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## DEFECTIVE ENGRAFTMENT OF C3aR<sup>-/-</sup> HEMATOPOIETIC STEM PROGENITOR CELLS REVEALS A NOVEL ROLE OF THE C3a-C3aR AXIS IN BONE MARROW HOMING

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### Abstract

We reported that complement (C) becomes activated and cleaved in bone marrow (BM) during preconditioning for hematopoietic transplantation and the third C component (C3) cleavage fragments C3a and <sup>desArg</sup>C3a increase responsiveness of hematopoietic stem/progenitor cells (HSPCs) to stromal derived factor-1 (SDF-1). We also demonstrated that this homing promoting effect is not C3a receptor (C3aR) dependent. Herein, we report our new observation that transplantation of C3aR<sup>-/-</sup> HSPCs into lethally irradiated recipients results in: 1) ~5-7 day delay in recovery of platelets and leukocytes; 2) decrease in formation of day 12 colony-forming units-spleen (CFU-S); and 3) decrease in the number of donor-derived CFU-granulocyte-macrophage (GM) progenitors detectable in the BM cavities at day 16 after transplantation. In agreement with the murine data, blockage of C3aR on human umbilical cord blood CD34<sup>+</sup> cells by C3aR antagonist SB290157 impairs their engraftment in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. However, HSPCs from C3aR<sup>-/-</sup> mice stimulated by C3a still better responded to SDF-1 gradient, after exposure to C3a, they secrete less matrix metalloprotease-9 (MMP-9) and show impaired adhesion to stroma cells. We conclude that C3a, in addition to enhancing responsiveness of HSPCs to SDF-1 gradient in a C3aR independent manner, may also directly modulate HSPC homing by augmenting C3aR-mediated secretion of MMP-9 and cell adhesion.

### Keywords

C3; C3aR; CXCR4; SDF-1; stem cells

### Introduction

The collective term “homing” refers to the early stages of bone marrow (BM) seeding by hematopoietic stem/progenitor cells (HSPCs) after transplantation, which precedes

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engraftment, proliferation, and differentiation of these cells. In general, homing, engraftment, and expansion of HSPCs after transplantation are closely related processes, although all the factors involved have not been identified (1, 2). However, it is well demonstrated that  $\alpha$ -chemokine stromal-derived factor-1 (SDF-1) - chemokine receptor CXCR4 axis and vascular cell adhesion molecule 1 (VCAM-1) - very late antigen-4 (VLA-4) interactions play important roles in these processes. While SDF-1 secreted by BM stroma chemoattracts intravenously infused CXCR4<sup>+</sup> HSPCs, VCAM-1 expressed on BM endothelium interacts with and tethers VLA-4 ( $\alpha$ 4 $\beta$ 1 integrin)<sup>+</sup> HSPCs (3-11).

It is well known that conditioning for transplantation by chemo/radiotherapy destroys old hematopoiesis and by emptying hematopoietic niches, makes space available for transplanted cells and highly upregulates expression of SDF-1 in the BM microenvironment, which creates a proper chemoattracting gradient for HSPCs (12-14). In addition, SDF-1 activates adhesion molecules on HSPCs (e.g., VLA-4), which promotes their adhesion and upregulates secretion of metalloproteinases (e.g., matrix metalloproteinase-9 [MMP-9]) by these cells. All these processes orchestrate transendothelial migration of HSPCs into the BM microenvironment (3, 5, 6, 9-11, 15-17).

We have recently reported that myeloablative conditioning for transplants by radio/chemotherapy activates complement (C) cascade in BM as well (18-19). The protein components of C including the third C component (C3) are activated through proteolysis in a cascade-like fashion leading to the generation of "liquid phase" activation peptides with potent pro-inflammatory properties termed anaphylatoxins (C3a and <sub>desArg</sub>C3a) as well as activated/cleaved "solid phase" proteins that bind to the surface of C-activating cells (e.g., iC3b). C3 cleavage fragments bind to specific C3 receptors that are expressed on several types of cells including HSPCs (18-22). While C3a binds to G-protein coupled, seven transmembrane-spanning C3a receptor (C3aR) and C5L2, C3a<sub>desArg</sub> binds to C5L2 only. The receptor for iC3b is CR3 and is the  $\alpha$ <sub>M</sub> $\beta$ <sub>2</sub>-integrin, which is also known as CD11b/CD18 or Mac-1 (22-27).

We previously reported that C3 liquid phase cleavage fragments (C3a and <sub>desArg</sub>C3a) enhance responsiveness of HSPCs to SDF-1 gradient. The molecular explanation of this phenomenon is based on our observation that both C3a and <sub>desArg</sub>C3a increase incorporation of CXCR4 into membrane lipid rafts, thus facilitating its more effective assembly with downstream signaling molecules (18, 22, 23, 28). In addition, C3a, but not <sub>desArg</sub>C3a, enhances secretion of MMP-9 in HSPCs and increases adhesion of these cells to VCAM-1. As a result, HSPCs primed before transplantation by C3a but not <sub>desArg</sub>C3a home and engraft better (23). On the other hand, iC3b deposited on cells in the BM microenvironment tethers HSPCs via CR3. To support this, we found that C3 deficient mice engraft poorly with HSPCs (18, 22, 29).

Because C3aR<sup>-/-</sup> cells show normal priming effect after C3a stimulation (18, 22, 29), we hypothesized that this phenomenon does not involve C3aR. However, to better address whether C3aR is also directly involved in engraftment, we employed HSPCs from C3aR<sup>-/-</sup> mice and compared their homing and engraftment characteristics to wildtype (WT) counterparts. Our data provides the first evidence that C3a increases homing responses of

HSPCs by direct interaction with C3aR and that another C3a binding receptor (C5L2 receptor) cannot compensate for C3aR deficiency. We conclude that C3a plays an important but underappreciated role in engraftment of HSPCs by increasing non-C3aR-dependent responsiveness of HSPCs to SDF-1 gradient and by C3aR-mediated increase in MMP-9 secretion and cell adhesion.

## Materials and Methods

### Murine BM-derived cells

Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Louisville. Murine BM mononuclear cells (BMMNCs) were flushed from the femora of BALB/c C3aR<sup>+/+</sup> (The Jackson Laboratory, Bar Harbor, ME) and BALB/c C3aR<sup>-/-</sup> (kind gift from Dr. Rick A Wetzels, University of Texas, Houston). The protocols used were approved by the appropriate institutional ethics review boards. MNCs were depleted of adherent cells (A<sup>-</sup>) and then enriched for light-density MNCs by Ficoll-Paque centrifugation. Sca-1<sup>+</sup> cells were isolated using paramagnetic mini-beads (Miltenyi Biotec, Auburn, CA). The purity of isolated BM Sca-1<sup>+</sup> cells was >95%, as determined by fluorescein-activated cell sorting (FACS). For some of transplant experiments, Sca-1<sup>+</sup>kit<sup>+</sup>lin<sup>-</sup> (SKL) cells were purified by employing MoFlo sorter as described (18).

### Human umbilical cord blood (UCB)—derived cells

Human light-density UCB cells were obtained after informed consent; the protocols used were approved by the appropriate institutional ethics review boards. Light-density cells were depleted of adherent cells and T lymphocytes (A-T- MNCs) and enriched for CD34<sup>+</sup> cells by immunoaffinity selection with MiniMACS paramagnetic beads (Miltenyi Biotec). The purity of isolated BM CD34<sup>+</sup> cells was >95%, as determined by FACS analysis using a FACScan (Becton Dickinson, San Jose, CA). Human BM CD34<sup>+</sup> transplants were performed on non-obese diabetic/severe combined immunodeficient (NOD/SCID) inbred mice (The Jackson Laboratory).

### Adhesion Assay

WT BM stroma was grown to confluence and WT or C3aR<sup>-/-</sup> Sca-1<sup>+</sup> cells were non-stimulated or stimulated by C3a (1 µg/ml) or desArg C3a (1 µg/ml) and added to the cultures for either 1 or 3 hours. Non-adhered cells were washed off. Subsequently, the entire cultures were trypsinized and plated in methylcellulose cultures and stimulated to grow colony-forming units-granulocyte-macrophage (CFU-GM) colonies as described (18). The number of colonies was scored under an inverted microscope.

### Zymography

To evaluate MMP-9 secretion by Sca-1<sup>+</sup>, 2 × 10<sup>6</sup> cells/mL were incubated (for 24 h at 37°C in 5% CO<sub>2</sub>) in the absence or presence of C3a or desArg C3a (1 µg/ml), and cell-cultured media were analyzed immediately by zymography as previously described by us (23). The intensity of the bands was quantified using the NIH ScionImage for Windows software (Scion Corp., Frederick, MD).

### Short term homing assays

Mice were irradiated with a lethal dose of  $\gamma$ -irradiation (900 cGy). After 24 hours, the mice were transplanted (by tail vein injection) with  $2 \times 10^5$  BM Sca-1<sup>+</sup> cells. At 6 or 24 hours post transplant, BM was harvested from the femurs and plated in serum-free methylcellulose cultures and stimulated to grow CFU-GM colonies by stimulation with granulocyte macrophage-colony stimulating factor (GM-CSF) (25ng/mL) + Interleukin (IL)-3 (10 ng/ml).

### BM transplantation

For transplant experiments, mice were irradiated with a lethal dose of  $\gamma$ -irradiation (900 cGy). After 24 hours, mice were transplanted with  $10^5$  BM Sca-1<sup>+</sup> cells or  $10^4$  Sca-1<sup>+</sup>kit<sup>+</sup>lin<sup>-</sup> (SKL) cells by tail vein injection. Anesthetized transplanted mice were bled at various intervals from the retro-orbital plexus to obtain samples for leukocytes and platelets.

### Evaluation of engraftment

**CFU-spleen (S) assay**—For the CFU-S assays, mice were transplanted with  $5 \times 10^4$  Sca-1<sup>+</sup> marrow cells. At day 12, spleens were removed, fixed in Telesynczky's solution, and CFU-S colonies were counted on the surface of the spleen as described (18).

**Number of CFU-GM/femur**—Femora of transplanted mice were flushed with phosphate-buffered saline (PBS) at day 16 post-transplant. Subsequently, Sca-1<sup>+</sup> cells were purified and plated in serum-free methylcellulose cultures and stimulated to grow CFU-GM colonies by granulocyte-colony stimulating factor (G-CSF; 25 ng/ml) + IL-3 (10 ng/ml).

### 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

### Delayed engraftment after transplantation of C3aR<sup>-/-</sup> HSPCs

We reported that C3aR<sup>-/-</sup> mice or WT mice in which C3aR is blocked by small molecular antagonist SB 290157 are easy mobilizers in response to G-CSF (30, 31). This could indicate that C3aR plays an important role in retention of HSPCs in the BM microenvironment.

To better address this question, we performed hematopoietic transplantation experiments using HSPCs from C3aR<sup>-/-</sup> and WT mice to both WT and C3aR<sup>-/-</sup> animals. As shown in Figure 1 **panel A**, the kinetics of white blood cell (WBC) and platelet counts revealed delayed engraftment of C3aR<sup>-/-</sup> HSPCs. Next, to exclude a potential contribution of C3aR deficiency on cells in the hematopoietic environment, we performed transplants into C3aR<sup>-/-</sup> recipients (Fig. 1 **panel B**) and noticed that C3aR<sup>-/-</sup> HSPCs also show delayed engraftment as compared to WT HSPCs. Thus, regardless of the genetic background of the recipient animal, C3aR<sup>-/-</sup> HSPCs do not engraft as well as their WT counterparts.

Our WBC and platelet counts recovery kinetic data were subsequently confirmed by evaluating the number of: a) day 11 CFU-S (Fig. 2 **panel A**) and b) cells able to grow CFU-GM colonies that were recovered at day 16 from the femora of transplanted mice (Fig. 2 **panel B**) or in WT mice transplanted either with C3aR<sup>-/-</sup> or WT HSPCs. Next, similar results were obtained after transplantation of HSPCs from C3aR<sup>-/-</sup> or WT mice to C3aR<sup>-/-</sup> deficient animals (not shown). Thus, our data revealed an engraftment defect of C3aR<sup>-/-</sup> HSPCs and show that this defect does not depend on C3aR expression in the BM microenvironment. Therefore, these data clearly demonstrate that C3a modulates engraftment and that some of its effects are mediated by its direct interaction with C3aR on HSPCs.

### **C3aR<sup>-/-</sup> cells show defective homing to BM**

Homing is the first step of BM seeding by HSPCs after transplantation, which precedes their engraftment, proliferation, and differentiation. Because we demonstrated that the C3a-C3aR axis is not involved in proliferation of HSPCs (23), we asked if delayed recovery of hematopoiesis in mice transplanted with C3aR<sup>-/-</sup> cells could be explained by their defective homing and lodgment to the BM microenvironment.

To address this issue, we employed short-homing assay to evaluate lodging of HSPCs to BM. Accordingly, WT mice were lethally irradiated and, 24 hours later, they were transplanted with C3aR<sup>-/-</sup> or WT HSPCs. Subsequently, animals were sacrificed 6 or 24 hours after transplantation, the femora were removed, and BM cells were isolated and plated in methylcellulose cultures to grow CFU-GM colonies (Fig. 2 **panel A**). By employing this assay, we found that C3aR<sup>-/-</sup> HSPCs show defective homing and lodging to the BM microenvironment. This defective lodging could be the result of defective transendothelial migration of HSPCs to cells in the BM microenvironment.

To further address this question, we performed in vitro adhesion studies with sorted Sca-1<sup>+</sup> cells. Accordingly, Sca-1<sup>+</sup> cells isolated from the BM of C3aR<sup>-/-</sup> or WT mice were non-exposed, or exposed for 30 minutes to C3a or <sub>desArg</sub>C3a and subsequently plated over BM-derived irradiated stroma feeder layers established in well plates. Subsequently, at 1 and 3 hours later, plates were gently shaken and non-adherent cells were discarded. Finally, cells remaining in the wells were trypsinized and plated in methylcellulose to grow CFU-GM colonies. We noticed that C3a, but not <sub>desArg</sub>C3a, increased adhesion in WT but not C3aR<sup>-/-</sup> HSPCs (Table 1). Based on this, the C3a-C3aR axis seems to be involved in increasing adhesion of HSPCs. Because stimulation by <sub>desArg</sub>C3a was ineffective to compensate the C3aR deficiency in C3aR<sup>-/-</sup> cells that express C3a and <sub>desArg</sub>C3a binding C5L2 receptor, we excluded the potential involvement of C5L2 in this phenomenon.

### **C3a-C3aR axis regulates secretion of MMP9 by HSPCs**

Following adhesion to endothelium, HSPCs have to migrate through the endothelial basement membrane in a process that involves secretion of proteolytic enzymes. MMP-9 was shown to play a pivotal role here. In our previous studies, we demonstrated that C3a stimulates secretion of MMP-9 by HSPCs (23). To address the importance of C3aR in this phenomenon, we exposed BM-derived Sca-1<sup>+</sup> cells isolated from C3aR<sup>-/-</sup> and WT to C3a

or  $\text{desArgC3a}$  and evaluated MMP-9 expression at the mRNA (Fig. 4 **panel A**) and protein (Fig. 4 **panel B**) levels. We noticed that C3a, but not  $\text{desArgC3a}$ , upregulates expression of MMP-9 in WT but not  $\text{C3aR}^{-/-}$  cells. Again, because stimulation by  $\text{desArgC3a}$  was ineffective to compensate the C3aR deficiency in  $\text{C3aR}^{-/-}$  cells that express C3a and  $\text{desArgC3a}$  binding C5L2 receptor, we excluded the potential involvement of C5L2 in this phenomenon.

### C3aR also plays a role in engraftment of human HSPCs

Finally, to address the role of C3aR in homing of human HSPCs, we performed xenotransplants in NOD/SCID mice (Fig. 5). Human UCB purified human  $\text{CD34}^{+}$  cells were pre-incubated or not exposed to C3aR antagonist SB 290157 and transplanted into lethally irradiated NOD/SCID mice 24 hours after an irradiation that eliminated murine endogenous HSPCs. Subsequently, 24 hours after transplantation of human UCBs, we recovered cells from the femora of transplanted mice and plated them in methylcellulose cultures to growth colonies in the presence of human hematopoietic factors. We noticed that the number of human CFU-GM progenitors recovered from murine femora was significantly lower in animals transplanted with UCB  $\text{CD34}^{+}$  cells in which C3aR was blocked by SB 290157 (Fig. 5), which also implicates a role of C3aR in homing of human HSPCs. Of note, in control experiments, no CFU-GM colonies were grown from BM cells recovered from lethally irradiated NOD/SCID mice that did not receive human UCB cells (not shown).

## Discussion

The  $\alpha$ -chemokine SDF-1 is upregulated in the BM microenvironment after conditioning for transplantation by radio-chemotherapy, which is a crucial chemoattractant for intravenously infused (1, 2, 4, 12, 13). However, the biological activity of the SDF-1-CXCR4 axis is modulated by several factors. For example, data from our laboratory demonstrated that during conditioning for transplantation, C cascade is activated. While C3 liquid phase cleavage fragments (C3a and  $\text{desArgC3a}$ ) prime and enhance responsiveness of HSPCs to SDF-1 gradient (22, 23, 28, 29), solid phase iC3b tethers HSPCs to BM stroma (18).

In agreement with this, we have previously shown that HSPCs poorly engraft after transplantation into  $\text{C3}^{-/-}$  mice (18). We have explained this defective engraftment in  $\text{C3}^{-/-}$  mice as a lack of generated C3a and  $\text{desArgC3a}$ , which enhance responsiveness of HSPCs to SDF-1 gradient (18, 28). To our surprise, however, we noticed that C3 cleavage fragments increase responsiveness of HSPCs to SDF-1 gradient in vitro and that this effect does not seem to be C3aR dependent. In support of this, we demonstrated that cells from WT and  $\text{C3aR}^{-/-}$  mice respond equally to priming effect to both C3a and  $\text{desArgC3a}$  (18).

On the other hand, we have also previously shown that  $\text{C3aR}^{-/-}$  mice as well as WT animals exposed to C3aR antagonist SB290157 are easy G-CSF mobilizers (30, 31). This latter observation prompted us to investigate whether C3a-C3aR may play a role in the retention of HSPCs in BM, which could be a potentially novel role of C3a independent of its priming effect on HSPCs.

Here, we show for the first time that transplantation of murine C3aR<sup>-/-</sup> HSPCs results in delayed engraftment and hematopoietic recovery of lethally irradiated mice. Because C3aR<sup>-/-</sup> animals engraft normally with WT cells, this defect is intrinsic to HSPCs and does not depend on C3aR expressed on cells in the hematopoietic microenvironment. More importantly, our in vivo lodging experiments suggest that this defect is related to impaired homing of transplanted cells. Furthermore, our short-term lodging xenotransplants experiments in NOD/SCID mice revealed that this novel role for the C3aR-C3a axis also seems to be relevant for homing of human CD34<sup>+</sup> cells. Thus, the C3a-C3aR axis plays an underappreciated role in homing of both human and murine HSPCs.

Based on these data, we postulate that activation of C cascade and release of C3a in BM after conditioning for transplantation not only primes responsiveness of HSPCs to SDF-1 gradient by a C3aR independent lipid raft formation-dependent mechanism (18, 22, 23, 29), but in addition, C3a directly engages C3aR on the surface of HSPCs and thus enhances homing of these cells to BM.

To better address the role of the C3aR-C3a axis in the early steps of homing and engraftment, we report here that murine HSPCs stimulated by C3a, but not <sub>desArg</sub>C3a, secrete more MMP-9 and adhere better to BM stroma. Thus, C3a-C3aR plays an important role in increasing secretion of MMP-9, which facilitates transmigration of HSPCs through the endothelial barrier as well as by increasing the interaction of HSPCs with cells in the hematopoietic microenvironment. Furthermore, because <sub>desArg</sub>C3a was not able to compensate for the C3aR deficiency, we excluded that another C3aR, the C5L2 receptor, is not involved in these phenomena.

In our previous work, we have also shown that another C3 cleavage fragment, iC3b, which is deposited during activation of C on the surface of damaged stroma cells in BM, increases tethering of HSPCs via the CR3 (CD11b/CD18) receptor. This dual role of C3 cleavage fragments, i.e., liquid phase C3a and solid phase iC3B, may explain why the engraftment defect in C3<sup>-/-</sup> mice was somehow slightly more pronounced than that observed after transplantation of WT cells into C3aR<sup>-/-</sup> mice. It is obvious that C3aR<sup>-/-</sup> mice still express and activate C3 and thus iC3b is normally deposited on BM stroma cells, providing a proper tethering for transplanted HSPCs via CR3 (CD11b/CD18) receptor (18).

Our data also explain our previous priming studies in vivo (22, 23, 29) showing that stimulation of HSPCs before transplantation with C3a, but not <sub>desArg</sub>C3a, accelerated engraftment of HSPCs into lethally irradiated WT mice. Because <sub>desArg</sub>C3a does not activate C3aR, the short exposure of HSPCs before transplantation to C3a accelerates engraftment by activating the homing function of C3aR. To support this and as we have demonstrated in this work, C3a, but not <sub>desArg</sub>C3a, increased MMP-9 secretion and enhanced adhesion of HSPCs to BM stroma cells. By employing C3aR<sup>-/-</sup> mice in this study, we clearly demonstrate that this effect is related to C3aR and another C3a binding receptor, C5L2, which also binds <sub>desArg</sub>C3a, cannot compensate for the C3aR deficiency. However, further research is required to identify if other proteases are released from HSPCs in addition to MMP-9 and to identify which adhesion molecules are directly affected by the C3a-C3aR axis.

In conclusion, we show for the first time that the C3a-C3aR axis plays an underappreciated role in homing of HSPCs to BM. By employing C3aR<sup>-/-</sup> mice, we excluded the involvement of the C5L2 receptor. Our data show that defective homing and engraftment of C3aR<sup>-/-</sup> HSPCs could be explained by the C3a-C3aR axis modulating secretion of MMP-9 by HSPCs as well as their adhesion. Thus, C3a plays a dual role in homing and engraftment (Figure 6). As reported previously, the first is by enhanced responsiveness of HSPCs to SDF-1 gradient through a C3aR independent mechanism (18, 22, 28, 29). Secondly and as shown here by engaging C3aR, it modulates several functions pertinent to transendothelial migration and homing (e.g., secretion of proteases and adhesion).

## Acknowledgments

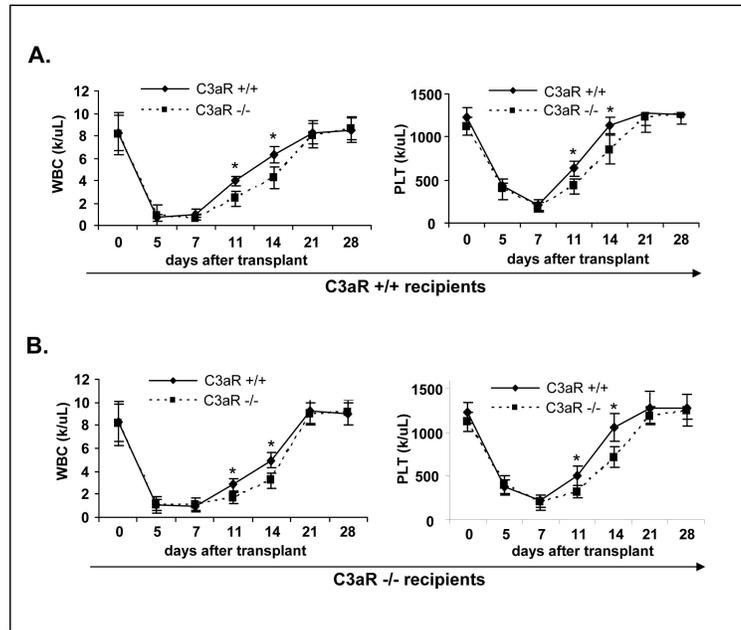
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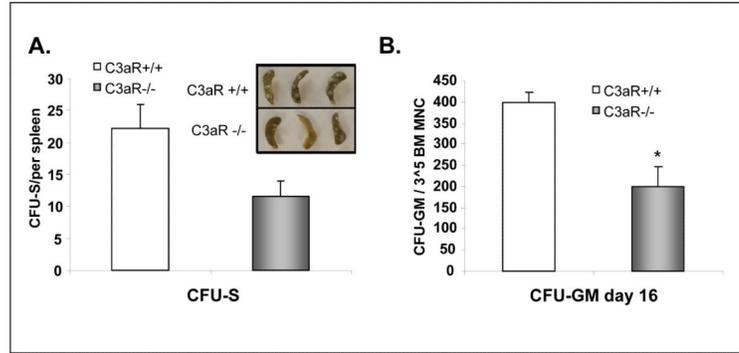
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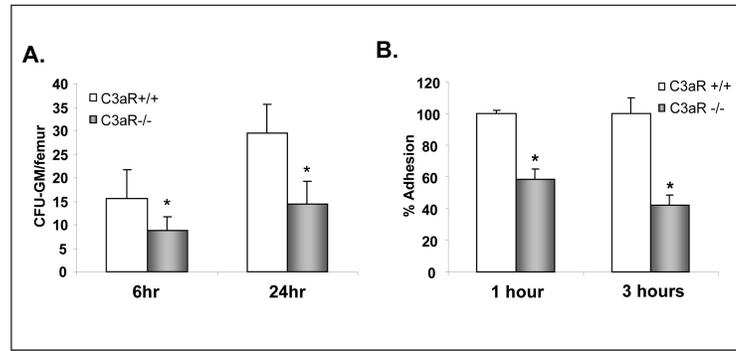
**Figure 1. HSPCs from C3aR<sup>-/-</sup> mice show defective engraftment**

This is demonstrated by a delayed recovery of platelets and leukocytes. **Panel A.** Lethally irradiated WT mice were transplanted with SKL cells ( $10^4$ /mouse) isolated from C3aR<sup>-/-</sup> and WT animals. Recovery of platelet and leukocyte counts was evaluated at days 0, 5, 7, 11, 16, and 28. **Panel B.** Lethally irradiated C3aR<sup>-/-</sup> mice were transplanted with SKL cells isolated from C3aR<sup>-/-</sup> and WT animals. Recovery of platelet and leukocyte counts was evaluated at days 0, 5, 7, 11, 16, and 28. The data shown represent the combined results from three independent experiments performed with 10 mice/group. \* $p < 0.0001$ . All mice in irradiation control (non-transplanted) died before day 14 (not shown).



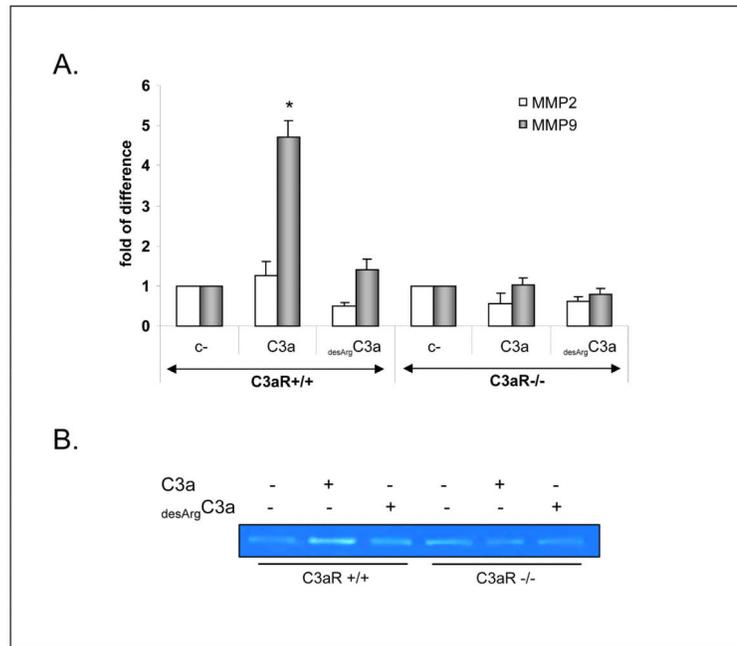
**Figure 2. HSPCs from C3aR<sup>-/-</sup> mice show decrease in day 11 CFU-S formation and content of day 16 CFU-GM/femur**

**Panel A.** Lethally irradiated WT mice were transplanted with Sca-1<sup>+</sup> cells isolated from C3aR<sup>-/-</sup> and WT animals. CFU-S colonies were counted 11 days after transplantation. **Inset.** Examples of spleens with CFU-S colonies. The data shown represent the combined results from three independent experiments performed with 10 mice/group. \* $p < 0.0001$ . All mice in irradiation control (non-transplanted) did not have any endogenous CFU-S colonies and died before day 14. **Panel B.** Lethally irradiated WT mice were transplanted with Sca-1<sup>+</sup> cells isolated from C3aR<sup>-/-</sup> and WT animals. BM from femora of transplanted mice were isolated and the contents of CFU-GM and femurs was evaluated in secondary in vitro cultures. The data shown represent the combined results from three independent experiments performed with 6 mice/group. \* $p < 0.003$ . All mice in irradiation control (non-transplanted) died before day 14.



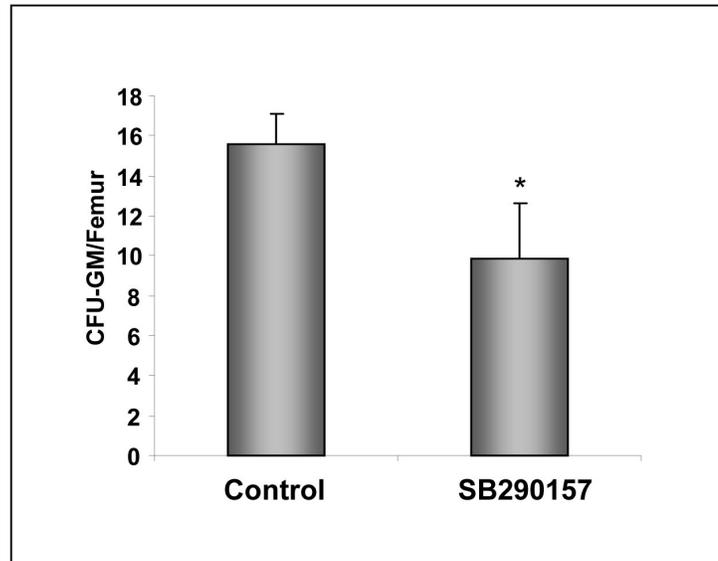
**Figure 3. HSPCs from C3aR<sup>-/-</sup> mice show defective homing in vivo**

**Panel A.** Lethally irradiated WT mice were transplanted with Sca-1<sup>+</sup> cells isolated from C3aR<sup>-/-</sup> or WT animals. At 6 and 24 hours later, BM from femora of transplanted mice were isolated and the contents of CFU-GM and femurs were evaluated in secondary in vitro cultures. The data shown represent the combined results from three independent experiments performed with 6 mice/group. \* $p < 0.0237$  for 6 hours and \* $p < 0.0016$  for 24 hours. No colonies grew from lethally irradiated and non-transplanted animals.



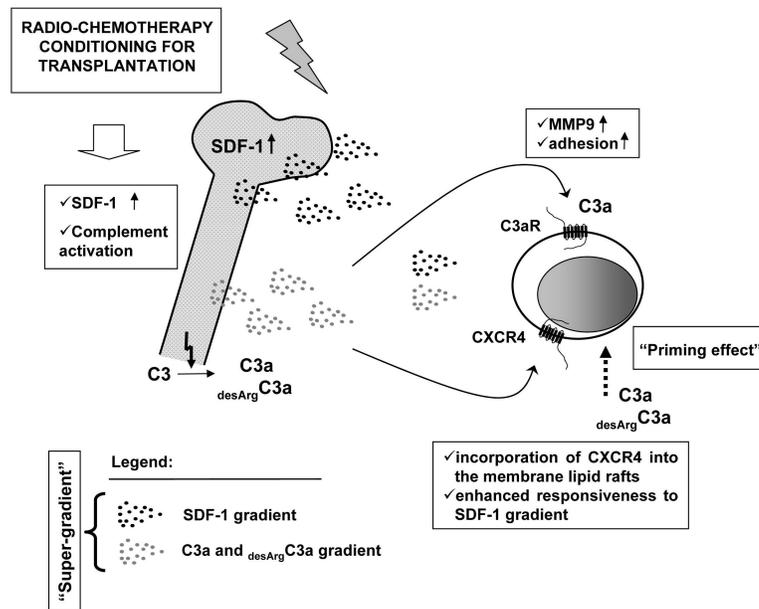
**Figure 4. C3a-C3aR axis enhances expression of MMP-9 by HSPC**

Sca-1<sup>+</sup> BM cells from C3aR<sup>-/-</sup> or WT mice were non-stimulated or stimulated overnight by C3a (1 µg/ml) or desArgC3a (1 µg/ml) and mRNA was isolated for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of MMP-9 and MMP-2 expression (**Panel A**). Supernatants were collected for MMP-9 analysis by zymography (**Panel B**). The data shown represent the combined results from three independent experiments. \**p*<0.0001 for real-time quantitative (RQ)-PCR. One representative zymography study is shown.



**Figure 5. Blocking of C3aR on human UCB CD34<sup>+</sup> cells impairs homing in NOD/SCID mice model**

Human UCB CD34<sup>+</sup> cells were exposed or not exposed to C3aR antagonist SB 290157 and transplanted into lethally irradiated NOD/SCID mice. At 24 hours later, BM from femora of transplanted mice was isolated and the contents of human CFU-GM and femurs were evaluated in secondary in vitro cultures. The data shown represent the combined results from three independent experiments performed with 6 mice/group each. \* $p < 0.001$ . No colonies grew from lethally irradiated and control animals not transplanted with UCB cells (n=5).



**Figure 6. SDF-1 and C3 cleavage fragments create “super-gradient” for HSPC**  
 Conditioning for transplantation by radio-chemotherapy upregulates in BM **i)** expression of SDF-1 (12) and **ii)** activates complement cascade (18, 22, 23, 28, 29). While SDF-1 directly chemoattracts HSPCs by interacting with CXCR4 receptor, our work revealed that the homing function of the SDF-1-CXCR4 axis is modulated by C3 cleavage fragments (C3a and <sub>desArg</sub>C3a) — both in C3aR-dependent and C3aR-independent manner. In a first mechanism, C3a (but not <sub>desArg</sub>C3a) activates C3aR and increases adhesiveness of HSPCs as well as secretion of MMP-9 by these cells. In a second one (C3aR independent) both C3a and <sub>desArg</sub>C3a enhance/prime responsiveness of HSPCs to SDF-1 gradient by increasing incorporation of CXCR4 into membrane lipid rafts. CXCR4 incorporated in lipid rafts is better connected with signal transduction proteins what explains why CXCR4<sup>+</sup> HSPCs respond more robust to SDF-1. However, the identification of receptor/receptors? involved in this C3a and <sub>desArg</sub>C3a mediated “priming” effect requires further studies.

**Table 1**  
**The C3a-C3aR axis enhances adhesion of BM-derived Sca-1<sup>+</sup> cells to BM stroma**

The number of CFU-GM from Sca-1<sup>+</sup> cells from WT mice that adhered to WT stroma stimulated for 30 min by C3a was arbitrarily assumed to be 100% in 1 hour and 3 hour adhesion assays. Data are pooled from quadruplicate samples from three independent experiments (n=12).

Source of Sca-1 <sup>+</sup> cells [adhesion time]	Non-stimulated	Stimulated by C3a (1µg/ml, 30 min)	Stimulated by <sup>desArg</sup> C3a (1µg/ml, 30 min)
WT [1 hr]	58+/-11 *	100+/-21	67+/-15 *
C3aR <sup>-/-</sup> [1 hr]	62+/-19 *	64+/-17 *	62+/-17 *
WT [3 hrs]	49+/-11 *	100+/-8	46+/-13 *
C3aR <sup>-/-</sup> [3 hrs]	53+/-16 *	49+/-20 *	51+/-7 *

\* p<0.00001

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