Mesenchymal Stromal Cells Engage Complement and Complement Receptor Bearing Innate Effector Cells to Modulate Immune Responses

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Abstract

Infusion of human third-party mesenchymal stromal cells (MSCs) appears to be a promising therapy for acute graft-versus-host disease (aGvHD). To date, little is known about how MSCs interact with the body’s innate immune system after clinical infusion. This study shows, that exposure of MSCs to blood type ABO-matched human blood activates the complement system, which triggers complement-mediated lymphoid and myeloid effector cell activation in blood. We found deposition of complement component C3-derived fragments iC3b and C3dg on MSCs and fluid-phase generation of the chemotactic anaphylatoxins C3a and C5a. MSCs bound low amounts of immunoglobulins and lacked expression of complement regulatory proteins MCP (CD46) and DAF (CD55), but were protected from complement lysis via expression of protectin (CD59). Cell-surface-opsonization and anaphylatoxin-formation triggered complement receptor 3 (CD11b/CD18)-mediated effector cell activation in blood. The complement-activating properties of individual MSCs were furthermore correlated with their potency to inhibit PBMC-proliferation in vitro, and both effector cell activation and the immunosuppressive effect could be blocked either by using complement inhibitor Compstatin or by depletion of CD14/CD11b-high myeloid effector cells from mixed lymphocyte reactions. Our study demonstrates for the first time a major role of the complement system in governing the immunomodulatory activity of MSCs and elucidates how complement activation mediates the interaction with other immune cells.

Introduction

Based on their immunomodulatory properties, mesenchymal stem or stromal cells (MSCs) are under investigation as treatment for acute graft-versus-host disease (aGvHD) and other types of hematopoietic stem cell transplantation-related disorders [1,2,3]. Their production of trophic factors also makes MSCs valuable candidates for many types of tissue repair applications [4,5]. The unique potential of MSCs to provide therapeutic options for thus-far untreatable human diseases is overshadowed by their difficult handling [6]. It is not clear yet how to assess the therapeutic potency of these cells before clinical administration to individual donors. Thus, it becomes clear that a more thorough mechanistic characterization of the most effective cell therapy product is urgently needed [5].

The exact mechanism by which MSCs elicit the broad immunomodulatory effect in vivo is at present unclear [5]. It has been observed that intravenous administration of MSCs promotes a beneficial effect on damaged tissues by inhibiting apoptosis, stimulating cell regeneration, and increasing angiogenesis [5,7]. It appears that MSCs reprogram recipient immune cells [8,9,10], for generating a complex immunosuppressive milieu consisting of a multitude of factors with complementary functions [6]. MSCs thereby synergize with the host’s immune system to potently suppress acute immune responses, in a fashion similar to that described for the process of tumor immune modulation [11]. The complement system serves as an important signalling system for modifying immune responses [12], e.g., in modulating the anti-tumor immune response [13,14]. Complement integrates the interaction between innate and adaptive immunity, it may be a key mediator of the broad immune modulation elicited by the therapeutic application of these cells, and it may possibly contribute to the generation of the immunosuppressive environ-
treatment [14]. It has recently been suggested that complement anaphylatoxins C3a and C5a participate in activation and recruitment of MSCs to sites of tissue damage and repair [15].

MSCs, like many other cell therapy treatments, can be applied via intravenous infusion into the blood circulation. These treatments are generally characterized by a high rate of cell loss [16]. This may be due to the instant blood-mediated inflammatory reaction (IBMIR) [17], which is characterized by a rapid destruction of the infused cells due to complement-, coagulation- and platelet activation. Complement rapidly reacts against foreign pathogens and cooperates with innate immune cells to clear these alien structures [18]. The central step in complement activation, regardless of the triggering event, is the proteolytic cleavage of complement component C3 (187 kDa) into C3b (177 kDa) and C3a (9 kDa) [19]. This cleavage reaction leads to disruption of the highly reactive internal thioester group and allows the subsequent covalent attachment of C3b to the triggering surface. C3b can then undergo a series of proteolytic cleavages to produce the surface-bound fragments iC3b and C3dg. These cell-bound fragments are ligands for immune cells bearing complement receptor type 1 (CR1; CD35), CR2 (CD21), CR3 (CD11b/CD18), and CR4 (CD11c/CD18); with CR3 being most prominent on monocytes, macrophages and NK-cells. Once complement activation occurs, the soluble anaphylatoxins C3a and C5a are released, which attract and activate leukocytes [12]. C5a-receptor signalling leads to up-regulation of CD11b on myeloid cells, to promote the interaction with its ligand iC3b [20]; this reaction can be blocked with a small cyclic C5a-receptor antagonist [21], or by inhibiting cleavage of C3 with the cyclic peptide Compsstatin [22].

Recently, complement activation was identified to be a major component in acute GvHD refractory to standard therapy in 28 patients, and treatment were failure of standard treatment approaches for tissue injury after HSCT (hemorrhagic cystitis and pneumomediastinum) in 14 patients. Patients received MSC-infusions from 3rd party unrelated donors (n = 50), from haploidentical related donors (n = 11) and from HLA identical siblings (n = 3). Patients received MSCs from passage 1 to 4 (P1-P4) in doses of approximately 1.0 to 3.0 x 10^6 cells/kg. All MSC donors (n = 53) were considered healthy after assessment of medical history, physical examination, and serological screening for HIV and hepatitis viruses. For fast availability, most of the applied cells were stored in liquid nitrogen and freshly thawed for IV-infusion. The MSC suspensions were culture-negative for bacteria and fungi, and polymerase chain reaction negative for Mycoplasma pneumoniae [2].

### Isolation and culture of cells for experiments

To isolate MSCs, bone marrow mononuclear cells were separated over a gradient of Redigrad (GE Health Care, Uppsala, Sweden), washed and resuspended in DMEM low-glucose medium (DMEM-LG; Gibco, Paisley, UK), supplemented with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal calf serum (FCS; Gibco), and plated at 1.6 x 10^5 cells/cm^2. When the cultures neared confluence (>80%), the cells were detached by treatment with trypsin and EDTA (Invitrogen, Grand Island, NY) and replated/passaged at a density of 4.000 cells/cm^2. HUVECs (Promocell, Heidelberg, Germany) were grown in endothelial cell growth medium (Promocell), supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin and replated at 10,000 cells/cm^2. Cells for experiments were detached with trypsin, viability was assessed by trypan blue exclusion (generally >95%), and cell suspensions were adjusted to 1.2 x 10^6 cells/ml.

### Blood and serum preparations

Fresh non-anticoagulated human blood was obtained from healthy volunteers who had given informed consent in accordance with the Helsinki Protocol and received no medication for at least 10 days. Complement-active normal human AB serum (NHS) was processed within 1 h of blood collection and stored at -70°C, to maintain the complement activity. In all experiments that made use of human serum, the final concentration of NHS or NHS/EDTA was 50% (v/v). Whole blood flow cytometry was performed with blood that had been anticoagulated by using 0.05 mg/ml lepirudin to (Refludan; Hoechst, Frankfurt am Main, Germany), a specific thrombin inhibitor that maintains the complement function in blood. To block complement function, blood or NHS was treated with cyclic Compsstatin Ac-I[CV(1MeWQDWAHCR)](1628Da) [22] or a inactive linear control peptide Ac-IAVVOQDWGHHRRAT (1532Da) and with C5aR antagonist Ac-F-[OPiChaWR] (896Da) [21] or its respective control peptide Phe-[Orn-Pro-dCha-Ala-D-Arg]. The inhibitors and the control peptides were produced in the laboratory of J. D. Lambris.

### Complement- and antibody-binding assays

The cells were mixed with an equal volume of NHS or NHS/EDTA in sterile polystyrene FACS tubes (BD, Franklin Lakes, NJ). Non-serum-treated or NHS/EDTA-treated cells were used as negative/washing controls, respectively. Incubation was carried out for 20 min at 37°C. Complement activity was stopped by adding EDTA (final concentration 10 mM). Supernatants were harvested after pelleting the cells by centrifugation at 900 g for 5 min, and frozen at -70°C. In experiments requiring the presence of Ca^2+ (detection of MBL, Clq, and annexin-V), cells were prepared under non-chelating conditions with binding buffer (10 mM HEPES/NaOH, pH 7.4, with 140 mM NaCl and
2.5 mM CaCl$_2$ from BD [24]. Pellet fractions were used as positive controls for immunodetection.

**Flow cytometry**

- **A. Phenotypic characterization of MSCs.** Cell suspensions were labeled with respective antibodies (Ab), washed and analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA). Cell acquisition was performed in a forward/side scatter (FSC/SSC) dot plot, cell debris was excluded with FSC, and dead cells were identified with propidium iodide. Fluorescence signals from 10,000 – 25,000 events were counted, with detection of median fluorescence intensity (MFI), and analyzed with Summit v4.1 software (Dako, Fort Collins, CO). The relative fluorescence intensity (RFI) was calculated by dividing the MFI of serum-treated cells by the MFI of non-serum-treated cells. MSCs/ECs were labeled with the following mouse anti-human mAbs (all from BD): isotype controls IgG1-FITC, and IgG2a-PE; negative controls CD45-FITC, and CD14-PE; positive controls CD90-FITC, and CD105-PE (Ansell, Bayport, MN); and complement regulatory proteins CD46-FITC, CD55-FTC, and CD59-PE (Ancell, Bayport, MN). Purified C3b, iC3b, and C3d (1 μg/lane) were used as positive controls for immunodetection.

- **B. Whole-blood analysis.** Whole-blood flow cytometric analysis was performed according to Mollnes et al. [20]. Blood was distributed equally into surface-heparinized FACS tubes (Corline Systems, Uppsala, Sweden), and treated with PBS, EDTA, antagonists, or matched control peptides. The final concentrations of inhibitors in blood were as follows: EDTA, 10 mM; Complementin or its control peptide, 60 μM; and C5aRA or its control peptide, 10 μM. The blood was split into two tubes for each condition and either MSCs or a similar volume of PBS was added (100 μl/ml). Different doses of MSCs were tested (0.1-1.0×10$^6$ cells/ml). The samples were incubated at 37°C, and remaining complement activity was stopped after 40 min by the addition of EDTA. Sample aliquots of 100 μl blood were collected after gentle mixing, labeled for 20 min with 5 μl of antibody (anti-C3c-FITC, or CD11b-FITC and respective isotype controls), and lysed for 5 min by adding 2 ml FACS lysis solution (BD Biosciences). The lysed samples were centrifuged at 900 g for 5 min, the supernatants discarded, and the cells were washed once again with 3 ml of PBS; 50,000 events were analyzed. The remaining blood sample volumes were diluted in an equal volume of 10 mM PBS/EDTA and centrifuged for 5 min at 2000 g to yield plasma supernatants for use in ELISA analysis. These supernatants were stored at -70°C until use.

**Mixed lymphocyte reactions**

Mixed lymphocyte reactions (MLRs) were performed as described elsewhere [25]. Responder PBMCs were stimulated with either PHA-mitogen, or alloantigen-stimulated with a pool of allogeneic donors (n = 5), and irradiated third-party MSCs (P3-5) were added at a 1:10 ratio to PBMCs. To block complement function different types of antagonists were added to MLRs: linear Compstatin (20 μM; cyclic Compstatin, 20 μM; and cyclic C5aR-antagonist, 5 μM). MACs-depletion was used to remove the CD14/CD11b-high fraction from PBMCs (Miltenyi Biotech, Germany); additionally blocking experiments of complement receptor 3 (CD11b/CD18) were performed with anti-CD11b mAb (5 μg/ml; Acris Biotechnology, Germany) or respective isotype control IgG (5 μg/ml). MSC-mediated suppression of alloantigen-stimulated PBMC proliferation was assessed at day 5 to 6 with [³H]thymidine incorporation (18 h) as counts per minute (cpm).

**ELISA analysis**

C3a and sC5b-9 generation in plasma supernatants was measured by ELISA according to the method of Nässén Ekdahl et al. [26]. Values are expressed in ng/ml and AU/ml, respectively, if not otherwise indicated.

**Statistical analysis**

Statistical analyses were performed using Student’s t-test or ANOVA. If the data did not fit normal distribution the Mann-Whitney test or the Wilcoxon matched pairs test was used (two-tailed confidence intervals, 95%; P<0.05 was considered statistically significant; Prism 5.0; Graphpad Software).

**Results**

MSCs and ECs display differential complement activating and regulatory properties

Flow cytometry was used to characterize the binding of complement factor C3 fragments to MSCs and HUVECs after the cells were incubated with complement-active normal human serum (NHS). MSCs showed a signal shift for anti-C3c detection relative to non-serum-treated cells or HUVECs treated in a similar fashion (Fig. 1A). Binding of C3 fragments was only observed after incubation with complement-active NHS, but not after treatment with EDTA-inactivated NHS (NHS/EDTA), demonstrating that C3 fragments only bound when complement was active. Significantly higher levels of C3 fragments were bound to the cell surface of MSCs, as compared to HUVECs (P<0.001, Fig. 1B), which was accompanied by C3a formation in the supernatants (P<0.05). Activation and subsequent binding of C3 to the surface of MSCs could be prevented by pre-treatment of NHS with Comstatin in a dose-dependent manner (Fig. 1C). NHS-treatment did not result in increased propidium iodide incorporation, Annexin-V-binding, or lysis of MSCs, as compared to NHS/EDTA treated cells (data not shown).

In order to study the intrinsic complement regulatory capacity of the two cell types, we analyzed the expression of complement regulatory proteins CD46, CD55, and CD59. Tryptsin detached
ECs strongly expressed all three regulatory molecules, whereas low to medium passage MSCs (P4-6) which had been harvested in a similar fashion expressed only CD59, but showed a relative lack for expression of CD46 and CD55 (both P<0.01, Fig. 2A), which suggests a substantial lack in complement regulatory capacity for culture expanded MSCs. We also studied the differential cell surface binding of complement activating factors (Fig. 2B). Both cell types bound small amounts of immunoglobulins (IgG and IgM) and specific C3 fragments (iC3b, C3d, C3c) was analyzed after cell treatment with NHS or NHS/EDTA and labeling with specific antibodies directed against the following epitopes: IgG (n=8), or IgM (n=8); iC3b (n=5), C3d (n=9), C3c (n=15). (C) Representative western blot for detection of C3c, iC3b and C3d epitopes bound to NHS or NHS/EDTA treated cells. Purified C3b, iC3b and C3d served as positive controls. The data in figure A and B are means±SEM, with: *P<0.05, **P<0.01, ***P<0.001.

Figure 1. MSCs and ECs activate complement to different degrees. Freshly trypsinized MSCs (black) and HUVECs (white) were exposed to complement active normal human serum (NHS) in order to study the cell surface binding of complement activation products with flow cytometry. (A) Histogram overlays for binding of anti-C3c antibody to non-serum-treated cells (empty, dotted), NHS (black), or NHS/EDTA-treated cells (shaded) is shown after a 20 min incubation with the respective sera at 37˚C. Complement-inactivated NHS (NHS/EDTA) served as negative control. (B) Box plots (whiskers min/max) for anti-C3c binding (RFI, left panel, n=11) and C3a generation in supernatants (ng/ml, right panel, n=8) after incubation of cells with NHS, the relative fluorescence intensity (RFI) was calculated compared to non-serum-treated cells. The data are expressed as means±SEM, **P<0.05, and ***P<0.001. (C) Inhibition of C3 fragment binding after pre-treatment of NHS with different doses (5-120 μM) of specific C3-inhibitor Compstatin (n=5).

doI:10.1371/journal.pone.0021703.g001

Figure 2. MSCs and ECs differ in their complement regulatory activity. (A) Expression of complement regulatory proteins MCP (CD46), DAF (CD55), and CD59 on MSCs and HUVECs (n=11 each) was quantified with flow cytometry (MFI, median fluorescence intensity). (B) Cell surface binding of immunoglobulins (IgG and IgM) and specific C3 fragments (iC3b, C3d, C3c) was analyzed after cell treatment with NHS or NHS/EDTA and labeling with specific antibodies directed against the following epitopes: IgG (n=8), or IgM (n=8); iC3b (n=5), C3d (n=9), C3c (n=15). (C) Representative western blot for detection of C3c, iC3b and C3d epitopes bound to NHS or NHS/EDTA treated cells. Purified C3b, iC3b and C3d served as positive controls. The data in figure A and B are means±SEM, with: *P<0.05, **P<0.01, ***P<0.001.

doI:10.1371/journal.pone.0021703.g002

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In order to clarify the specific profile of C3 fragments bound to the MSC-surface we performed flow cytometric analysis with antibodies which have reactivity against the C3c-, iC3b- and C3d- epitopes within the C3 molecule. We found that MSCs bound higher amounts of all three fragments on their cell surface (P<0.05 and P<0.001, Fig. 2B), which did not occur with EDTA-inactivated NHS. The presence of iC3b on MSCs was furthermore suggested by western blot experiments using polyclonal anti-C3c and C3d antibodies (Fig. 2C, lane 1). Bands with strong reactivity for C3 epitopes were detected on NHS-treated, but not on NHS/EDTA-treated MSCs. These bands corresponded to the 63- and 40-kDa fragments of the beta- and alpha-chain of control iC3b, respectively. Higher molecular weight bands (>110 kDa) larger than the native C3 alpha-chain were also found. No iC3b was found on HUVECs. Most interestingly, freshly thawed MSCs (as prepared for clinical use) displayed much higher degrees of complement fragment binding than did freshly trypsinized cells (data not shown), which indicates that minor damage to the cell-surface integrity/polarity as a result of the freeze/thaw procedure might have affected their complement triggering/regulating properties.

Complement-mediated modulation of MSC-induced effector cell priming in human blood

Since clinical MSCs are applied via systemic infusion to our HSCT patients, we simulated the exposure of MSCs to lepirudin-anticoagulated ABO-compatible human blood in vitro. Lepirudin
inhibits thrombin, but maintains complement activity in blood. In agreement with our prior findings in NHS, exposure of lepirudin-blood to MSCs revealed a significant increase in generation of complement activation product C3a (P<0.001, Fig. 3A) and soluble C5b-9 complex (P<0.01, Fig. 3B), which is indicative for formation of C5a. Flow cytometric analysis of MSCs and different blood effector cells (Fig. 3C), confirmed the activation and binding of C3 fragments to MSCs in blood (shown in red), which was accompanied by triggering of CD11b on neutrophil granulocytes (shown in green, P<0.001, Fig. 3D) and monocytes (data not shown). We furthermore analyzed the general activation of effector cells by detecting morphological changes with flow cytometry (Table S1), which revealed a significant reduction in the number of resting monocytes and lymphocytes (P<0.001 and P<0.01), and an increase in activated lymphocytes and monocytes (both P<0.01) in response to MSCs. But no significant changes in percentage of gated MSCs was found when active blood was compared to EDTA-inactivated control blood (8.1±4.4 vs. 7.3±4.6), which indicates that no immediate complement or effector cell-mediated lysis of MSCs had taken place (Fig. 3E). To verify if the activation of effector cells after contact with MSCs is mediated via complement signalling we blocked complement function by pre-treatment of blood with Compstatin, which lead to strongly reduced formation of both C3a and sC5b-9 (P<0.05, Fig. 4A and 4B). Compstatin furthermore abrogated triggering of CD11b on effector cells (P<0.05, Fig. 4C, left panel), which could also be blocked with a specific C5a receptor antagonist (P<0.05, Fig. 4C, right panel). But neither formation of C5a, sC5b-9, or triggering of CD11b was affected by the corresponding control peptides.

The complement-activating properties of MSCs affect their immunomodulatory profile

Many clinical applications of MSCs are based on their immunomodulatory properties and tests to determine the potency of MSCs to eliciting desired clinical responses would be anticipated. We therefore screened the suppressive effect of more than 60 individual MSCs in mixed lymphocyte reactions (MLRs), and found that MSCs show a broad donor specific variation in their suppressive properties, with an average inhibition of about 60% (Fig. 5A). Whilst some MSCs were highly suppressive in a consistent fashion (e.g. Kd086, K03, K22, K29, L32, L113), others showed strong variation (e.g. K01, L10, L43, L61, L11B), some suppressed poorly (e.g. Kd029, KD050, K14, K15, L10), whereas...
Figure 5. The complement activating properties of MSCs direct their interaction with complement receptor bearing effector cells and their immunomodulatory properties. (A) Suppression of PBMC-proliferation by MSCs was tested in MLRs. Retrospective analysis is shown.
(2003 – 2011), with an average of n = 5 MLR-experiments for each individual MSC-donor versus 3–5 random PBMC donors, the dotted line indicates overall average suppression (~60%); (B) Average suppression of MSCs in MLRs and resulting clinical response to MSCs therapy (PD, progressive disease; SD, stable disease; PR, partial response; and CR, complete response); (C) MSC-donor specific differences in cell surface binding of C3-fragments after incubation with NHS as detected by binding of anti-C3c with flow cytometry (RFI, n ≥ 5 each donor), dotted line indicates threshold for C3-low (average RFIC3c < 10) and C3-high cells (RFIC3c > 10); (D) Statistical comparison for suppressive potency of MSCs in MLRs, when grouped into C3-low and C3-high cells (n = 17 each); (E) Flow cytometric analysis on CD11b-triggering activity of MSCs in human blood (MFI CD11b on PMNs), when compared for C3-low (n = 6) and C3-high cells (n = 14), relative to PBS- or EDTA-treated control blood. (F) MACs depletion of CD14+ cells from PBMCs efficiently removes of CD11b-high monocytes but not CD11b-low NK cells from MLRs (n = 5). (G) Suppression of PBMC-proliferation by MSCs in MLRs (n = 3 experiments) with various combinations of MSCs (n = 5 donors), responder PBMCs (n = 2), and 2 different pools of allogeneic stimulator PBMCs (n = 5 donors), which were tested in the presence of different inhibitory treatments: untreated MLRs (n = 13, white), control peptide (linear Complement Mediates Suppressive Effects of MSCs

In recent years, the interaction of MSCs with the adaptive immune system has been extensively studied [27]. However, their relationship to the innate immune system has scarcely been addressed so far, focusing primarily on innate effector cells and toll like receptors [9,27]. In the present study, we have investigated the interactions between culture-expanded MSCs and the complement system, which plays an important role in host defense, and in modifying immune responses in vivo [12,13]. Here, we report for the first time that exposure of MSCs to complement-active human serum and blood leads to deposition of complement activation products on the cell surface of MSCs and generation of soluble anaphylatoxins. This process led to a complement-mediated triggering of effector cell activation, via the engagement of complement receptor type 3 (CD11b/CD18). The complement-activating properties of these cells were correlated with their immunomodulatory capacity to suppress PBMC-proliferation in vitro. The suppressive effect of MSCs could be blocked by inhibiting complement function or by removal of myeloid effector cells from MLRs. Thus, it appears that complement activation plays an important role in mediating the activation and interaction of MSCs with different types of complement receptor-bearing effector cells in human blood, potentially triggering their own intrinsic immunosuppressive functions and that of other effector cells, to generate a complex immunosuppressive environment (Figure S1).

It is generally believed that bone marrow derived MSCs migrate throughout the vascular system and home to specific target sites in vivo, but whether trafficking of MSCs occurs via the blood stream in healthy adults remains controversial [28]. We speculated that MSCs have a certain degree of intrinsic blood compatibility, such as attributed to endothelial cells (ECs), and studied how they interact with the complement system upon exposure to human serum. In our first set of experiments, we saw an enhanced cell surface C3-fragement deposition (iC3b and C3dg) and fluid phase generation of C3a after exposure of MSCs to human serum. Interestingly, Schrautstatter et al. recently demonstrated that complement anaphylatoxins C3a and C5a bind to their concomitant receptors on MSCs, which are then quickly translated to the cell nucleus, where they trigger cell activation and chemotactic responses of these cells [15]. Furthermore, it was shown that complement modulates the inflammatory response of mesenchymal and more mature osteoblastic cells [29]. The activation of complement on the surface of culture expanded MSCs and the generation of anaphylatoxins in proximity to respective receptors on these cells may therefore provide a fast auto-activation loop, which could potentially trigger the immunosuppressive function of MSCs directly after systemic infusion. The requirement of this initial licensing step for MSC-function has already been well described for a number of pro-inflammatory cytokines such as interferon gamma [10]. Complement activation may therefore provide yet another powerful signal to activate the intrinsic defense mechanisms of MSCs after systemic infusion.

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Cells in contact with blood are normally equipped to down-regulate complement activation via expression of complement regulatory proteins such as MCP, DAF, and CD59, which are typically found on ECs [30]. Culture-expanded MSCs lacked MCP and DAF, but were protected from complement-mediated lysis by the expression of CD59. We investigated potential complement-triggering factors and the specific profile of C3 fragments deposited onto the surface of MSCs, by using methods
that paralleled those described in a previous study on islet cells [31]. Flow cytometry and western blot analysis showed cell-surface deposition of iC3b and C3dg on MSCs. Two typical mediators of complement activation are the recognition molecules C1q (classical pathway), which preferentially binds to antibodies, and MBL (lectin pathway), recognizing non-self carbohydrate ligands. Both cell types bound similar, but very low amounts of immunoglobulins. Scarcе amounts of C1q and MBL were found to be primarily associated with non-vital cells, in agreement with the literature; typically, only late apoptotic or necrotic cells are to be primarily associated with non-vital cells, in agreement with earlier observations by Groh et al. who found that MSCs engage monocytes to elicit their immunosuppressive effects [33]. To clarify if a highly suppressive MSC-phenotype is actually associated with a beneficial clinical response in vivo we repeatedly tested the suppressive effect of clinical MSCs in alloantigen- and PHA-stimulated MLRs. We thereby obtained their average suppressive activity, which was then correlated with the degree of their individual clinical response obtained in treatment of acute GvHD and hemorrhagic cystitis. It appeared that MSCs with a medium suppressive activity are therapeutically more beneficial then highly MLR-suppressing MSCs, which might indicate that the strongly complement depositing phenotype is less favourable for therapeutic use.

A growing body of evidence suggests that the clinical infusion of MSCs can transform a pro-inflammatory environment into a milieu that favors healing and the suppression of allogeneic responses. This effect might be mediated by soluble factors and the generation of suppressive myeloid and lymphoid cell subsets. Many investigators have shown that MSCs inhibit the function of effector cells and may even polarize their phenotype to an immunosuppressive one, which possibly augments their own intrinsic immunosuppressive function [27]. Recent studies have pointed to the generation of suppressive myeloid cells after close interaction with MSCs, but the nature of this interaction was found to be elusive [8,9]. This report shows that complement activation serves as a mediator between MSCs and complement receptor bearing immune cells, such as CD11b+myeloid and NK cells, and that complement activation augments the immunomodulatory activity of MSCs in vivo. Our findings also demonstrate that strongly complement activating MSCs might not essentially be more valuable for therapeutic use, since average suppressors appeared to yield the most beneficial therapeutic effect in vivo. Our results may contribute to the understanding and interpretation of complement mediated interactions of MSC with other immune cells in vitro and in vivo.

**Supporting Information**

**Figure S1** The complement-activating properties of mesenchymal stem cells (MSCs). Triggering of complement activation on the surface of MSCs leads to C3 convertase-
mediated cleavage of complement factor C3 into its active fragments C3a and C3b. The covalently bound C3b can be degraded to iC3b by factor I. C3b and its degradation products mediate phagocytosis and immune responses via complement receptors, such as CR3 (CD11b/CD18), on host cells. Accumulation of C3b leads to assembly of C5 convertases that activate C5 to C5a and C5b, which may eventually lead to formation of the lytic membrane attack complex (MAC). However, cell lysis can be prevented by the complement regulatory function of membrane protein CD59. Anaphylatoxins C3a and C5a induce cell activation and chemotactic responses by binding to their receptors C3aR and C5aR on host cells and MSCs, which may promote interaction with various types of CR-bearing cells. Activated MSCs may reprogram host cells to synergistically produce an anti-inflammatory microenvironment composed of many different factors (iNOS/NO, IDO/kynurenine, HO-1/biliverdin and CO, PGE2, Galectin-1, TSG-6, shHLA-G5, HGF, IL6, IL10, TGFβ, IL1Rag), and may suppress alloimmune responses in vivo.

(TIF)

Table S1 MSC-induced effector cell activation in whole blood. * Percentage (means±SD, n = 14) of resting or activated effector cells is shown for blood treated w/wo MSCs. *P<0.05, **P<0.01, and ***P<0.001 relative to non-MSC-treated blood.

(DOCX)

Author Contributions
Conceived and designed the experiments: GM KNE KLBN. Performed the experiments: GM RJ LB ID DM. Analyzed the data: GM OR ID KLBN. Contributed reagents/materials/analysis tools: JDL GE LL BS. Wrote the paper: GM IRD OR KLBN.

References
1. Le Blanc K, Frassoni F, Ball I, Locatelli F, Roedel H, et al. (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371: 1579–1586.
2. Le Blanc K, Samuelson H, Gustafsson B, Remberger M, Sundberg B, et al. (2007) Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia 21: 1733–1738.
3. Ringdén O, Uzunel M, Sundberg B, Lonnies L, Nava S, et al. (2007) Tissue repair using allogeneic mesenchymal stem cells for hemorrhagic cystitis, pneumonieusthmatisum and perforated colon. Leukemia 21: 2271–2276.
4. Caplan AI (2009) Why are MSCs therapeutic? New data: new insight. J Pathol 217: 318–324.
5. Ankrum J, Karp JM Mesenchymal stem cell therapy: Two steps forward, one step back. Trends Mol Med 16: 203–209.
6. Pittenger M (2009) Shrouding the source of regeneration by MSCs. Cell Stem Cell 5: 8–10.
7. Prockop DJ, Olson SD (2007) Clinical trials with adult stem/progenitor cells for tissue repair: let’s not overlook some essential preconditions. Nature 451: 3147–3151.
8. Ohtaki H, Ylostalo JH, Foraker JE, Robinson AP, Reger RL, et al. (2008) Stem/progenitor cells from bone marrow decrease neuronal death in global ischemia by modulation of inflammatory/immune responses. Proc Natl Acad Sci U S A 105: 14638–14643.
9. Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, et al. (2009) C3a and C5a modulate osteoclast formation and inflammatory response of murine bone marrow stromal cells. Proc Natl Acad Sci U S A 106: 1348–1353.
10. Prockop DJ, Olson SD (2007) Clinical trials with adult stem/progenitor cells for tissue repair: let’s not overlook some essential preconditions. Nature 451: 3147–3151.
11. Koutoulaki A, et al. (2008) Modulation of the antitumor immune response by complement factor C5a. J Immunol 178: 34–41.
12. Ricklin D, Lambris JD (2007) Complement-targeted therapeutics. Nat Rev Drug Discov 6: 513–525.
13. Koutoulaki A, et al. (2008) Modulation of the antitumor immune response by complement factor C5a. J Immunol 178: 34–41.
14. Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, et al. (2009) Bone marrow stromal cells attenuate sepsis by modulation of inflammatory/immune responses. Proc Natl Acad Sci U S A 106: 14638–14643.
15. Prockop DJ, Olson SD (2007) Clinical trials with adult stem/progenitor cells for tissue repair: let’s not overlook some essential preconditions. Nature 451: 3147–3151.
16. Le Blanc K, Tammik I, Sundberg B, Haynesworth SE, Ringdén O (2003) Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 57: 11–20.
17. Vermees I, Haenen C, Steffen-Nakken H, Reutelingsperger C (1995) A novel assay for apoptosis.Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. J Immunol Methods 184: 39–51.
18. Nilsson B, Nilsson Ekhdal K, Avila D, Nilsson UR, Lambreis JD (1990) Neutrophins in complement component C3 as detected by monoclonal antibodies. Mapping of the recognized epitopes by synthetic peptides. Biochem J 268: 53–61.
19. Jansen BJ, Huizinga EG, Raatjmakers HC, Roos A, Daha MR, et al. (2005) Structures of complement component C3 provide insights into the function and evolution of immunity. Nature 437: 505–511.
20. Komell TE, Brekke OL, Fung M, Eure H, Christiansen D, et al. (2002) Essential role of the C5a receptor in E.coli-induced oxidative burst and phagocytosis revealed by a novel lepinidin-based human whole blood model of inflammation. Blood 100: 1869–1877.
21. Finch AM, Wong AK, Paczkowski NJ, Wadi SK, Craik DJ, et al. (1999) Low-molecular-weight peptidic and cyclic antagonists of the receptor for the complement factor C5a. J Med Chem 42: 1963–1974.
22. Katragadda M, Magotti P, Styrova G, Lambreis JD (2006) Hydrophobic effect and hydrogen bonds account for the improved activity of a complement inhibitor, compstatin. J Med Chem 49: 4616–4622.
23. Le Blanc K, Tammik I, Sundberg B, Haynesworth SE, Ringdén O (2003) Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 57: 11–20.
24. Vermees I, Haenen C, Steffen-Nakken H, Reutelingsperger C (1995) A novel assay for apoptosis.Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. J Immunol Methods 184: 39–51.
25. Nilsson B, Nilsson Ekhdal K, Avila D, Nilsson UR, Lambreis JD (1990) Neutrophins in complement component C3 as detected by monoclonal antibodies. Mapping of the recognized epitopes by synthetic peptides. Biochem J 268: 53–61.
26. Nilsson Ekhdal K, Nilsson B, Pekna M, Nilsson UR (1992) Generation of C3a at the interface between blood and gas. Scand J Immunol 35: 85–91.
27. Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol. 8: 155–165.
28. Fox JM, Chamberlain G, Ashton BA, Middleton J (2007) Recent advances into antibody-mediated complement activation mediates lysis of pancreatic islets cells and may suppress allogeneic immune responses in vivo. J Immunol 178: 267–296.
29. Ricklin D, Lambris JD (2007) Complement-targeted therapeutics. Nat Biotechnol 25: 1265–1275.
30. Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtensteiner SK, Koutoulaki A, et al. (2008) Modulation of the antitumor immune response by complement. Nat Immunol 9: 1225–1235.
31. Ouward-Rosenberg S (2008) Cancer and complement. Nat Biotechnol 26: 1348–1349.
32. Schraufstatter IU, Disciplo RG, Zhao M, Khaldoyanidi SK, et al. (2009) C3a and C5a are chemotactic factors for human mesenchymal stem cells, which cause prolonged EKK1/2 phosphorylation. J Immunol 182: 3827–3836.
33. Le Blanc K, Pittenger M (2005) Mesenchymal stem cells: progress toward promise. Cytotherapy 7: 36–45.
34. Bennett W, Sundberg B, Groth CG, Brendel MD, Brandhorst D, et al. (1999) Incompatibility between human blood and isolated islets of Langerhans: a finding with implications for clinical intraportal islet transplantation? Diabetes 48: 1907–1914.
35. Markiewski MM, Nilsson B, Ekhdal KN, Kothles TE, Lambris JD (2007) Complement and coagulation: strangers or partners in crime? Trends Immunol 28: 184–192.
36. Jansen BJ, Huizinga EG, Raatjmakers HC, Roos A, Daha MR, et al. (2005) Structures of complement component C3 provide insights into the function and evolution of immunity. Nature 437: 505–511.
37. Komell TE, Brekke OL, Fung M, Eure H, Christiansen D, et al. (2002) Essential role of the C5a receptor in E.coli-induced oxidative burst and phagocytosis revealed by a novel lepinidin-based human whole blood model of inflammation. Blood 100: 1869–1877.