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C3a and C5a Are Chemotactic Factors for Human Mesenchymal Stem Cells, Which Cause Prolonged ERK1/2 Phosphorylation[1]

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Mesenchymal stem cells (MSCs) have a great potential for tissue repair, especially if they can be delivered efficiently to sites of tissue injury. Since complement activation occurs whenever there is tissue damage, the effects of the complement activation products C3a and C5a on MSCs were examined. Both C3a and C5a were chemoattractants for human bone marrow-derived MSCs, which expressed both the C3a receptor (C3aR) and the C5a receptor (C5aR; CD88) on the cell surface. Specific C3aR and C5aR inhibitors blocked the chemotactic response, as did pertussis toxin, indicating that the response was mediated by the known anaphylatoxin receptors in a G_i activation-dependent fashion. While C5a causes strong and prolonged activation of various signaling pathways in many different cell types, the response observed with C3a is generally transient and weak. However, we show herein that in MSCs both C3a and C5a caused prolonged and robust ERK1/2 and Akt phosphorylation. Phospho-ERK1/2 was translocated to the nucleus in both C3a and C5a-stimulated MSCs, which was associated with subsequent phosphorylation of the transcription factor Elk, which could not be detected in other cell types stimulated with C3a. More surprisingly, the C3aR itself was translocated to the nucleus in C3a-stimulated MSCs, especially at low cell densities. Since nuclear activation/translocation of G protein-coupled receptors has been shown to induce long-term effects, this novel observation implies that C3a exerts far-reaching consequences on MSC biology. These results suggest that the anaphylatoxins C3a and C5a present in injured tissues contribute to the recruitment of MSCs and regulation of their behavior. *The Journal of Immunology,* **2009, 182: 3827–3836.**

Mesenchymal stem cells (MSCs)[4] hold a great therapeutic potential for tissue regeneration, since they can differentiate into bone, cartilage, tendon, and muscle, including cardiac muscle, fat, and fibroblasts (1, 2). Adult bone marrow MSCs can also be harvested and expanded in vitro in sufficient cell numbers to hold promise for clinical applications of tissue repair. Interestingly, i.v. application of MSCs results in the specific recruitment of these cells to an area of tissue injury (2, 3), and circulating MSCs appear to represent a pool of cells constantly available for the repair of damaged tissues. However, the mechanisms that guide this attraction of circulating MSCs to areas of tissue injury are poorly understood, although a number of factors have been described, which are chemoattractants for MSCs (4). It appears that MSC recruitment to inflammatory sites occurs in a fashion reminiscent of leukocyte trafficking via the utilization of adhesion molecules including selectins and integrins, as well as chemotactic factors and their receptors (4), but there is some controversy about which specific chemotactic factors are involved.

While the chemotactic effect of several growth factors, including basic fibroblast growth factor (bFGF), platelet-derived growth factor, insulin-like growth factor 1, and hepatocyte growth factor, is documented for MSCs (5–7), the effect of chemotactic factors, which activate G protein-coupled receptors (GPCRs), such as chemokines and the anaphylatoxins C3a and C5a, has only recently received attention. Chemokine receptors such as the stromal cell-derived factor 1 (SDF-1) receptor (CXCR4) appear to play a role, although the CXCR4 is only expressed on the cell surface of a limited fraction of MSCs (4, 6, 8), and it remains controversial whether SDF-1 is a chemotactic factor for MSCs (4, 6, 8). The chemotactic effect of the anaphylatoxins C3a and C5a on MSCs has so far been completely overlooked. However, since complement is necessarily activated at the locale of tissue injury, the effect of C3a and C5a on MSC migration is of particular interest, since these anaphylatoxins may contribute to the mobilization and recruitment of MSCs to an injury site, thus facilitating wound healing and regeneration.

Complement activation from classical, alternative, and lectin pathways leads to production of the anaphylatoxins C3a and C5a, which are small (M_r of ~8,700–11,000) polypeptides released from their precursor proteins C3 and C5, respectively, by C3/5 convertases. Both C3a and C5a are well known as chemotactic, oxidant-inducing, and degranulating agents for myeloid cells (9–14).

The C3a receptor (C3aR) and the C5a receptor (C5a; CD88) are both GPCRs (15–19), which couple usually to G_i (13) and share ~40% sequence identity. There is a third anaphylatoxin receptor, C5L2, which binds C5a, but according to most reports its activation does not cause any cell stimulation (20), suggesting that this may be a scavenger receptor for C5a (21).

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[4] Abbreviations used in this paper: MSC, mesenchymal stem cell; bFGF, basic fibroblast growth factor; C3aR, C3a receptor; C5aR, C5a receptor (CD88); DAPI, 4′,6-diamidino-2-phenylindole; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; SDF-1, stromal cell-derived factor 1.

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Receptors for C3a and C5a are widely expressed and are not only found on cell types of myeloid lineage, but also on T lymphocytes, smooth muscle cells, neurons, and glial, endothelial, and epithelial cells (22–26). While C5a functions as a potent chemotactic factor for most leukocytes both in vitro and in vivo (9, 27–29), C3a is only a moderately effective chemoattractant for various leukocytes in vitro. Even cell types that showed a modest chemotactic response to C3a in vitro (i.e., eosinophils) failed to be chemoattracted by C3a in vivo (29). This may be explained in part by the presence of serum carboxypeptidase N, which inactivates C3a by cleaving the carboxyl-terminal arginine residue (30). Although carboxypeptidase N also converts C5a to C5a(desArg), this modified polypeptide still retains a reduced functional ability and affinity for the C5aR (9), whereas C3a(desArg) loses the complete ability to bind and activate the Ca3R (31). However, even in serum-free conditions, activation of the C3aR causes a transient response, and certain pathways that necessitate a more sustained response such as phosphatidylinositide production are not invoked by C3a in neutrophils (32). In comparison, activation of the C5aR on leukocytes shows a more robust respiratory burst, calcium mobilization, and chemotaxis (9, 29, 32).

Apart from its effect on myeloid cells, it has been described that C3a causes transient stress fiber formation and activation of the MAPK ERK1/2 in endothelial cells (26, 33). Finally, in mast cell lines C3a mediates its effects by transient calcium ion mobilization and, in contrast to what has been described for other cells, sustained ERK1/2 and Akt phosphorylation (34). Overall, there is limited information available on the signaling pathways activated by C3aR stimulation.

The signaling cascades, which are activated by stimulation of the C5aR, are better defined and include strong activation of ERK1/2 (35, 36) and of Akt (37, 38) and modest activation of the MAPK p38 (39), which has been described in multiple cell types, but mostly in cells of myeloid lineage. Additionally, in cells that express both the C5aR and the epidermal growth factor (EGF) receptor, C5a can induce transactivation of the EGF receptor, as has been described for endothelial cells (26).

The C3aR contains two interesting features. First, it has an unusual, very long second extracellular loop, of which sequences adjacent to the transmembrane domains are important for C3a binding (40). Second, an apparent nuclear localization signal sequence is located near the C terminus of the C3aR, FRKKAR, starting at aa 442 (41), but a functional significance for this observation was not discerned.

Although almost all GPCRs reside in the outer cell membrane, nuclear localization and nuclear translocation have been observed for only a few of them (42–44). For instance, the receptors for platelet-activating factor, prostaglandin E_2, and lysophosphatidic acid (43, 45) are located in the nucleus constitutively. These receptors are thought to be activated by endogenously produced, nonsecreted ligands. Other nuclear GPCRs, such as those for angiotensin II type 1 receptor (AT_1R), somatostatin, and neurotensin (46), are initially localized to the plasma membrane but are subsequently endocytosed and translocated to the nucleus following ligand binding (43). Endocytosis and nuclear relocalization of these GPCRs are thought to be necessary for transcriptional activation (43). While GPCR activation at the plasma membrane induces mostly short-term signaling events, nuclear translocation results in long-term effects, including prolonged nuclear ERK1/2 activation (47), leading to transcriptional activation (47), cell proliferation, and differentiation (42).

In this study we examined the expression of C3aR and C5aR in MSCs as well as their cell signaling and chemotactic responses. Apart from finding that C5a and more unusually C3a are effective chemotactic factors for MSCs, we made the interesting observation that the C3aR in MSCs is translocated to the nucleus following C3a stimulation, which was accompanied by prolonged phosphorylation and nuclear translocation of ERK1/2 and phosphorylation of the transcription factor Elk. In contrast, in other cell types that were tested the activated C3aR was retained in endocytic vesicles, and the cell signaling pathway activation was much more transient.

Materials and Methods

Materials and DNA constructs

Abs against phospho-ERK, phospho-Akt, and total and phospho-Elk were purchased from Cell Signaling Technology, and Abs against total ERK1/2, total Akt, lamin B, and β-actin were obtained from Santa Cruz Biotechnology.

The human C3aR and C5aR were expressed as fusion proteins with GFP. The cDNA for the C3aR in pcDNA3.1 (a gift of Z. K. Pan, Medical College of Ohio, Toledo, OH) was subcloned into the *Xho*I and *Kpn*I sites of pEGFP-N3 (Clontech). The C5aR-GFP construct has been previously described (48).

Human C3a was excised from human complement C3 using a fluid-phase C3 convertase, and the anaphylatoxin was purified on carboxymethyl-Sephadex as previously described (29). C5a was prepared by expression as an oligo(histidine)-tagged chimera in *Escherichia coli*, and after excision using thrombin, recombinant C5a was purified by carboxymethyl-Sephadex (R. G. DiScipio, S. K. Khaldoyanidi, and I. U. Schraufstatter, manuscript in preparation).

For inhibition of the C3aR, cells were incubated for 30 min with 1 μM SB290157 (Calbiochem) (49); for inhibition of the C5aR 1 μM W-54011 (Calbiochem) (50) was used also for 30 min, and G_i was inactivated with an overnight incubation with 100 ng/ml pertussis toxin (List Biological Laboratories).

Cell culture

Human MSCs provided by Dr. D. Prockop (Tulane University, New Orleans, LA) were cultured in α-MEM (Invitrogen) with mesenchymal stem cell stimulatory supplements (StemCell Technologies) and used up to passage 7.

HEK293 cells (human embryonic kidney cells; American Type Culture Collection), a commonly used cell type for signaling studies, were grown in DMEM containing 10% FCS. The DNA constructs for the C3aR and the C5aR were transfected into HEK293 cells using Lipofectamine (Invitrogen) as previously described (51). Stable cell lines were selected with G418.

Human peripheral monocytes were purified using Ficoll gradient centrifugation of blood from healthy volunteers (San Diego Blood Bank) as previously described (52).

RT-PCR

RNA from MSCs was isolated with the RNeasy kit (Qiagen). Complementary DNA was synthesized from MSC RNA using Omniscript reverse transcriptase (Qiagen). For the PCR, the primers used for detection of the C3aR were: forward, 5′-CAT CCT TGG ACA GTC CCC ACT GTC TTC C-3′ and reverse, 5′-GTC GAC TCA CCT AGT GAT CGT TAT TGC CAC GA-3′. Those used for the C5aR were: forward, 5′-GGA TCC AAC TCC TTC AAT TAT ACC ACC CCT GAT TAT G-3′ and reverse, 5′-GGC GCT GAT GGT GGC CAG GAG CAG GAT GCT-3′; those for the C5L2 were: forward, 5′-AAC GAT TCT GTC AGC TAC GAG TAT GGG-3′ and reverse, 5′-CGT AGA CCA CCA GGC AGG CCC GAG A-3′. Amplification was performed for 35 cycles. DNA derived from the PCR was cloned into the plasmid pCR II (Invitrogen) and was sequenced using a capillary ABI 3730 sequencer (Retrogen).

FACS analysis

FACS detection of C3aR and C5aR followed a general FACS protocol as previously described using anti-human C3aR Ab (BD Pharmingen) and FITC-conjugated anti-mouse IgG as the secondary Ab (Upstate Biotechnology) or C5aR Ab (BD Pharingen) and FITC-conjugated anti-rabbit IgG (Upstate Biotechnology). Isotype controls were from BD Biosciences. FACS analysis was performed on an LSR II (BD Biosciences) with FACSDiva software.

Confocal microscopy

Cells were cultured on collagen-coated glass coverslips, stimulated for the indicated times with 300 nM C3a or 100 nM C5a at 37°C, fixed with 4%

paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. Fc receptors on monocytes were blocked with 1/100 dilution of human plasma. After blocking with 2% FCS for 30 min at room temperature, cells were incubated with anti-C3aR or anti-C5aR for 2 h, followed by staining with Alexa Fluor 488 anti-mouse or anti-rabbit IgG Ab (Invitrogen) in 2% FCS for 1 h at room temperature. Cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 10 min and washed three times with PBS before mounting with AntiFade (Invitrogen). Images were taken on an Olympus FV1000 confocal microscope.

To detect actin polymerization, MSCs were serum-starved overnight, stimulated with 300 nM C3a or 100 nM C5a for 20 min, fixed, and permeabilized as just described. Polymerized actin was detected with Alexa Fluor 488-phalloidin (Invitrogen) as previously described (26), and confocal images were taken.

Transwell chemotaxis assay

A single-cell suspension of MSCs was loaded into the upper wells of 0.15% gelatin-coated Transwells (Costar; 8-μm pore size, 2×10^4 cells/insert). The lower wells contained no stimulus, a dose-response range of C3a, C5a, or bFGF (positive control; Santa Cruz Biotechnology) in media containing 0.2% BSA, but no growth factors. The assembled wells were incubated for 16 h in a tissue culture incubator, cells in the upper compartment were carefully removed, the filters were stained with DAPI, and the transmigrated cells were counted on a Nikon Eclipse TE200 inverted fluorescence microscope with a SPOT camera system (Diagnostics Instruments). Background chemotaxis of unstimulated cells was defined as 100%.

MSC protection from H_2O_2-mediated cell death

To determine protection from cell death, MSCs (5×10^3cells/well) were seeded into 12-well tissue culture plates in MesenPRO RS media containing growth supplements (Invitrogen). At 24 and 48 h, 300 nM C3a, 100 nM C5a, or 20 ng/ml bFGF were added to the wells followed by 100 μM H_2O_2 at 49 h. At 72 h, total cell numbers were counted and cell viability was assessed using a FITC-annexin V staining kit (Calbiochem) and FACS analysis.

Immunoblotting

Cell cultures were stimulated with C3a or C5a as described in the figure legends, then lysed with modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.4), 10% glycerol, 1% Nonidet P-40, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 0.2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 mM sodium pyrophosphate, 2 mM sodium vanadate, and 10 mM NaF), and clarified by centrifugation. The cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked with 4% dry milk in TBS-Tween 20, and exposed to specific primary Abs as described for each experiment. Ab binding was detected using HRP-conjugated goat anti-rabbit or anti-mouse secondary Abs and ECL (ECL Plus; GE Healthcare). Phosphoblots were reprobed with a second Ab (e.g., anti-ERK1/2 Ab) to assure equal loading. UN-SCAN-IT gel digitizing software (Silk Scientific) was used to quantify results.

Subcellular fractionation

To isolate nuclear fractions, cells were vortexed in hypotonic buffer (10 mM HEPES (pH 8.0), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM DTT, 0.2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin) containing 0.5% Nonidet P-40, then microfuged at highest speed for 2 min. Supernatants representing the cytoplasmic fraction were retained, the pellets were washed twice with the same buffer, then resuspended in buffer B (20 mM HEPES (pH 8.0), 250 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM Na_3VO_4, 1 mM DTT, 0.2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin), incubated on ice for 15 min, and followed by another 2 min centrifugation at 4°C. The supernatants (nuclear fraction) were transferred to clean tubes containing 4× Laemmli sample buffer, boiled, and separated on SDS gels. The purity of the fractions was confirmed by Western blotting using lamin B as a nuclear marker and β-actin as a cytoplasmic marker.

Results

Expression of C3aRs and C5aRs by MSCs

Since the ability to migrate is an important function of MSCs, and because C3a and C5a, known chemotaxins for leukocytes, are generated at sites of trauma and infection, we tested MSCs for the expression of receptors for the anaphylatoxins C3a and C5a. Application of RT-PCR showed that MSCs expressed both the C3aR and the C5aR, but apparently not the nonsignaling C5a-binding receptor C5L2 (Fig. 1*A*). DNA sequencing verified that the two PCR products were identical to the published C3aR and C5aR sequences. Expression of the respective proteins could be shown by Western blotting (Fig. 1*B*), and finally cell surface expression was determined by FACS (Fig. 1*C*). Cell surface C3aRs and C5aRs were expressed in moderately high levels by the entire cell population (Fig. 1*C*), in contrast to cell surface expression of several chemokine receptors such as CXCR4, where a small fraction of MSCs expressed high levels of the receptor, but most cells expressed none (53). Following stimulation with C3a or C5a, surface expression of the respective receptor decreased due to receptor internalization (Fig. 1*D*), as has been described previously for C3aRs and C5aRs expressed by leukocytes (54, 55).

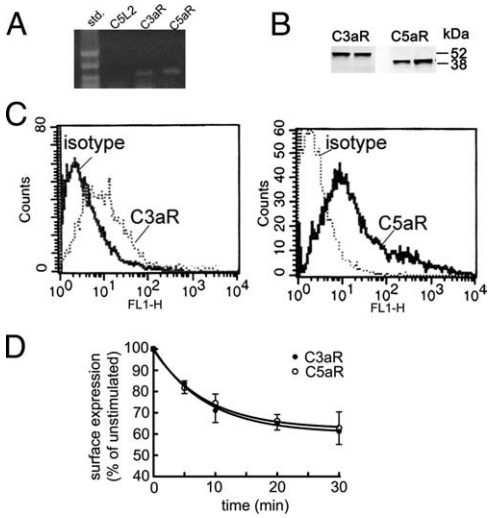

FIGURE 1. Expression of the C3aR and the C5aR by MSCs. *A*, RT-PCR of the C3aR and the C5aR. RNA was purified and reverse transcribed from MSCs as described in *Materials and Methods*. Products for the C3aR and the C5aR were consistently detected. *B*, Detection of the C3aR and C5aR protein by Western blotting. Cell lysates were prepared from MSCs and immunoblotted as described in *Materials and Methods*. One experiment representative of three is shown. *C*, Detection of cell surface C3aR and C5aR by FACS. MSCs were detached with cell dissociation buffer and stained with anti-C3aR/FITC-anti-mouse IgG or anti-C5aR/FITC-anti-rabbit IgG Ab and analyzed on an LSR II with FACSDiva software. One experiment representative of three is shown. *D*, Internalization of the C3aR and the C5aR following ligand activation. MSCs were stimulated with 300 nM C3a or 100 nM C5a for the indicated times at 37°C. Cell surface receptor expression was detected by FACS as described for *C*. The mean channel minus the mean channel of the isotype control was defined as 100% cell surface expression. Means ± SD of two experiments in duplicates are shown.

C3a and C5a mediated MSC migration

Since C5a and to a limited capacity C3a are chemotactic factors for a variety of cells, including neutrophils, eosinophils, monocytes, mast cells, and fibroblasts, it was next asked whether C3a and C5a could serve as chemotactic factors for MSCs, which could be an important physiological means of directing MSCs to an area of tissue injury, where complement activation occurs. First, actin polymerization, which is a precondition for cell migration, was detected by staining with Alexa Fluor 488-phalloidin. Since it was difficult to obtain complete quiescence in MSCs even with prolonged serum starvation, some stress fiber formation was evident even in unstimulated cells. However, in the presence of C3a, the

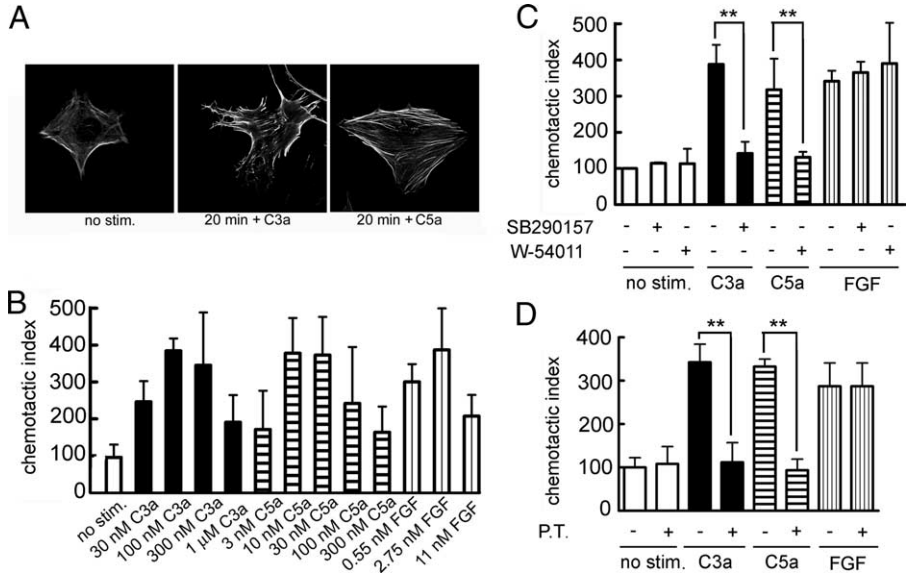

FIGURE 2. Cell migration of C3a- and C5a-stimulated MSCs. *A*, Actin polymerization in MSCs stimulated with 300 nM C3a or 100 nM C5a for 20 min at 37°C. Polymerized actin was stained with Alexa Fluor 488-phalloidin and images were taken with an Olympus FV1000 confocal microscope. MSCs stimulated with C3a showed pronounced lamellipodia and filopodia formation indicative of cells with high motility. *B*, Chemotaxis induced by C3a and C5a in MSCs. Chemotaxis was determined using 8-μm pore size Transwells as described in *Materials and Methods*. bFGF was used as a positive control. Means ± SD of four experiments in duplicate are shown. Chemotaxis values of all concentrations of C3a and C5a were statistically different from those of unstimulated cells (Student's *t* test, $p < 0.01$). *C*, Effect of specific inhibition of the C3aR and the C5aR on cell migration. MSCs were incubated in the presence or absence of SB290157 or W-54011 and chemotaxis was determined as in *B*. Means ± SD of triplicate samples are shown. **, $p < 0.01$ and *, $p < 0.05$ (Student's *t* test). *D*, Effect of inhibition of G_i on C3a- and C5a-mediated cell migration. MSCs were treated with pertussis toxin and chemotaxis was determined as in *B*. Means ± SD of triplicate samples are shown. **, $p < 0.01$ and *, $p < 0.05$ (Student's *t* test).

additional formation of lamellipodia and filopodia became apparent as shown for a 20-min incubation time in Fig. 2*A*, indicative of increased cell motility. While C5a caused some increase in fluorescence intensity (Fig. 2*A*), lamellipodia and filopodia formation were less pronounced. To determine whether the cytoskeletal response was associated with enhanced cell migration, chemotaxis of MSC toward the anaphylatoxins C3a and C5a was determined next using bFGF as the positive control (5). Both C3a and C5a were chemoattractants for MSCs (Fig. 2*B*) and showed the typical bell-shaped chemotaxis response, where chemotaxis reaches a maximum and decreases again with very high ligand concentrations (Fig. 2*B*). When the chemotactic response induced by optimal concentrations of C3a and C5a was compared with the chemotactic response to the known MSC chemoattractant bFGF at its optimal concentration, transmigration of at least as high a percentage of cells was observed with the anaphylatoxins. Although higher concentrations of C3a and C5a (than of bFGF) were necessary for an optimal response, the concentrations used are physiological concentrations of these factors considering that the plasma concentration of C3 is 1 mg/ml and that of C5 is 100 μg/ml.

Chemotaxis was specifically mediated by the C3aR and the C5aR as shown by the inhibitory effect of the specific C3aR inhibitor SB290157 and the specific C5aR inhibitor W-54011 on C3a- or C5a-mediated chemotaxis (Fig. 2*C*). This inhibition could not be attributed to any nonspecific effect of the inhibitors, as bFGF-mediated chemotaxis was not affected by SB290157 or W-54011 (Fig. 2*C*). Both the C3aR- and the C5aR-mediated responses were coupled to G_i, as indicated by the blockade of chemotaxis in the presence of pertussis toxin (Fig. 2*D*), which inhibited C3a- and C5a-mediated cell migration, but not the cell migration observed in the presence of bFGF (Fig. 2*D*), indicating that the C3aR and the C5aR in MSCs were coupled to the same G protein as in leukocytes.

C3a and C5a protect MSC from oxidative damage

C3a and C5a are chemoattractants for MSCs in vitro. Translated into the in vivo situation one would expect that the anaphylatoxins would recruit MSCs to areas of tissue injury, where these cells would encounter the production of oxidants due to neutrophil recruitment and reperfusion injury. It was therefore asked whether C3a or C5a would protect MSCs from oxidative damage. Pretreatment of MSCs with C3a or C5a decreased the extent of cell death in MSCs exposed to 100 μM H_2O_2 significantly (Fig. 3). Although a slight protection was also seen with bFGF, this attenuation did not reach statistical significance (Fig. 3).

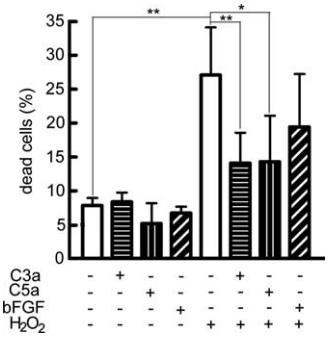

FIGURE 3. Attenuation of oxidative damage in MSCs stimulated with C3a or C5a. Effect of 300 nM C3a or 100 nM C5a on cell death caused by 100 μM H_2O_2. MSCs were treated as described under *Materials and Methods*, and cell viability was assessed by detecting the percentage of annexin V-positive cells. Means ± SD of three experiments are shown. **, $p < 0.01$ and *, $p < 0.05$ (Student's *t* test).

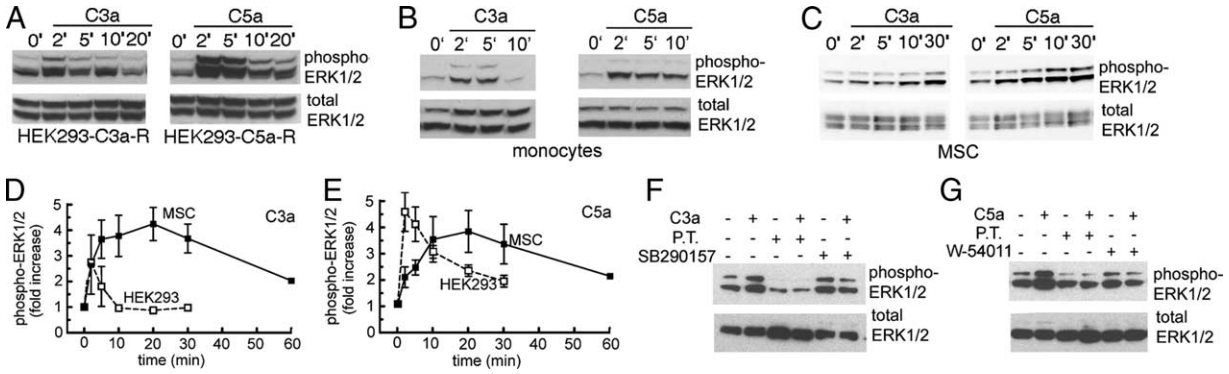

FIGURE 4. ERK1/2 phosphorylation in C3a- and C5a-stimulated MSCs, HEK293 cells, and monocytes. HEK293 cells stably expressed C3aR-GFP or C5aR-GFP. Cells were serum-starved overnight, then stimulated with 300 nM C3a or 100 nM C5a for the indicated times. Whole cell lysates were separated by SDS-PAGE, and ERK1/2 phosphorylation was determined by immunoblotting with antiphospho-ERK1/2 Ab (*top*) followed by total ERK1/2 detection (*bottom*) to ensure equal loading. *A*, Time course of ERK phosphorylation in whole cell lysates of HEK293 cells expressing the C3aR or the C5aR as indicated. Note that the response to C3a is considerably shorter than for C5a. *B*, Time course of ERK phosphorylation in whole cell lysates of monocytes stimulated with C3a or C5a as indicated. *C*, Time course of ERK phosphorylation in whole cell lysates of MSCs stimulated with C3a or C5a indicating a more prolonged response for both ligands in these cells. *D*, Quantification of the response to C3a in HEK293-C3aR-GFP cells and MSCs. Means ± SD of three experiments are shown. *E*, Quantification of the response to C5a in HEK293-C5aR-GFP cells and MSCs. Means ± SD of three experiments are shown. *F*, Inhibition of the response to C3a, which was added for 15 min, by pertussis toxin (P.T.) and SB290157. One experiment representative of three is shown. *G*, Inhibition of the response to C5a, which was added for 15 min, by pertussis toxin (P.T.) and W-54011. One experiment representative of three is shown.

C3a and C5a caused prolonged ERK1/2 phosphorylation in MSCs

As C5a is usually a more effective chemoattractant than C3a (10, 29), it was surprising that equal numbers of cells migrated toward C3a as toward C5a, and we thus asked whether C3a caused generally more prolonged signaling events in MSCs than in other cell types. To assess this, C3a-mediated ERK1/2 phosphorylation was determined in MSCs, in monocytes, and in HEK293 cells expressing the C3aR or the C5aR. Monocytes were used because they have been widely employed to determine the effect of the anaphylatoxins, and HEK293 cells were chosen because they are used extensively to determine cell signaling induced by activation of transfected receptors. C5a stimulation was used for comparison. Preliminary results had shown that 300 nM C3a and 100 nM C5a resulted in a maximal ERK1/2 response, which could not be further augmented with higher ligand concentrations, and these concentrations were therefore used in all of the signaling experiments. As anticipated, ERK1/2 phosphorylation was short-lived in HEK293 cells and monocytes stimulated with C3a (Fig. 4, *A* and *B*), which was consistent with a previous report on mast cells (56). In contrast, ERK1/2 phosphorylation was prolonged in C3a-stimulated MSCs (Fig. 4*C*). C5a induced prolonged ERK1/2 phosphorylation in all of the tested cell types (Fig. 4*A–C*), consistent with previous reports in other cell types, where C5a activation of various signaling pathways was more protracted than that caused by C3a (26). Quantification of the ERK1/2 phosphorylation in HEK293 cells and MSCs indicated that the response to both anaphylatoxins was longer lasting in MSCs (Fig. 4, *D* and *E*), but that the difference in the response was much more prominent for C3a. The response to C3a and C5a in MSCs was due to receptor coupling to G_i, as demonstrated by inhibition in the presence of pertussis toxin (Fig. 4, *F* and *G*) and mediated by the specific C3aR and C5aR, as it could be blocked with SB290157 or W-54011, respectively (Fig. 4, *F* and *G*). Although C3a failed to induce ERK1/2 phosphorylation in the presence of SB290157, SB290157 itself caused moderate ERK1/2 phosphorylation (Fig. 4*F*), which is consistent with an earlier report that indicated that SB290157 can be a partial agonist of the C3aR in the absence of ligand (57). Even if this activation in the absence of ligand was not the originally anticipated result, it nevertheless points to the involvement of the specific C3aR in mediating the ERK1/2 response.

Nuclear translocation of phospho-ERK1/2 in C3a- and C5a-stimulated MSCs

Since activation of ERK1/2, especially if it is prolonged, can result in the translocation of ERK1/2 to the nucleus, we next asked whether C3a and C5a were able to cause translocation of phospho-ERK to the nucleus and whether this response was more pronounced in MSCs with their more prolonged response, especially to C3a. This was an important question, since nuclear ERK1/2 translocation is required for G_1 phase cell cycle progression (58) and the activation of several transcription factors (59). As shown in Fig. 5, nuclear ERK1/2 phosphorylation in HEK293-C3aR-GFP cells stimulated with C3a was negligible (Fig. 5*A*). In contrast, C5a caused nuclear translocation of ERK1/2 in HEK293-C5aR-GFP cells (Fig. 5*B*). In MSC both C3a and C5a caused ERK1/2 phosphorylation in the nucleus and in the cytoplasm (Fig. 5, *C* and *D*). Quantification of these results again indicated the transience of the response to C3a in HEK293 cells compared with the prolonged and more robust response in MSCs seen both in the nucleus and in the cytoplasm (Fig. 5, *E* and *G*). In contrast, C5a induced similar kinetics for nuclear and cytoplasmic ERK phosphorylation in both cell types (Fig. 5, *F* and *H*). In agreement with these results, phosphorylation of Elk, which is a transcription factor activated by nuclear phospho-ERK1/2, was seen in C3a-stimulated MSCs (Fig. 5*I*), and to a lesser degree in C5a-stimulated MSCs (Fig. 5*I*), but not in HEK293 cells (results not shown).

Prolonged Akt phosphorylation in C3a- and C5a-stimulated MSCs

Since Akt activation has been shown to be another important signaling pathway for MSCs (7, 60), Akt phosphorylation was also assessed. Again, Akt phosphorylation was transient in HEK293 cells stimulated with C3a (Fig. 6, *A* and *C*), but it was prolonged in MSCs (Fig. 6, *B* and *D*). Again, C5a caused prolonged Akt phosphorylation in both cell types (Fig. 6*A–D*). As shown above for other functions, Akt phosphorylation was due to activation of the specific C3aR and C5aR as indicated by its inhibition in the

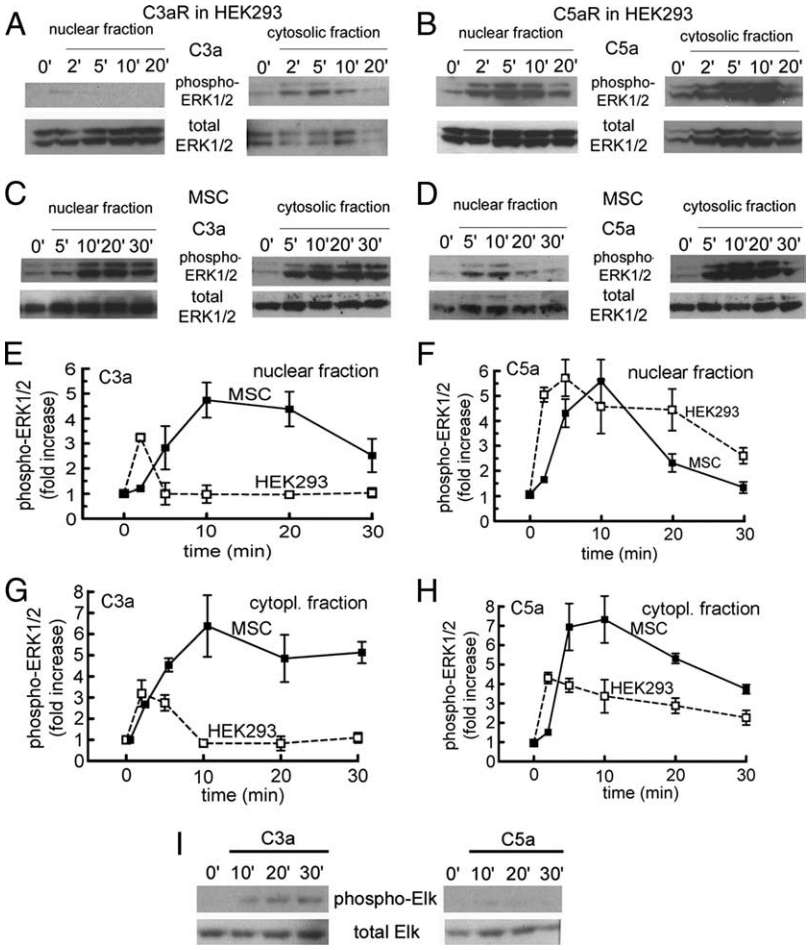

FIGURE 5. C3a and C5a cause nuclear translocation of ERK1/2, resulting in phosphorylation of the transcription factor Elk. Western blots of nuclear and cytoplasmic fractions were prepared as described in *Materials and Methods* and probed with anti-phospho-ERK1/2 and anti-total-ERK1/2. *A*, Time course of C3a-induced ERK1/2 phosphorylation in nuclear and cytoplasmic fractions prepared from HEK293-C3aR-GFP cells stimulated with 300 nM C3a. *B*, Time course of C5a-induced ERK1/2 phosphorylation in nuclear and cytoplasmic fractions prepared from HEK293-C5aR-GFP cells stimulated with 100 nM C5a. *C*, Time course of C3a-induced ERK1/2 phosphorylation in nuclear and cytoplasmic fractions prepared from MSCs stimulated with 300 nM C3a. *D*, Time course of C5a-induced ERK1/2 phosphorylation in nuclear and cytoplasmic fractions prepared from MSCs stimulated with 100 nM C5a. *E*, Quantification of the response to C3a in the nuclear fraction of HEK293-C3aR-GFP cells and MSCs. Means ± SD of three experiments are shown. *F*, Quantification of the response to C5a in the nuclear fraction of HEK293-C5aR-GFP cells and MSCs. Means ± SD of three experiments are shown. *G*, Quantification of the response to C3a in the cytoplasmic fraction of HEK293-C3aR-GFP cells and MSCs. Means ± SD of three experiments are shown. *H*, Quantification of the response to C5a in the cytoplasmic fraction of HEK293-C5aR-GFP cells and MSCs. Means ± SD of three experiments are shown. *I*, Western blots of nuclear extracts prepared from MSCs stimulated with 300 nM C3a or 100 nM C5a were probed with anti-phospho-Elk and reprobed with anti-total Elk Ab. One experiment representative of three is shown.

presence of SB290157 or W-54011 (Fig. 6, *E* and *F*), and for this signaling pathway SB290157 did not show any agonistic effect on its own. As anticipated, pertussis toxin inhibited the response to both C3a and C5a (Fig. 6, *E* and *F*).

In summary, several activation pathways showed prolonged responses in C3a-stimulated MSCs compared with other cell types, while the response to C5a was similar in different cell types.

Nuclear translocation of the C3aR in C3a-stimulated MSCs

When expression and internalization of the C3aR was verified by confocal microscopy using an anti-C3aR Ab, the C3aR was detected on the cell surface before stimulation (Fig. 7*A*), as was expected from the FACS results (Fig. 1*C*). Following C3a stimulation, the C3aR was internalized but was not detected in the

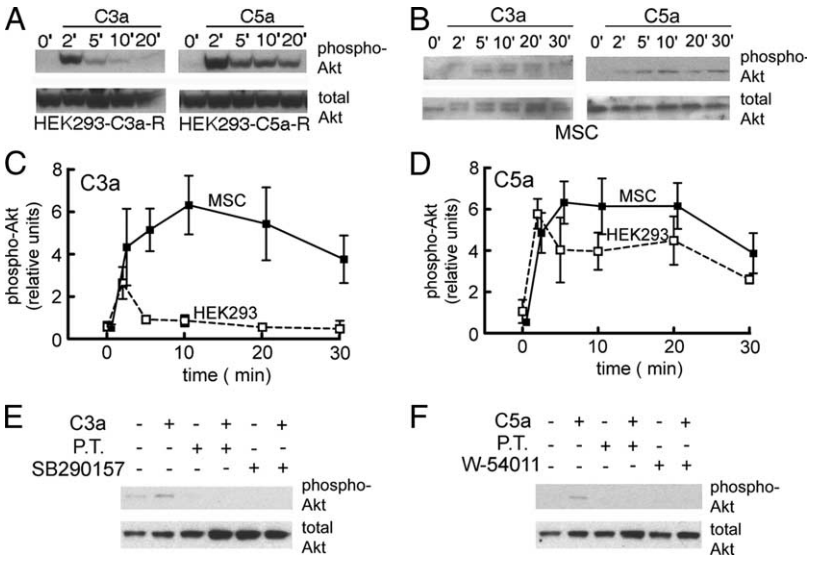

FIGURE 6. Akt phosphorylation in C3a- and C5a-stimulated HEK293 cells expressing the C3aR-GFP or C5aR-GFP and in MSCs. Cell lysates and immunoblots were prepared as described in Fig. 4, but anti-phospho-Akt and total Akt were used as the primary Abs. *A*, Time course of Akt phosphorylation in HEK293 cells. *B*, Time course of Akt phosphorylation in MSCs. *C*, Quantification of Akt phosphorylation in MSCs and HEK293-C3aR-GFP cells stimulated with C3a. Means ± SD of three experiments are shown. *D*, Quantification of Akt phosphorylation in MSCs and HEK293-C5aR-GFP cells stimulated with C5a. Means ± SD of three experiments are shown. *E*, Inhibition of the response to C3a, which was added for 15 min, by pertussis toxin (P.T.) and SB290157. One experiment representative of three is shown. *F*, Inhibition of the response to C5a, which was added for 15 min, by pertussis toxin (P.T.) and W-54011. One experiment representative of three is shown.

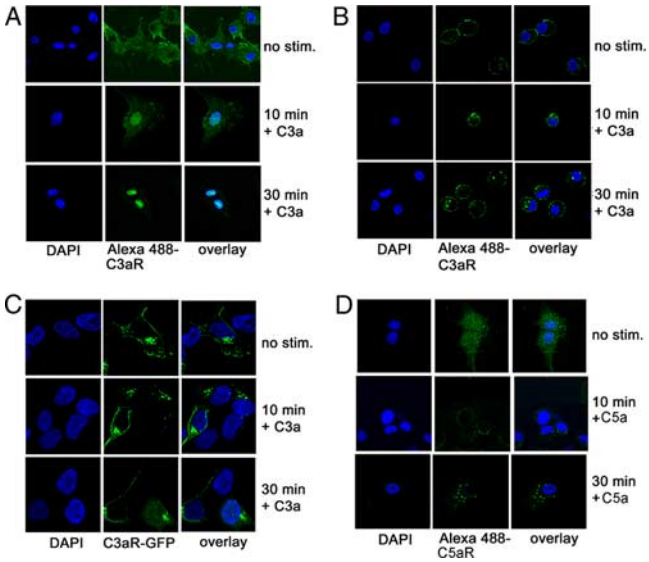

FIGURE 7. Nuclear translocation of the C3aR in MSCs. C3aR internalization in MSC, monocytes, and HEK293 cells was determined by confocal microscopy. Monocytes were used for comparison because C3aR activation is best documented for myeloid cells (11, 14), and HEK293 cells were used because they are among the most commonly used cells to determine the function of transfected receptors. The different cell types were stimulated with 300 nM C3a or 100 nM C5a for the indicated times and stained as described for each panel. Following counterstaining with DAPI, confocal images were acquired on an Olympus FV1000 microscope with a ×60 oil immersion lens. *A*, Internalization of the C3aR in MSCs. The C3aR was stained with mouse anti-C3aR/anti-mouse Alexa Fluor 488-IgG (Invitrogen) as described in *Materials and Methods*. By 10 min a large portion of the C3aR colocalized with DAPI in the nucleus and was retained there for at least for 30 min. One experiment representative of five is shown. *B*, Internalization of the C3aR in monocytes, which were stained as described in *A*. Following C3a stimulation, a portion of the C3aR was internalized into endocytic vesicles, but not into the nucleus. *C*, Internalization of the C3aR in HEK293 cells expressing C3aR-GFP. As in the monocytes, the C3aR was internalized into endocytic vesicles although there were occasional cells that showed faint translocation to the nucleus as, for example, the cell in the lower right quadrant of the 30 min time point. *D*, Internalization of the C5aR in MSC. The C5aR was stained with rabbit anti-C5aR/anti-rabbit Alexa Fluor 488-IgG (Invitrogen). Following C5a stimulation, the C5aR was internalized into endocytic vesicles.

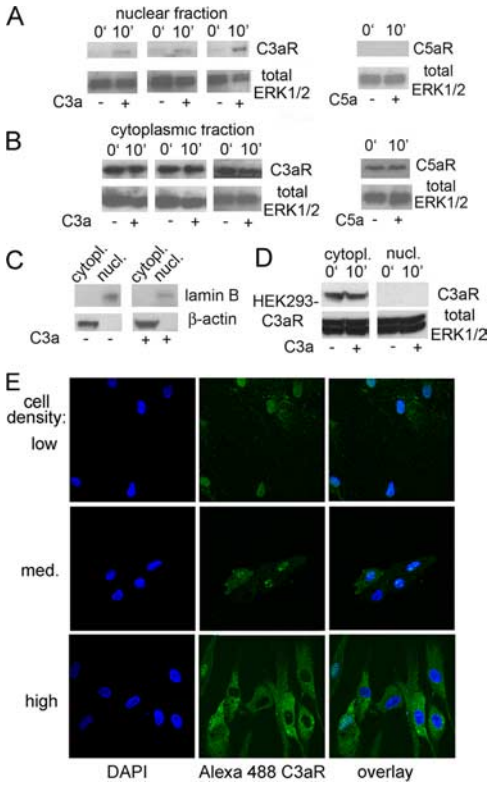

FIGURE 8. Detection of nuclear translocation of the C3aR in MSCs by cell fractionation. MSCs or HEK293 cells expressing C3aR-GFP were stimulated with 300 nM C3a for 20 min. Nuclear extracts were prepared as described in *Materials and Methods*, and nuclear and cytoplamic fractions were resolved by SDS-PAGE. Western blots were developed using anti-C3aR Ab, followed by reprobing with total anti-ERK. *A* and *B*, Western blot detecting the C3aR in nuclear (*A*) and cytoplasmic fractions (*B*) of MSCs, indicating nuclear translocation of the C3aR. *C*, To assure the purity of the nuclear and cytoplasmic fractions from MSCs, Western blots of both fractions were probed with anti-lamin B, stripped, and reprobed with anti-β-actin as indicated. *D*, Western blot detecting the C3aR in nuclear and cytoplasmic fractions of HEK293-C3aR-GFP cells, where no nuclear translocation could be detected. *E*, Confocal microscopy images of MSCs stimulated with C3a for 20 min. Note that nuclear translocation of the C3aR is cell density-dependent and much more prominent at low cell density.

intracellular endosomal compartment, as is generally seen when GPCRs are stimulated with their ligand, but rather it was translocated to the nucleus (Fig. 7*A*), where it largely colocalized with DAPI. This was unexpected, since the C3aR in monocytes was internalized into endocytic vesicles (Fig. 7*B*). Furthermore, in HEK293 cells transfected with a C3aR-GFP construct, C3a stimulation also caused internalization of the C3aR-GFP in endocytic vesicles (Fig. 7*C*), although there were occasional cells in which a minor fraction of the receptor appeared in the nucleus (Fig. 7*C*). Such different receptor internalization routes depending on the cellular make-up are a highly unusual observation, which may account for the observed effects.

When MSCs were stimulated with C5a followed by immunofluorescence staining for the C5aR, no nuclear translocation of the receptor was detected, but immunostaining of endocytic vesicles was observed (Fig. 7*D*). This was also true for monocytes and HEK293 cells expressing C5aR-GFP (results not shown).

To ascertain by biochemical means that the C3aR was translocated to the nucleus of MSCs, MSCs were stimulated with C3a and nuclear extracts were prepared. As shown in Fig. 8*A*, translocation of the C3aR to the nuclear fraction was seen consistently following stimulation with C3a, although the fraction of the receptor that was translocated was not as prominent as expected from the confocal microscopy. The fraction of C3aR translocated to the nucleus in the cell fractionation assay was not large enough to be apparent as a loss of C3aR in the cytoplasmic fraction (Fig. 8*B*); nevertheless, nuclear translocation was observed on all occasions (Fig. 8*A*). Western blots detecting the cytoplasmic marker β-actin and the nuclear protein lamin B indicated that there was no cross-contamination between the two fractions (Fig. 8*C*). In contrast, in HEK293 cells stably expressing C3aR-GFP, no nuclear translocation could be detected (Fig. 8*D*), and the C3aR was retained in the cytoplasmic fraction. This does not contradict the endocytosis observed by confocal microscopy (Fig. 7*C*), since the method used does not discriminate between plasma membrane and the endocytic membrane compartment. This behavior was not due to overexpression of the C3aR, since the small copy number of C3aRs, which these cells express constitutively, was not translocated to the nucleus either (results not shown). One explanation for the more prominent nuclear translocation of the C3aR observed by confocal microscopy in contrast to cell fractionation and Western blotting could be that microscopy depends on the presence of aggregates of

protein. A second possible explanation originally described for the lysophosphatidic acid receptor-1 (61) is that nuclear translocation is cell density-dependent, with less nuclear accumulation at high cell densities, and cultures had to be grown to ~80% confluence for the cell fractionation experiments, but were kept at low densities for the microscopy. When confocal microscopy was performed on MSC cultures grown to different cell densities, it was indeed noted that nuclear translocation of the C3aR was more prominent at lower cell densities (Fig. 8*E*).

Conversely, stimulation of the C5aR did not result in nuclear translocation in MSCs (Fig. 8*D*), consistent with the fact that in contrast to the C3aR, the C5aR does not contain a nuclear localization signal.

Discussion

Although the ability to migrate is a quintessential function of MSCs, without which they could not fulfill their role in tissue repair, there is sparse information on the receptors that are used for this purpose. Only recently has it been reported that MSCs express chemokine receptors (8, 53), and the presence of the anaphylatoxin receptors had not been previously recognized in these cells. Here we could show that human MSCs express functional receptors for C3a and C5a, and that both anaphylatoxins cause a chemotactic response in MSCs, which is an important function of MSCs necessary to attract these cells to an area of tissue injury, where they can contribute to tissue repair.

It is important that both C3a and C5a were chemotactic for MSCs (Fig. 2*B*). Since MSCs, which are ubiquitous in many body tissues, play an important role in tissue repair following injury or any other insult, and since C3a and C5a are produced in areas of tissue injury, it is highly likely that the anaphylatoxins are chemotactic factors for MSCs under a variety of conditions of tissue insult, including injury, infections, various inflammatory diseases, and cancer, and during ischemia/reperfusion (62). However, for C5a this potential beneficial effect is Janus-faced, as it is accompanied by the potent inflammatory response C5a causes (62), such that it would not be desirable to therapeutically augment C5a concentrations. In contrast, C3a fails to induce leukocyte influx in vivo (29), and means of up-regulating C3a concentrations could potentially be useful to attract MSCs for tissue repair. Importantly, the protection from oxidative damage seen in MSCs stimulated with C3a or C5a could be of physiological relevance, since MSCs are attracted to areas of tissue damage, where they would be exposed to an oxidative milieu.

Chemotaxis induced by C3a and C5a was mediated by the specific C3aR and C5aR as indicated by the ability to block this response with receptor-specific inhibitors (Fig. 2*C*). Although SB290157 had some agonistic effect of its own, specifically of ERK1/2 phosphorylation, which would make it an undesirable inhibitor for in vivo studies, its effect on MSCs only strengthens the conclusion that C3a acts through the C3aR in these cells, since SB290157 is specific for this receptor (49). This effect of SB290157 differed from the situation in hematopoietic stem and progenitor cells, where the C3a-mediated response could apparently not be blocked by SB290157 (63). However, there may still be parallels between the response to C3a in MSCs and hematopoietic stem and progenitor cells: C3a increased the sensitivity to SDF-1 in hematopoietic stem and progenitor cells, thus contributing to the retention of these cells in the bone marrow niche (64). It is certainly a possibility that a similar cooperation between C3a and SDF-1 exists in MSCs with potentially the same biological consequence of retaining MSCs within the bone marrow under homeostatic conditions.

Apart from the effect of the anaphylatoxins on cell migration of MSCs, the most intriguing observation made was the demonstration that activation of the C3aR in MSCs causes nuclear translocation of the C3aR (Figs. 7 and 8), an observation that is consistent with the presence of a nuclear translocation signal located near the C terminus in this receptor. However, in contrast, in monocytes, which constitutively express the C3aR, and in HEK293 cells, which were transfected with a C3aR-GFP construct, C3a stimulation led to receptor accumulation in the intracellular endosomal compartment (Fig. 7). In this intracellular endosomal compartment the C3aR colocalized with β-arrestin (I. U. Schraufstatter, R. G. DiScipio, M. Zhao, and S. K. Khaldoyanidi, unpublished results). It has been described for a few GPCRs, including the angiotensin II type 1 receptor (AT_1R) and the somatostatin receptors, which are expressed in the plasma membrane, that ligand stimulation can lead to translocation of the receptor to the nucleus (43, 46). However, it is highly unusual that the trafficking of the C3aR depended on the specific cell type in which the receptor was expressed, and we are aware of only one precedence of such differential GPCR trafficking; that is, the apelin receptor was translocated to the nucleus in neuronal cells, but not in HEK 293 cells (41), which was not due to any cell-specific defect in HEK293 cells, since the bradykinin receptor was translocated to the nucleus in these cells (41). It is interesting that the C3aR can traffic to different intracellular structures depending on the specific cell type in which the receptor is expressed, and it is intriguing that the cellular make-up of a cell can determine what the fate of the activated C3aR and the downstream signaling sequelae will be. It remains to be determined whether there is something specific to the stem cell nature of MSCs that contributes to the nuclear translocation of the C3aR in these cells. Nuclear translocation was more prominent at lower cell concentrations, but since MSCs are rare cells in the bone marrow niche, one would hypothesize that C3a stimulation in vivo may well result in nuclear translocation of the receptor. This distinct behavior in MSCs also deserves further investigation at the molecular level, especially since Ser^{449}, next to the nuclear localization signal sequence, was shown to be important for G protein coupling (65), and the C3aR in MSCs was coupled to G_i, as indicated by the inhibition with pertussis toxin. This nuclear localization signal sequence is also in close proximity to serine and threonine residues in the C terminus of the C3aR, which are phosphorylated following ligand activation and play a role in β-arrestin association, signal termination, and receptor internalization (65).

Nuclear translocation of the C3aR in MSCs correlated with prolonged phosphorylation and nuclear translocation of ERK1/2 and with phosphorylation of the transcription factor Elk. In contrast, in other cell types that were tested the activated C3aR was retained in endocytic vesicles, and the cell signaling pathway activation was much more transient. Further investigation is warranted to determine if the nuclear translocation of the C3aR is responsible for the prolonged response to C3a seen in MSCs. Although uncommon, nuclear localization, constitutive or following ligand binding, has been described for a limited number of GPCRs (41, 44, 46, 66). While GPCR activation at the plasma membrane generally leads to short-term signaling events, nuclear GPCR translocation causes long-term effects, including nuclear ERK phosphorylation (46), transcriptional activation (66), cell proliferation, and differentiation (42, 43, 67), all of which are important for MSC biology. Prolonged ERK1/2 phosphorylation has been associated with transcriptional activation, which is consistent with the nuclear translocation of ERK1/2 observed in MSCs (Fig. 5) and the phosphorylation of Elk. Elk was chosen as a target here because it is directly phosphorylated by activated nuclear ERK1/2, but a more general screen for transcription factor activation is justified.

The response to C3a in MSCs was emphasized in this discussion because it differs from that generally seen with this ligand, while the response to C5a followed expectations for activation of this receptor in other cell types. C5aR lacking a nuclear translocation signal was endocytosed to endoplasmic vesicles after C5a stimulation in all cell types tested, which is the general internalization route for GPCRs, but the response to C5a appears to be intrinsically more prolonged than that to C3a. It is important to recognize that the spatial and temporal regulation of ERK1/2 activation, regulated by the formation of multiprotein signaling complexes, is a means by which the same signaling pathway can be used to result in opposite functional responses, such as proliferation vs senescence (68). In these signaling complexes GPCRs can cross-talk with receptor tyrosine kinases, which serve as a scaffold for the assembly of the proteins of the ERK cascade (69, 70), and the activation of which amplifies the signal. For instance, activation of the C5aR transactivates the EGF receptor in endothelial cells (26). Since MSCs express EGF receptors (71), it is highly likely that this is also the case with MSCs. Additionally, some GPCRs can also activate ERK1/2 independently of G proteins by using β-arrestin as a scaffold associated with the GPCR (72). Since pertussis toxin blocked the response to both C3a and C5a in MSCs, this pathway does not appear to be involved, however.

In various scenarios, activation of ERK1/2 and Akt in MSCs was important for cell proliferation (73) and protection from apoptosis (74). Prolonged inhibition of ERK1/2 has furthermore been shown to lead to adipogenic differentiation (75). Since both C3a and C5a induced prolonged activation of ERK1/2 and Akt in MSCs (Figs. 5–7), we hypothesize that C3a and C5a may be useful as stimuli that improve functions of MSCs that are important for better transplantation results of MSC (e.g., in ischemia models). Such a nongenetic approach that does not involve retroviral vectors would be preferable for clinical applications.

Although Akt activation has been described as such an important signaling pathway in MSCs (76, 77, 78), we found that these cells expressed low levels of Akt when compared with other cell types. However, it has been shown that MSCs grown under hypoxic conditions, greatly up-regulating Akt expression (79). Since the environment of MSCs in their bone marrow niche would be hypoxic, and since MSCs would often be attracted to hypoxic areas following ischemic injury, examination of the combined effect of the anaphylatoxins and hypoxic conditions on MSCs may reveal more pronounced Akt activation.

In summary, stimulation of the C3aR and the C5aR caused chemoattraction of MSCs and prolonged activation of multiple signaling pathways, which indicates that the anaphylatoxins are important for MSC behavior.

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Disclosures

The authors have no financial conflicts of interest.

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