final) to solubilize rafts. Western

blot analysis was carried out using standard techniques with a CXCR4 antibody (Serotec, Oxford, UK) and Lyn antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

### Statistical analysis

Arithmetic means and standard deviations were calculated using Instat 1.14 software (Graphpad, San Diego, CA). Statistical significance was defined as $P<0.05$. Data were analyzed using Student's t-test for unpaired samples.

## Results

### C5-deficient mice are poor mobilizers

Both $C5^{+/+}$ and $C5^{-/-}$ mice were mobilized by long-term (6 days) G-CSF- or short-term (1 h) zymosan-induced protocol. Subsequently, after the last G-CSF injections and 1 h after zymosan administration, we evaluated the number of circulating neutrophils, CFU-GM progenitors, and SKL cells in mobilized animals. We noticed that $C5^{-/-}$ mice show impaired mobilization response to G-CSF (Figure 1A) as well as to zymosan (Figure 1B).

These observations were subsequently confirmed by transmission electron microscopy (TEM). Figure 2A shows egress of wild type (wt) nucleated cells from BM into PB. This egress was impaired in $C5^{-/-}$ animals (Figure 2B), however, cells accumulated in the BM microenvironment in these mice around sinusoids. This indicates that the defect for their egress is somehow related to migration over the endothelial barrier.

### Effects of C5a and $_{desArg}$C5a on chemotaxis of BMNCs

Because $C5^{-/-}$ mice are poor mobilizers, we hypothesized that CC cleavage fragments C5a and $_{desArg}$C5a anaphylatoxins may play an important role here. To address this question, we first performed chemotactic assays of wt BMNCs to C5 and $_{desArg}$C5a gradient. Next, because another anaphylatoxin C3a (C3 cleavage product) enhances responsiveness of BMNCs to a low gradient of SDF-1,10,16 we evaluated whether a similar phenomenon will occur for C5a and $_{desArg}$C5a. Figure 3A shows that $_{desArg}$C5a, but not C5a, chemoattracts BMNCs alone or in additive manner with SDF-1. In contrast, no effect of C5a and $_{desArg}$C5a was observed on CFU-GM chemotaxis (Figure 3B). In control experiments, C3a did not chemoattract CFU-GM progenitors alone, however as expected it increased responsive if BMNCs to low dose of SDF-1 (Figure 3B). These data show that $_{desArg}$C5a, but not C5a, chemoattracts BMNCs, however both C5 cleavage fragments do not affect CFU-GM migration.

It is also known that C5a and $_{desArg}$C5a bind to two G-protein-coupled C5aR and C5L2 receptors. To address the importance of both receptors in this process, we performed chemotaxis with BMNCs isolated from wild type (wt) and $C5aR^{-/-}$ animals. Because $C5aR^{-/-}$ BMNCs did not respond to $_{desArg}$C5a in contrast to wt BMNCs (Figure 3C and D), this fact demonstrates that C5aR, but not C5L2, is involved in migration of BMNCs.

Finally, we analyzed expression of both C5 cleavage fragment receptors (C5aR and C5L2) on murine BMNCs. Figure 4A shows that murine SKL cells that are enriched for HSPCs, in contrast to neutrophils ($Gr\text{-}1^{+}$ cells) and monocytes ($CD14^{+}$ cells), do not express mRNA

for C5aR and C5L2 receptors. Finally, FACS analysis confirmed no C5aR expression on murine SKL cells (Figure 4B).

Together, these data show that $_{desArg}$C5a chemoattracts neutrophils and that this effect is mediated by C5aR.

## Granulocytes are the first cells to egress from BM and release factors that increase responsiveness of HSPCs to SDF-1 gradient

As reported previously, CC becomes activated during mobilization in PB and cleavage of C5 releases C5a (short half-life) that is immediately converted to $_{desArg}$C5a (long half-life). Based on our data that $_{desArg}$C5a chemottracts and activates granulocytes, we became interested in a potential role of these cells in egress of HSPCs from BM. First, by employing kinetic studies, we confirmed that granulocytes are the first cells that egress from BM during mobilization (data not shown) preceding egress of HSPCs. We assume that granulocytes, which are enriched in several proteolytic enzymes, somehow "pave the way" to facilitate egress of HSPCs. To support this our real time RT-PCR data revealed that Gr-1$^+$ BMNCs express MMP-9 at > than 100 times higher level than purified SKL cells and that this expression is additionally upregulated ~ 2 times after C5a and $_{desArg}$C5a stimulation.

Next to evaluate whether granulocytes after release into BM vessels may secrete some factors affecting subsequent egress of HSPCs, we purified Gr-1$^+$ cells from BM by FACS. Subsequently, these Gr-1$^+$ cells were non-stimulated (control) or stimulated with C5a or $_{desArg}$C5a and conditioned media (CM) were harvested and employed in chemotactic assays. We noticed that while all these CM alone had a very weak chemotactic activity (data not shown), CM harvested from C5a, but not $_{desArg}$C5a, stimulated Gr-1$^+$ BM-derived cells enhanced SDF-1-mediated chemotaxis of BMNCs and CFU-GM (Figure 5A and B). As a similar effect was not observed for CM harvested from Gr-1$^+$ BM-derived isolated from C5aR$^{-/-}$ animals (Figure 5C and D), we concluded that this effect was C5aR-dependent and C5L2 could not compensate for the C5aR absence on Gr-1$^+$ cells.

## Cationic peptides released from granulocytes enhance responsiveness of HSPCs to SDF-1 gradient

To address which factors released from granulocytes stimulated by C5a may increase responsiveness of HSPCs to SDF-1 gradient, we focused on granulocyte-derived cationic peptides that, similarly as reported by us, C3a may interact with the cell membrane surface in a non-receptor-mediated manner.10,14,28

We selected four potential candidates: lactoferrin (LF); β-defensin-1 (hBD-1); β-defensin-2 (hBD-2); and active fragment of cathelicidin (LL-37). Figure 5 shows that LL-37 and hBD-2, which alone do not show chemotactic activity (data not shown), enhance/prime migration of BMNCs (Figure 5E) and CFU-GM (Figure 5F) to low doses of SDF-1. To our surprise, this hBD-2 and LL-37 "priming" effect was several times higher as those described by us previously for C3a.10,16

We reported in the past that enhanced responsiveness of HSPCs to SDF-1 gradient in the presence of C3a (priming effect) is mediated by C3a non-receptor-mediated incorporation of

CXCR4 into membrane lipid rafts.10,14 We postulated that CXCR4 incorporated in membrane lipid rafts is better connected to downstream signaling machinery and responds robustly to an SDF-1 gradient. Figure 6A shows that both hBD-2 and LL-37 are also able to increase incorporation of CXCR4 into membrane lipid rafts. This was subsequently confirmed in chemotactic assays after perturbation of lipid raft formation by exposure of cells to cell membrane cholesterol-depleting agent methyl-β-cyclodextran (MβCD). Figure 6B shows that exposure of cells to MβCD inhibited LL-37-enhanced responsiveness to SDF-1 gradient. In control experiments this pretreatment did not affect N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) mediated migration (data not shown), which use G-protein independent and non-lipid raft associated fMLP receptor.29

## Discussion

The process of HSPC mobilization is still not fully understood and a significant number of patients, particularly those with previous histories of chemotherapy, are poor mobilizers.30,31 G-CSF is currently the most frequently employed clinical drug that may efficiently mobilize HSPCs after a few consecutive daily injections.5-7 Mobilization could also be induced within hours after administration of some chemokines [e.g., IL-8, growth-related oncogene protein-beta (Gro-β, macrophage inhibitory protein-1 alpha (MIP-1α)] or small molecular CXCR4 receptor antagonists (e.g., AMD3100, T140).32 Additionally, in experimental animals, mobilization also occurs within 1 h after a single injection of selected polysaccharides (e.g., zymosan, fucoidans).33,34

Several host-related factors have been described to regulate mobilization, including: i) release of proteases in the BM microenvironment by activated myeloid cells, which perturb interactions of SDF-CXCR4, VLA-4-VCAM-1, and KL-c-KitR axes9,20,21; ii) release of neurotransmitters in the BM microenvironment by neural fibers that innervate BM35; iii) downregulation of SDF-1 expression in osteoblastic stem cell niches36,37; iv) activation of osteoclasts38; and v) upregulation of serpin family protease inhibitors.39,40 Recent evidence demonstrates that mobilization of HSPCs is also regulated by several components of innate immunity including CC10-12,14,16,17 and granulocytes.13,14,41

Accordingly, we reported that, e.g., G-CSF and cyclophosphamide or zymosan infusion strongly activate CC in BM by a classical pathway due to exposure during mobilization of neo-epitope on the surface of damaged BMNCs.11,42,43 This neo-epitope binds naturally occurring antibodies (NAbs) and neo-epitope-NAb complexes circulating in PB via C1q trigger the classical immunoglobulin (Ig)-dependent pathway of CC activation. In addition, because G-CSF activates the coagulation system, thrombin has recently been identified as an underappreciated CC activator.44 In contrast, Zymosan as a polysaccharide may directly activate CC by employing factor B and D involving the Ig-independent alternative CC activation pathway. The crucial role of CC in mobilization of HSPCs was demonstrated in experiments in mice with various defects of complement components.10,12,14,16,17 We noticed that various CC components may play different roles and that mobilization could be regulated differently depending on the level of CC activation. For example, we reported that $C3^{-/-}$ mice are easy mobilizers17 and, in contrast, $C5^{-/-}$ mice are poor mobilizers.12,14

Granulocytes are another important component of innate immunity required for HSPC mobilization; however, the mechanisms by which granulocytes induce mobilization are still not completely understood. On one hand, they were postulated to be a source of proteolytic enzymes in the BM that perturb HSPC retention signals.9,21,22 On the other, in several mobilizing protocols, granulocytes were described as the first cells to egress from BM. To support this notion, mobilization of HSPCs is severely reduced in neutropenic mice.13,22,23

In the current work, we wanted to elucidate molecular mechanisms underlying poor mobilization observed in $C5^{-/-}$ animals. Because C5-derived anaphylatoxins (C5a and $_{desArg}$C5a) bind to G-protein-coupled, seven-transmembrane span C5a receptors (C5aR and C5L2) that are expressed on granulocytes, we became interested in a link between major components of innate immunity, such as C5a and $_{desArg}$C5a anaphylatoxins and egress of granulocytes, and subsequent mobilization of HSPCs.

Our C5aR and C5L2 receptor expression analysis revealed that hematopoietic stem cells do not express C5 cleavage fragments receptors on the surface. This was subsequently supported by functional chemotaxis studies to C5a and $_{desArg}$C5a that revealed that BMNCs but not purified SKL cells show chemotaxis to C5 clevage fragments. Furthermore, both C5 cleavage fragments, in contrast to C3 cleavage fragments,10,16 did not enhance (prime) chemotactic responsiveness of HSPCs to low/threshold levels of SDF-1.

However, both C5 cleavage fragments affected several important neutrophil-dependent steps of HSPC mobilization (Figure 7). First, in addition to their ability to increase secretion of proteases by granulocytes,9,21,22 C5 cleavage fragments induce their egress from BM ($_{desArg}$C5a). Subsequently, these fragments in BM vessels (C5a) stimulate the release of several factors that, as we demonstrated in this paper, enhance responsiveness of HSPCs to SDF-1 gradient. Because these effects were not visible in $C5aR^{-/-}$ mice, this suggests a crucial involvement of C5aR, but not the C5L2 receptor in this phenomenon.

Based on this, we envision that granulocytes are the first cells to egress from BM in response to $_{desArg}$C5a. Since they highly express various proteases (e.g., metalloproteinases) necessary for cell migration through the endothelial barrier, as such, they somehow facilitate subsequent egress of HSPCs, which express these enzymes at much lower levels (“ice breaker mechanism”) (Figure 7). However, we are aware that in addition to $_{desArg}$C5a, other factors may also facilitate egress of neutrophils from BM (e.g., IL-8, NAP-2, Gro-β). Therefore, some compensatory overlapping mechanisms exist that still maintain some level of granulocyte egress and HSPCs mobilization in $C5^{-/-}$ animals

Of importance in this report, we identified for the first time that several plasma-derived cationic peptides (cathelicidin and β-defensin-2) that are released by activated granulocytes (e.g., after stimulation by C5a)14,45,46 show strong priming effects that increase responsiveness of HSPCs to SDF-1 gradient. This phenomenon, as initially described for C3 cleavage fragments (C3a and $_{desArg}$C3a anaphylatoxins),10,28,47 is based on non-receptor-mediated interactions of these proteins, which also possess a cationic structure, with the cell membrane. We reported that this C3a and $_{desArg}$C3a-mediated interaction leads to lipid raft formation on the surface of target cells.10,11,16

Because CXCR4 receptor requires incorporation into membrane lipid rafts for optimal signaling, priming by C3a, $_{desArg}$C3a, or, as shown here for the first time, by granulocyte-derived cathelicidin and β-defensin-2 increases incorporation of this receptor into lipid rafts. As a result, CXCR4 is better associated with downstream signal transduction proteins and responds more robustly to low/threshold levels of SDF-1. Thus, based on this our data indicate that cathelicidin and β-defensin-2 released from granulocytes in a C5a-C5aR-dependent manner facilitate egress of HSPCs from BM by enhancing lipid raft-dependent priming of HSPCs to plasma SDF-1 levels (Figure 7). Finally, further work is needed to evaluate the direct effect of C5 cleavage fragments on the permeability of the BM endothelium, which is currently being investigated in our laboratory.

In conclusion, our data demonstrate a central role of innate immunity and better explain involvement of granulocytes and C5 cleavage fragments in mobilization of HSPCs. Granulocytes and C5 cleavage fragments orchestrate this process and, in light of our presented data, mobilization of HSPCs could be envisioned as a part of the innate immunity response. With CC activation also observed during inflammation16,48 and tissue/organ injury,49,50 this phenomenon has broader implications. While HSPCs mobilized from BM during infection may patrol peripheral tissues and thus perform an important role in host defense,1,2 other types of BM-derived stem cells may be released to act in regeneration of damaged tissues.3,4,43 To support this concept in all these processes, both CC activation and granulocytosis is involved.

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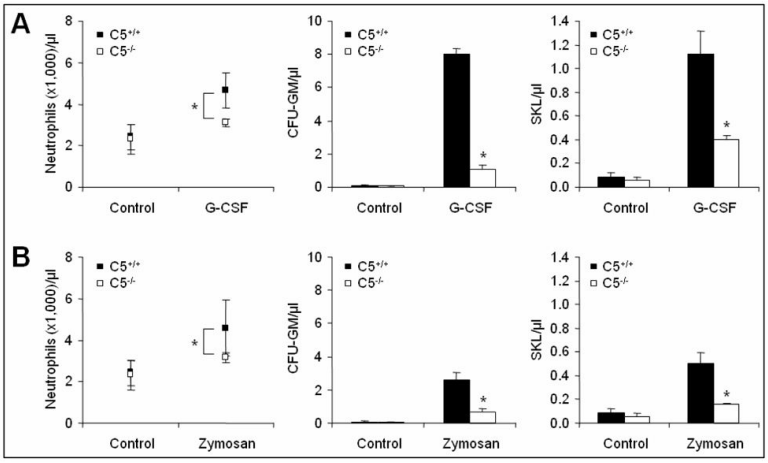


**Figure 1. Impaired mobilization of HSPCs in C5-deficient mice**

$C5^{-/-}$ as well as age- and sex-matched $C5^{+/+}$ wt mice were mobilized for 6 days with G-CSF (250 µg/kg s.c. per day; n=5 mice per group) (A) or for 1 h with zymosan (0.5 mg/kg i.v.; n=5 mice per group) (B). Number of circulating neutrophils (left), CFU-GM progenitors (middle), and SKL cells (right) per microliter of PB. * P<.05 as compared with wt $C5^{+/+}$ mice. Data show representative results from three independent experiments.

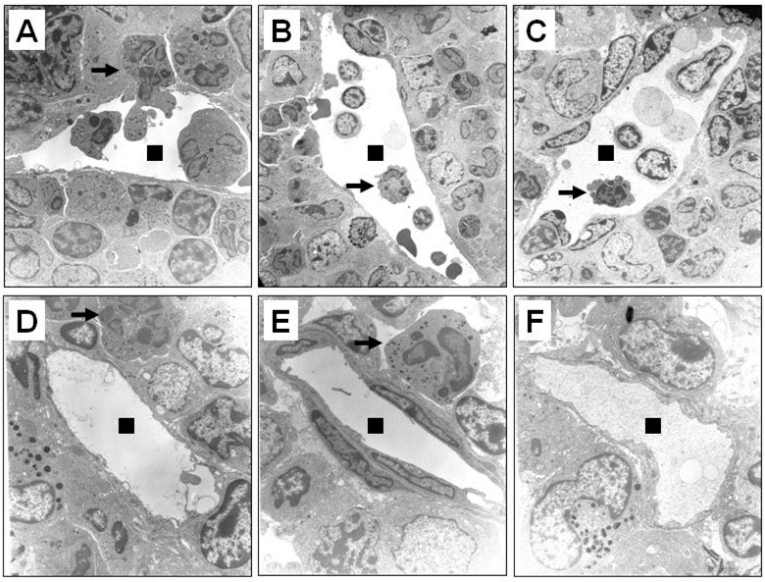


**Figure 2. TEM analysis of BM tissue**

Granulocytes (arrow) in wt $C5^{+/+}$ mouse BM show trans-endothelial migration from BM stroma to sinusoids (■) after 6-day G-CSF administration (A-C). In contrast, granulocytes in $C5^{-/-}$ mouse BM are accumulated around the endothelial layer and retained in the BM stroma without entering into sinusoids (D-F). Original magnification = x 2,000. Representative TEM pictures are shown.

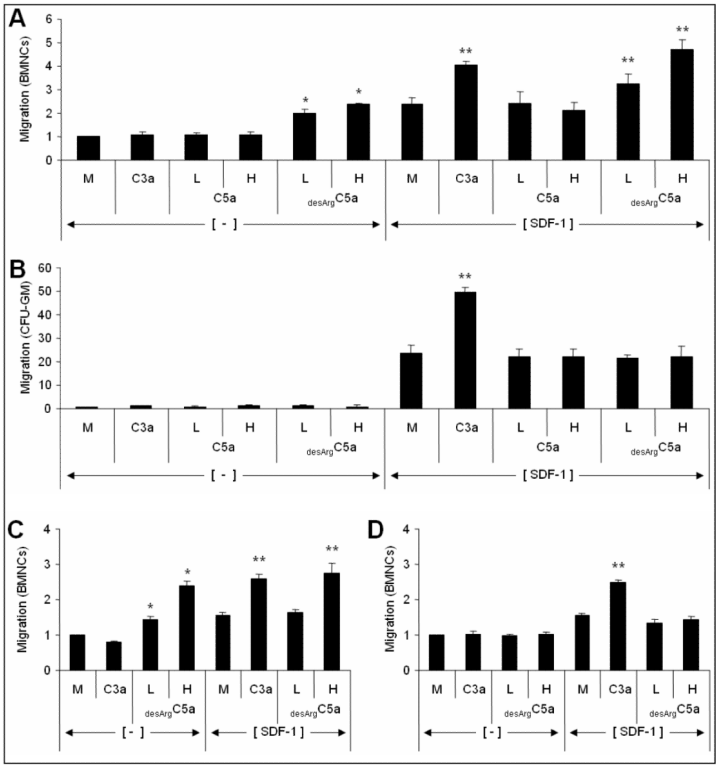


**Figure 3. Chemotactic effect of C5a and $_{desArg}$C5a on mouse BMNCs**

$_{desArg}$C5a, but not C5a, directly chemoattract BMNCs in a C5aR-dependent manner, but do not induce migration of CFU-GM progenitors. (A) Migration of whole BMNCs (A) and CFU-GM progenitors (B) in response to C5a and $_{desArg}$C5a in the absence or presence of SDF-1. (C) $_{desArg}$C5a directly chemoattract Balb/c mice BMNCs (C). In contrast, it has no chemotactic effect on C5aR$^{-/-}$ mouse BMNCs (D). Values are the fold increases of migrated cells compared to media alone. M, media alone; C3a (1 μg/ml); SDF-1 (50 ng/ml); C5a and $_{desArg}$C5a (L, 70 ng/ml; H, 140 ng/ml). * $P<0.05$ as compared with media alone control; ** $P<0.05$ as compared with SDF-1 (50 ng/ml) alone. The data shown represent the combine results from three independent experiments carried out in triplicate per group (n=9).

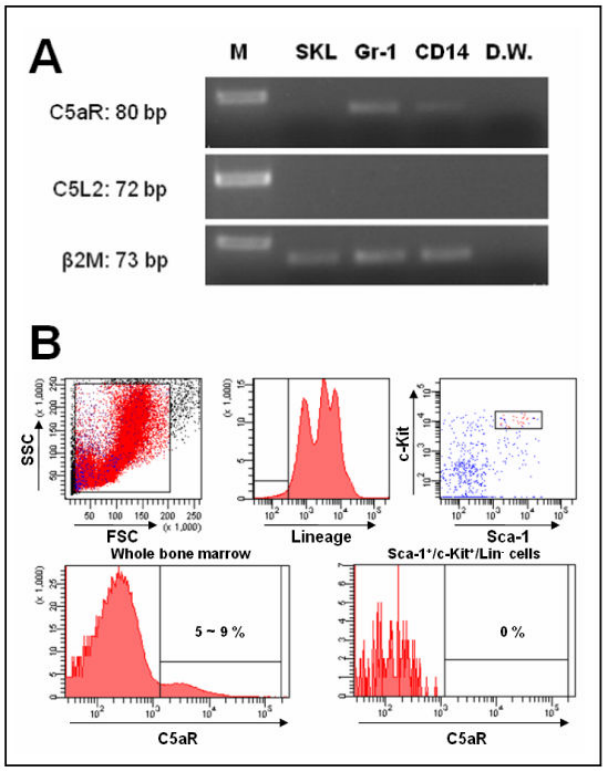


**Figure 4. C5aR expression on mouse BMNCs**

(A) RT-PCR analysis of C5aR mRNA expression on purified mouse BMNCs (M, size marker; SKL, Sca-1$^+$/c-Kit$^+$/Lin$^-$ cells; Gr-1, granulocytes; CD14, monocytes; D.W., $H_2O$ instead of cDNA). The experiment was repeated twice on two different batches of sorted cells with similar results. (B) FACS analysis of C5aR expression on mouse BMNCs. C5aR was expressed on mouse BMNCs (6~9%) including granulocytes and monocytes, but not on SKL cells. The experiment was repeated three times on cells from three independent donors with similar results. Representative staining is shown.

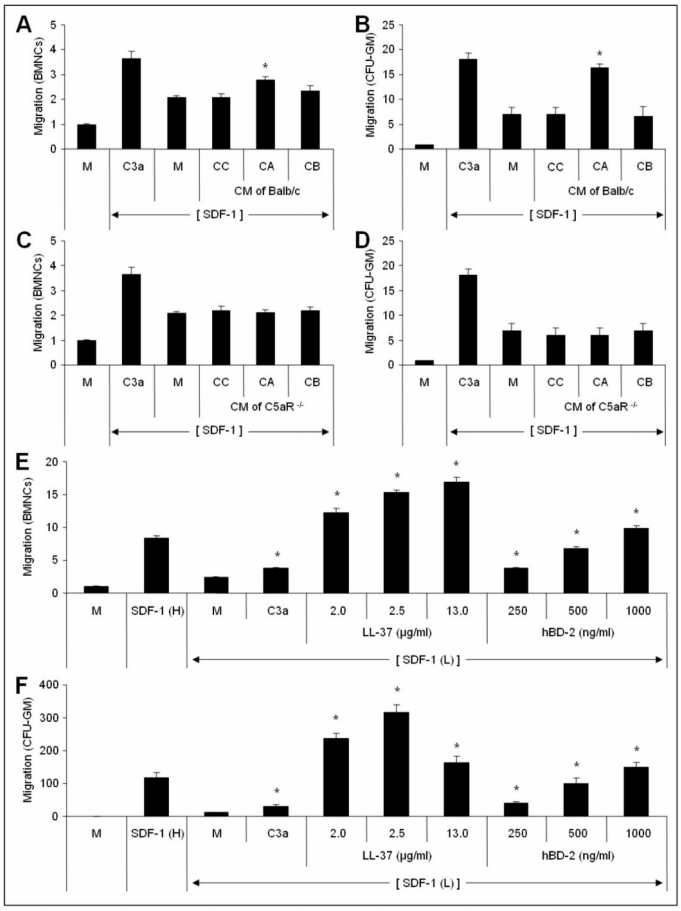


**Figure 5. Cationic peptides released from granulocytes enhance responsiveness of HSPCs to SDF-1 gradient**

CM, prepared by incubating Gr-1$^+$ cells ($2\times10^6$/ml) for 1 h with C5 cleavage fragments (CC, no stimulation; CA, stimulated with C5a; CB, stimulated with $_{desArg}$C5a) was added into lower wells (final concentration 50%) to evaluate the priming effect of granules released from granulocytes. CM of Balb/c mice granulocytes, stimulated with C5a but not $_{desArg}$C5a, enhanced responsiveness of C5aR$^{-/-}$ BMNCs (A) and CFU-GM progenitor cells (B) to SDF-1 gradient. In contrast, CM of C5aR$^{-/-}$ mice granulocytes did not enhance responsiveness of C5aR$^{-/-}$ BMNCs (C) or CFU-GM progenitor cells (D). Recombinant cationic peptides LL-37 and hBD-2 strongly enhanced migration of BMNCs (E) and CFU-GM progenitor cells (F) in response to SDF-1 gradient. Values are the fold increases of migrated cells compared to media alone. M, media alone; SDF-1 (L), 50 ng/ml; SDF-1 (H), 300 ng/ml; C3a, 1 μg/ml. * $P<0.05$ as compared with SDF-1 (50 ng/ml) alone. The data shown represent the combine results from four independent experiments carried out in triplicate per group (n=12).

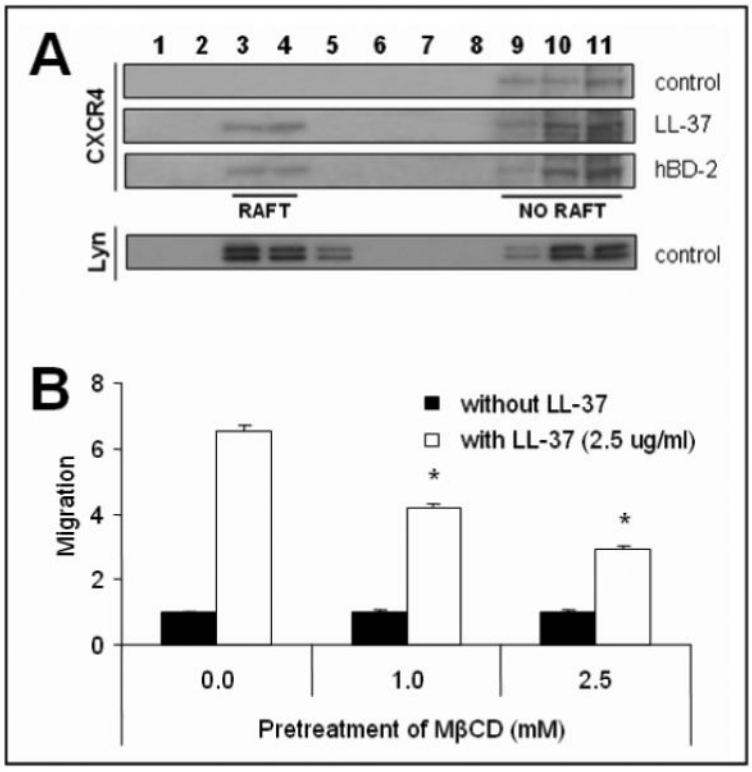


**Figure 6. Priming effect of cationic peptides is dependent on enhanced incorporation of CXCR4 into lipid rafts**

(A) Western blot analysis of the localization of CXCR4 in various fractions of cell membranes. Membranes enriched in lipid rafts (fractions 3-5) and depleted of lipid rafts (fractions 9-11). Human pre-B cell line, Nalm-6, was stimulated with LL-37 (5 μg/ml) or hBD-2 (250 ng/ml) or was not stimulated (control). CXCR4 was detected in these membrane fractions by Western blot along with Lyn, a marker of lipid rafts. Experiments were performed three times with similar results. (B) LL-37-induced enhancement of THP-1 cell migration to SDF-1 gradient was inhibited by 1 h pretreatment with MβCD (1.0 or 2.5 mM). * $P<0.05$ as compared with migration of control cells in the absence of LL-37 (2.5 μg/ml). Lipid raft formation was analShown are representative of three independent experiment carried out in triplicate per group. The data shown represent the combine results from four independent experiments carried out in triplicate per group (n=12).

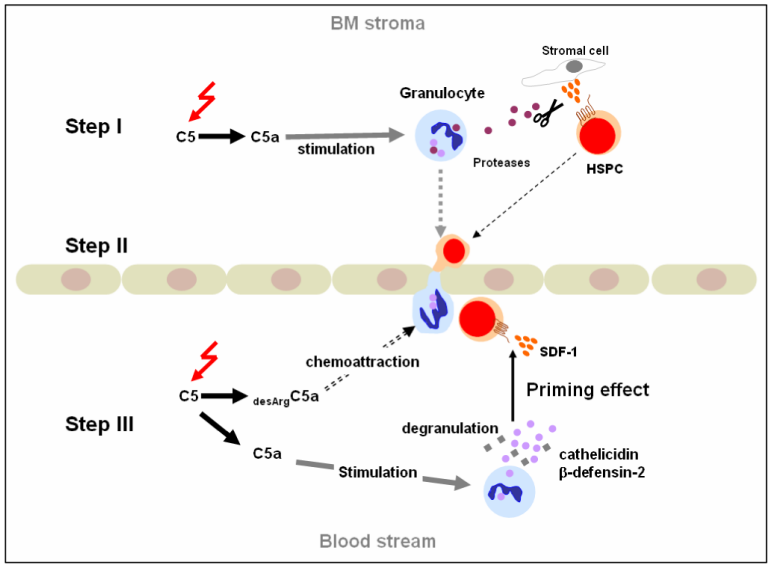


**Figure 7. C5 cleavage fragments play an important role in granulocyte egress and HSPCs mobilization from BM**

There are three levels at which C5 cleavage fragments (C5a and $_{desArg}$C5a) may affect granulocytes and thus affect HSPC mobilization. First, stimulation of granulocytes in the BM microenvironment by C5a and $_{desArg}$C5a enhances secretion of proteolytic enzymes, which perturb HSPCs retention signals (e.g., SDF-1-CXCR4 and VLA-4-VCAM-1 interactions) (**Step I**). Second, serum $_{desArg}$C5a chemoattractants induce egress of HSPCs from BM into PB. Migrating from BM into PB, granulocytes rich in metalloproteinases "pave the way" for HSPCs, which follow granulocytes and migrate through the endothelial barrier ("ice breaker phenomenon") (**Step II**). Finally, after egress from BM, granulocytes are stimulated in BM vessels by C5a and release several cationic peptides, some of which (e.g., cathelicidin, β-defensin-2) enhance/prime responsiveness of HSPCs to serum levels of SDF-1 (**Step III**). Finally, we cannot exclude the potential direct effect of C5 cleavage fragments on permeabilization of the endothelium, which will require further studies.