Complement C3a and C5a Modulate Osteoclast Formation and Inflammatory Response of Osteoblasts in Synergism With IL-1β

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ABSTRACT

There is a tight interaction of the bone and the immune system. However, little is known about the relevance of the complement system, an important part of innate immunity and a crucial trigger for inflammation. The aim of this study was, therefore, to investigate the presence and function of complement in bone cells including osteoblasts, mesenchymal stem cells (MSC), and osteoclasts. qRT-PCR and immunostaining revealed that the central complement receptors C3aR and C5aR, complement C3 and C5, and membrane-bound regulatory proteins CD46, CD55, and CD59 were expressed in human MSC, osteoblasts, and osteoclasts. Furthermore, osteoblasts and particularly osteoclasts were able to activate complement by cleaving C5 to its active form C5a as measured by ELISA. Both C3a and C5a alone were unable to trigger the release of inflammatory cytokines interleukin (IL)-6 and IL-8 from osteoblasts. However, co-stimulation with the pro-inflammatory cytokine IL-1β significantly induced IL-6 and IL-8 expression as well as the expression of receptor activator of nuclear factor-kappaB ligand (RANKL) and osteoprotegerin (OPG) indicating that complement may modulate the inflammatory response of osteoblastic cells in a pro-inflammatory environment as well as osteoblast–osteoclast interaction. While C3a and C5a did not affect osteogenic differentiation, osteoclastogenesis was significantly induced even in the absence of RANKL and macrophage-colony stimulating factor (M-CSF) suggesting that complement could directly regulate osteoclast formation. It can therefore be proposed that complement may enhance the inflammatory response of osteoblasts and increase osteoclast formation, particularly in a pro-inflammatory environment, for example, during bone healing or in inflammatory bone disorders. J. Cell. Biochem. 112: 2594–2605, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: COMPLEMENT; C3a; C5a; OSTEOBLAST; MESENCHYMAL STEM CELL; OSTEOCLAST; INTERLEUKIN

There is a steadily increasing interest for the interaction of the skeletal and the immune system, a scientific field known as osteoimmunology [Takayanagi, 2007]. In particular, the crucial cytokines for the crosstalk between bone-forming osteoblasts and bone resorbing osteoclasts, macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-kappaB ligand (RANKL) closely link the bone and the immune system. While M-CSF is a growth factor for macrophages as well as for osteoclast

precursors, RANKL plays a role in T-cell activation and also regulates osteoclast activity [Takayanagi, 2007]. Furthermore, inflammatory cytokines, for example, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF), enhance the activity of osteoclasts and modulate bone regeneration [Bar-Shavit, 2007; Nakashima and Takayanagi, 2009]. Whereas the role of cytokines in bone is being extensively explored, little is known about the relevance of the complement system, a crucial part of innate immunity and the main trigger for local and systemic inflammation.

The functions of the complement system include the opsonization of antigens, the lysis of microorganisms, the support of phagocytosis, and the induction of inflammatory reactions. Following tissue damage or detection of pathogen-associated molecular patterns by immune cells, the enzymatic cascade of the complement system consisting of over 30 proteins can be activated by four pathways: classical; lectin; alternative; extrinsic [Ricklin et al., 2010; Ehrnthaller et al., 2011]. All four pathways lead to the production of the central anaphylatoxins C3a and C5a, which are small polypeptides generated by proteolytic cleavage by C3/5 convertases from their respective precursor proteins C3 or C5. C3a and C5a can act via their corresponding receptors C3aR and C5aR, respectively, inducing the migration of phagocytes, degranulation of mast cells, cytokine release, and respiratory burst reaction, as well as regulating apoptosis in inflammatory cells [Flierl et al., 2006; Ehrnthaller et al., 2011]. The excessive activation of complement can also provoke detrimental side effects. Therefore, to strictly control its activation and to protect healthy cells against a complement attack there are many fluid phase complement regulators as well as membrane-bound regulatory proteins (complement receptor 1/CR1/CD35; membrane cofactor protein/MCP/CD46; decay accelerating factor/DAF/CD55, protectin/CD59) [Kim and Song, 2006; Zipfel and Skerka, 2009].

There is evidence for complement playing a regulative role in bone biology. The starting enzyme of the classical pathway, C1s, which, after activation by C1r, cleaves C2 and C4 to form C3 convertase, was found in hypertrophic chondrocytes in the primary ossification center of human and hamster femurs. Due to its potency in degrading collagen type I and II, C1s was suggested to participate in cell disintegration and matrix degradation during enchondral bone formation [Sakiyama et al., 1994, 1997]. Andrades et al. [1996] reported the presence of C3, C5, C9, and factor B in the growth plate of rats and reasoned that cartilage-bone transformation might be influenced by the alternative pathway. It was shown that osteoblasts generate C3 in response to $1\alpha,25$-dihydroxyvitamin D_3 [Sato et al., 1991] and that C3 could potentiate osteoclast differentiation, indicating a regulatory function in osteoblast–osteoclast interaction [Sato et al., 1993; Tu et al., 2010]. Furthermore, the key receptor C5aR was expressed in osteoblast-like osteosarcoma cells (MG-63) and modulated the expression of IL-6 [Pobanz et al., 2000]. Recently, we demonstrated that C5aR was expressed in a distinct spatial and temporal pattern by osteoblasts, osteoclasts, and chondroblasts in the fracture callus during intramembranous and enchondral ossification [Ignatius et al., 2011]. We found that C5aR was strongly up-regulated during osteogenic differentiation and that its activation by C5a-induced osteoblast migration suggesting that complement might regulate their recruitment during bone healing [Ignatius

et al., 2011]. These findings support the hypothesis of complement playing a crucial role in bone formation.

To further elucidate the role of complement in bone cells, we investigated the expression of receptors (C3aR, C5aR), anaphylatoxin precursors (C3, C5), and complement regulatory proteins (CD46, CD55, CD59) in human mesenchymal stem cells (MSC), in osteoblasts, as well as in osteoclasts. We studied whether osteoblasts and osteoclasts could directly activate complement by cleaving C3 and C5 and whether C3a and C5a could induce an inflammatory response in these cells by inducing the production and release of inflammatory cytokines. Furthermore, we asked whether C3a and C5a could induce osteoclast formation directly or indirectly via modulation of RANKL and osteoprotegerin (OPG) expression in osteoblasts.

MATERIALS AND METHODS

CULTIVATION OF HUMAN MSC, OSTEOBLASTS, AND OSTEOCLASTS

The isolation of human primary cells was approved by the Ethical Committee of the University of Ulm, Germany (01/08).

MSC were isolated from bone marrow aspirates obtained from surgical procedures on five male, donors (aged 20–38 years) by density gradient centrifugation and adhesion to tissue culture plastic material [Pittenger et al., 2000]. The cells were cultured in a basal medium consisting of Dulbecco's modified Eagle's medium (DMEM, Biochrom, Germany) containing 10% fetal calf serum (FCS; Cambrex, Belgium), 4 mM L-glutamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Biochrom). The isolation procedure is specific for adherent MSC and maintains their progenitor phenotype [Pittenger et al., 1999; Fickert et al., 2004; Dominici et al., 2006]. MSC of passages 2 or 3 were used for the experiments. To promote osteogenic differentiation 1×10^4 MSC/cm^2 were seeded in 24-well tissue culture plates or in 175 cm^2 flasks (Nunc, Wiesbaden, Germany) in basal medium supplemented with 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 0.2 mM ascorbate-2-phospate (all from Sigma–Aldrich, Germany). Von Kossa staining of calcium deposition and alkaline phosphatase staining (86R-1KT, Sigma–Aldrich) were used to confirm osteogenic differentiation. To investigate if osteogenic differentiation was influenced by the presence of anaphylatoxins, 1 μg/ml C3a or 0.1 μg/ml C5a were added to the differentiation medium. The concentrations were chosen according serum levels in patients with posttraumatic systemic inflammation [Gebhard et al., 2000].

Osteoblasts were isolated according to Robey and Termine [1985] from bone samples (femur or tibia) of four male and one female patients (aged 23–70 years) undergoing surgery for fracture repair. Briefly, bone pieces were subjected to collagenase digestion and cell isolation was performed in calcium-free DMEM to inhibit fibroblast growth. At sub-confluence, cells were sub-cultured by 0.05% trypsin/0.02% EDTA (Biochrom) or Accutase^{TM} (PAA, Germany) treatment. For expansion 5,000 cells/cm^2 were seeded in DMEM supplemented with 10% FCS (Biochrom), 4 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C, 8.5% CO$_2$, and saturated humidity. Cells of passages 3 or 4 were used for the experiments following culture for 14 days in osteogenic medium described above.

Osteoclasts were generated from peripheral blood mononuclear cells (PBMNC) isolated from blood samples of four male, healthy donors (aged 29–35 years) by density gradient centrifugation as previously described [Kreja et al., 2010]. Briefly, 5×10^5 PBMNC/cm^2 were seeded in 96-well tissue culture plastic plates (Nunc) in α-medium (Biochrom) supplemented with 10% selected FCS (Gibco, Germany), 4 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin (Biochrom), 20 ng/ml human recombinant human (rh) RANKL, and 10 ng/ml rh M-CSF (both Chemicon International, USA). The medium was changed twice a week. On day 21 cells were fixed with 4% buffered formaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) using the Acid Phosphatase Leukocyte Kit (Sigma–Aldrich, 387A). Osteoclasts were identified as TRAP-positive multinucleated cells containing at least three or more nuclei by light microscopy at 400-fold magnification [Buckley et al., 2005].

For the investigation of osteoclast resorption activity 5×10^5 PBMNC/cm^2 were cultured in the combined presence of 40 ng/ml RANKL and 20 ng/ml M-CSF in calcium phosphate-coated 16-well slides (BD BioCoat™ Osteologic™ Bone Cell Culture System; Becton Dickinson, Germany). After 28 days the wells were filled with pure water for 24 h to detach the cells and the remaining mineralized surface was visualized via von Kossa staining according to manufacturer instructions.

STIMULATION OF OSTEOBLASTS WITH C3a AND C5a

Osteoblasts were treated with C3a (1 μg/ml medium, Calbiochem, Germany) or C5a (0.1 μg/ml medium, Sigma–Aldrich) as well as with the pro-inflammatory cytokine IL-1β (0.1 ng/ml medium, Sigma–Aldrich) to investigate their ability to respond to inflammatory stimuli. In these experiments osteoblasts were seeded at 100,000 cells/cm^2 in DMEM supplemented with 10% FCS. After 24 h cells were stimulated for 24 h with the different factors in medium containing 2% heat-inactivated FCS (56°C for 30 min). The concentrations used were within the range of the patho-physiological concentrations measured in the sera of patients with polytrauma [Gebhard et al., 2000]. As a positive control cells were stimulated with 0.1 μg/ml lipopolysaccharide (LPS; E. coli serotype 055:B5; Sigma–Aldrich).

After 24 h supernatants were collected, centrifuged at 800g for 5 min and stored at -80°C. IL-6 and IL-8 release was assessed using enzyme-linked immunosorbent assay (ELISA; R&D Systems, United Kingdom) according to the manufacturer's instructions. Cell lysates were harvested to determine mRNA expression of IL-6, RANKL, M-CSF, and OPG.

ANALYSIS OF mRNA EXPRESSION

Effects on gene expression were examined by RT-PCR as previously described [Liedert et al., 2006]. Briefly, total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. One microgram RNA was transcribed into cDNA using the Omniscript RT Kit (Qiagen). Specific primer pairs (Table I) were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide database) and were synthesized by Thermo Electron Ulm (Germany). Amplification products were cloned and used as standards for RT-PCR (StepOnePlus™ Real-Time PCR System, Applied Biosystems, Germany). The amount of the respective amplification product was determined relative to the housekeeping gene GAPDH. Normalized values of stimulated cells were compared to the respective control group. Amplification products were separated on 2% agarose gel (Life Technologies, USA) and visualized by ethidium bromide staining.

IMMUNOSTAINING OF MSC, OSTEOBLASTS, AND OSTEOCLASTS

MSC and osteoblasts were seeded on 13 mm Ø microscope cover glasses or on eight-well Lab-Tek chamber slides (Nunc) with 4,000 cells/cm^2 2–3 days before staining. Osteoclasts were cultured for 21 days in Lab-Tek chamber slides. Cells were fixed with 4% phosphate-buffered formalin. Visualization of complement regulatory proteins and receptors was performed by detection of the primary antibodies: mouse-anti-human-CD88 (C5aR), -CD46, -CD55, and -CD59 (all from AbD Serotec, Germany), rabbit-anti-human-C3aR (Santa Cruz Biotechnology, Inc., Germany) by the binding of a fluorescence-labeled secondary antibody (rabbit anti-mouse IgG-Texas Red or goat anti-rabbit IgG-Texas Red, Abcam, UK). Cell nuclei were counterstained with Hoechst 33342 (Invitrogen, Germany). For negative controls the primary antibody was substituted with mouse immunoglobulin (mouse IgG1, AbD Serotec) or rabbit immunoglobulin (IgG: I5006, Sigma–Aldrich). Unspecific binding was blocked with 2% BSA in PBS.

To investigate C3aR and C5aR localization after stimulation with the respective ligands, osteoblasts were incubated with C3a and C5a,

TABLE I. Primer Sequences Used for Real-Time RT-PCR

mRNA	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR product size (bp)
C3	ACC AGC AGA CCG TAA CCA TC	GCA GCC TTG ACT TCC ACT TC	100
C5	TGT CGT CGC AAG CCA GCT CC	TGC CAA TGC CTT GAA TTT CCC AGG	215
C3aR	GCA GGT TCC TAT GCA AGC TC	GAA GAT TTC CCG GTA CAC GA	221
C5aR	CTC AAC ATG TAC GCC AGC AT	CAG GAA GGA GGG TAT GGT CA	168
CD46	GTG AGG AGC CAC CAA CAT TT	GCG GTC ATC TGA GAC AGG T	177
CD55	CAG CAC CACCAC AAA TTG AC	CTG AAC TGT TGG TGG GAC CT	215
CD59	CCG CTT GAG GGA AAA TGA G	CAG AAA TGG AGT CAC CAG CA	130
IL-6	AGG AGA CTT GCC TGG TGA AA	CAG GGG TGG TTA TTG CAT CT	180
M-CSF	GGA GAC CTC GTG CCA AAT TA	GGC CTT GTC ATG CTC TTC AT	223
OPG	AGG AAA TGC AAC ACA CGA CA	TAC TTT GGT GCC AGG CAA AT	168
RANKL	CCA GCA TCA AAA TCC CAA GT	CCC CAA AGT ATG TTG CAT CCT G	194
AP	GAA CGT ATT TCT CCA GAC CCA GA	GTG GTC TTG GAG TGA GTG AGT GA	224
BSP	CGA GGG GGA GTA CGA ATA CA	AGG TTC CCC GTT CTC ACT TT	79
OP	CTC AGG CCA GTT GCA GCC	GCC ACA GCA TCT GGG TAT TT	177

respectively, for 15 or 45 min before immunostaining. To detect the internalization of the receptors the cells were fixed, and incubated with the respective primary antibody for 60 min followed by 60 min incubation with a green fluorescence-labeled secondary antibody (rabbit anti-mouse IgG-FITC or goat anti-rabbit IgG-FITC; Abcam). Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and the procedure was repeated with the same primary antibody and a red fluorescence-labeled secondary antibody. Images were taken with a Zeiss Cell Observer miroscope using a Photometrics CoolSNAP EZ camera and version 7.5.5.0 of the metamorph software.

C3 AND C5 CLEAVAGE BY OSTEOCLASTS AND OSTEOBLASTS

PBMNC cultured for 14 days in 96-well plates in medium with 20 ng/ml RANKL and 10 ng/ml rh M-CSF were incubated for 4 h with 2.5 µg of purified human complement protein C5 or C3 (Quidel®, USA) in 50 µl FCS-free medium per well. Osteoblasts were seeded at 10,000 cells/cm^2 in 24-well plates and cultured in osteogenic medium for 14 days. Cells were then incubated for 4 h with 5 µg C3 or C5 in 250 µl FCS-free medium per well. In parallel cultures, cells were treated with C3 or C5 in the presence of 25 ng/ml phorbol 12-myristate 13-acetate (PMA) as a stimulus (Sigma–Aldrich). PMA, which activates the protein kinase C (PKC) is often used as an activator for neutrophils and macrophages. PMA was shown to activate human neutrophils and rat alveolar macrophages which then where able to effectively cleave C5 to C5a [Huber-Lang et al., 2002]. Supernatants were centrifuged at 800g for 5 min and stored at −80°C until use. The cleavage of C3 and C5 was analyzed using a C3a ELISA Kit (Quidel®, USA) or a C5a ELISA Kit (DRG Diagnostics, Germany), respectively, according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Experiments were performed independently 3–7 times in triplicate or quadruplicate cultures. The expression of complement components during osteogenic differentiation was evaluated using a non-parametric Wilcoxon signed rank test for paired samples. A non-parametric Mann–Whitney U-test for non-paired samples was performed to evaluate differences between control and cells treated with anaphylatoxins and IL-1β (SPSS Version 18.0.0, SPSS, Inc., Chicago, IL). To evaluate C3 and C5 cleavage activity of osteoclasts a Student's t-test was performed. Statistical differences of $P < 0.05$ were considered significant. Boxplots depict median, lower and upper quartiles as well as minimum and maximum; outlier values are indicated as single data points.

RESULTS

EXPRESSION OF COMPLEMENT REGULATORY PROTEINS

The cell bound complement regulatory proteins CD46, CD55, and CD59 were expressed by MSC and osteoblasts at the mRNA as well as the protein level as revealed by RT-PCR and immunostaining (Figs. 1A–C,F and 2). CD46 and CD59 mRNA expression was significantly up-regulated during osteogenic differentiation compared to undifferentiated MSC (Fig. 1A,C). Mature osteoblasts were intensively stained for CD46, CD55, and CD59 (Fig. 2). Osteoclasts also expressed all regulatory proteins but the staining was weaker compared to osteoblasts (Figs. 1G and 2).

EXPRESSION AND LOCALIZATION OF COMPLEMENT RECEPTORS

C5aR and C3aR were expressed by undifferentiated and differentiated MSC (Figs. 1D,E and 2). The mRNA expression of the complement receptor C5aR was significantly up-regulated during osteogenic differentiation (Fig. 1E). C3aR mRNA expression was slightly but not significantly increased after 28 days of osteogenic differentiation (Fig. 1D). Immunostaining of C3aR revealed no considerable difference between differentiated and undifferentiated MSC whereas the staining for C5aR was stronger on differentiated MSC. The receptors were strongly expressed in primary osteoblasts (Figs. 1F and 2) and osteoclasts (Figs. 1G and 2). PBMNC expressed both receptors. The C5aR mRNA expression was in tendency up-regulated up to 2.3-fold, the C3aR mRNA expression up to 20-fold during the formation of osteoclast-like cells (Fig. 1G).

Figure 3 demonstrates the presence and localization of C3aR and C5aR in osteoblasts without and after a 45 min treatment with C3a and C5a, respectively. The second column of Figure 3 shows the green labeling of the receptors before membrane permeabilization. Only membrane-bound receptors are stained, because the antibody cannot penetrate the cell membrane. The third column demonstrates the staining after cell permeabilization; because the antibody could permeate the cell, extra- as well as intracellularly located receptors are visible. In the overlay (fourth column) the yellow color indicates the membrane bound receptor, the red the intracellular receptor. In unstimulated osteoblasts C3aR was mainly localized on the cell surface but was also detected in the nucleus after membrane permeabilization (first row of Fig. 3). C5aR was found extra- as well as intracellularly in the cytoplasm but not in the nucleus (third row). After stimulation with C3a the majority of C3aR was internalized into the cells (second row). Stimulation with C5a led to complete internalization of C5aR (forth row). C5aR was completely internalized even after 15 min of stimulation with C5a (data not shown). The nuclei were not stained suggesting that C5aR was not translocated to the nucleus.

EXPRESSION OF THE COMPLEMENT FACTORS C3 AND C5

MSC as well as primary osteoblasts expressed C3 and C5 mRNA (Fig. 1H). The expression of both molecules did not change during osteogenic differentiation of MSC. PBMNC expressed no C5 but some C3 (Fig. 1H). Osteoclasts lacked C5 expression whereas C3 expression was increased in comparison to their precursor cells (PBMNC).

CLEAVAGE OF C3 AND C5

Both osteoblasts and osteoclasts were able to actively cleave native C5, which was added to the cell culture medium osteoclasts being more effective (Fig. 4). C5a could be detected in supernatants of both cultures incubated for 4 h with C5. In osteoclasts but not in osteoblasts C5 cleavage was significantly increased in the presence of a PMA stimulus. There was no C5a found in cultures lacking C5. In contrast, no C3 cleavage was evident either in osteoblasts or in osteoclasts as no C3a was detected in the supernatants.
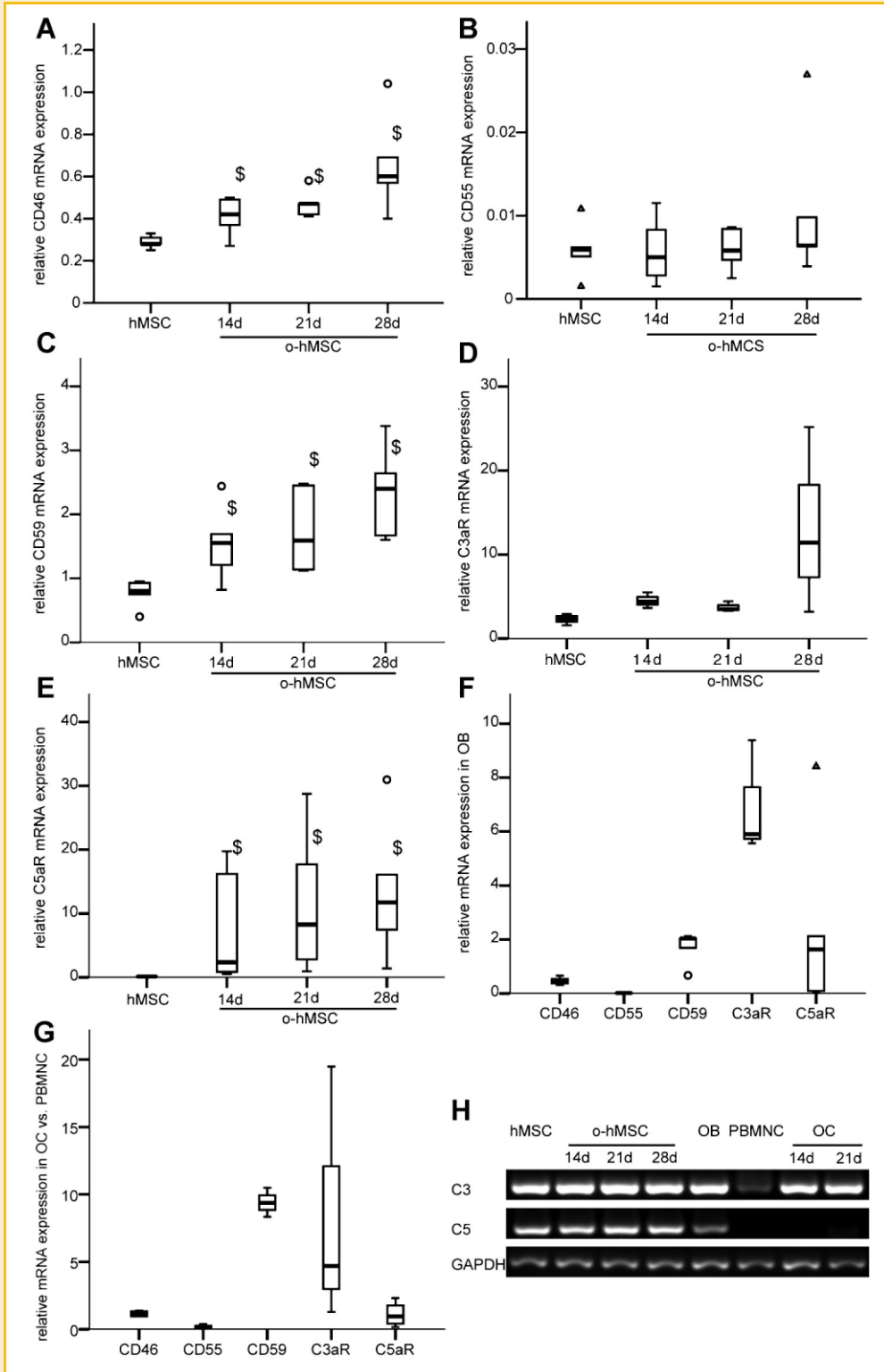
Fig. 1. Expression of complement regulatory proteins (A–C,F), complement receptors (D–F), and C3 and C5 (H) in bone cells: hMSC, o–hMSC, OB, and OC. mRNA expression of the respective gene was related to the housekeeping gene GAPDH. G: Increase in mRNA expression after OC formation from PBMNC. H: Representative agarose gels, C3, and C5 expression in hMSC, o–hMSC, OB, PBMNC, and OC. Experiments were performed independently 3–7 times in triplicate or quadruplicate cultures; $P < 0.05$. hMSC, human mesenchymal stem cells; o–hMSC, hMSC cultivated under osteogenic conditions for 14, 21, and 28 days; OB, primary osteoblasts; OC, osteoclasts, multinucleated tartrate–resistant acid phosphatase (TRAP)-positive cells generated from peripheral blood mononuclear cells (PBMNC).
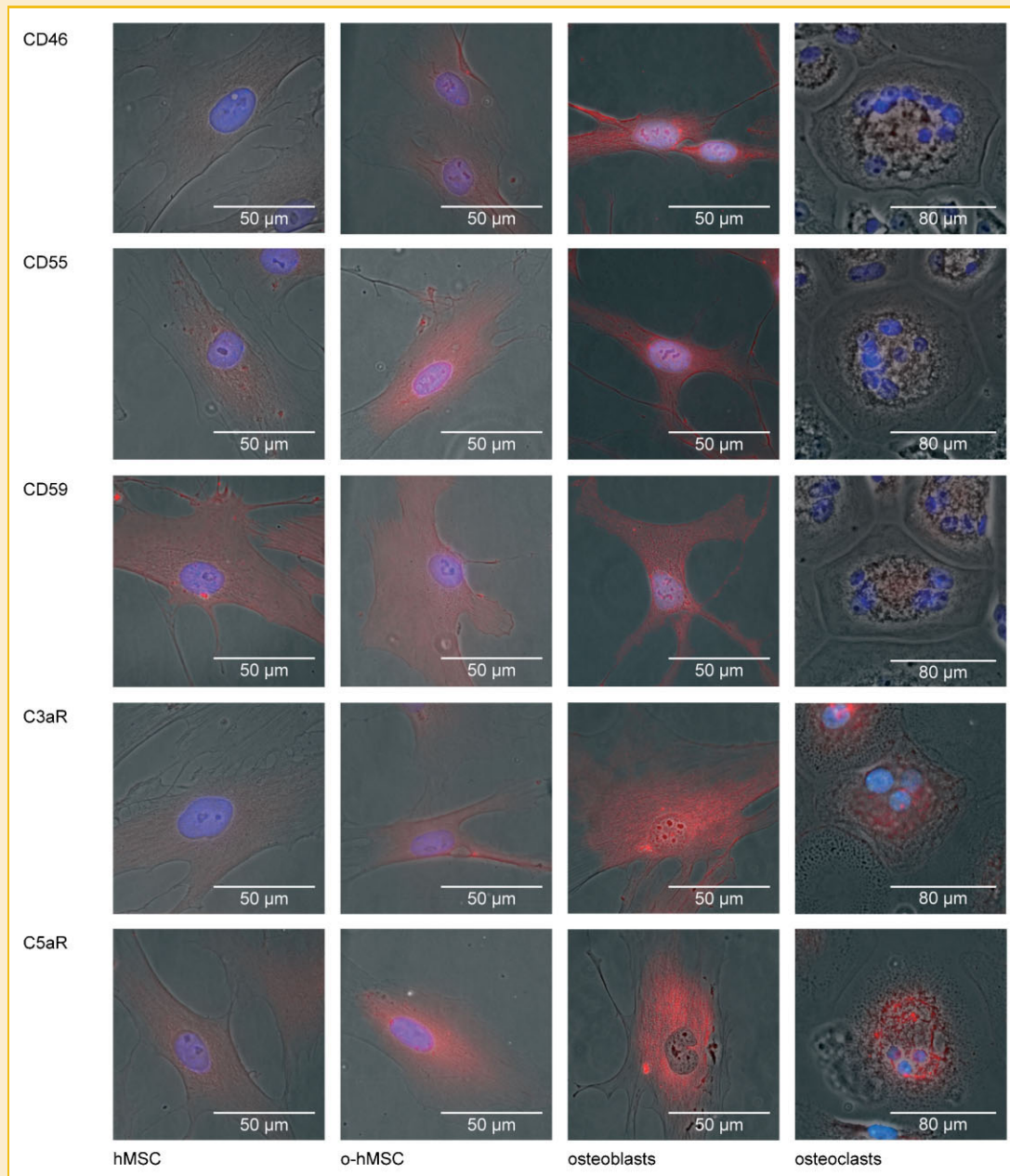
Fig. 2. Fluorescence images of immunostained complement regulators proteins (CD46, CD55, CD59) and receptors (C3aR, C5aR) in bone cells.

STIMULATION OF OSTEOBLASTS WITH C3a AND C5a

To investigate whether C3a or C5a can act as an inflammatory stimulus osteoblasts were treated with C3a, C5a, or the pro-inflammatory cytokine IL-1β alone or with a combination of IL-1β/C3a and IL-1β/C5a for 24 h. LPS was used as a positive control. Untreated cells were used as a negative control. After 24 h we quantified IL-6 and IL-8 by ELISA (Fig. 5A). The treatment with LPS significantly increased IL-6 and IL-8 release in comparison to untreated cells (Fig. 5A). Stimulation with the individual factors IL-1β, C3a, or C5a, respectively, did not significantly induce IL-6 or IL-8 secretion in comparison to untreated cells. In contrast, co-stimulation with IL-1β/C3a or IL-1β/C5a, respectively, significantly up-regulated IL-6 and IL-8 production (Fig. 5A). The data for IL-6 release were confirmed by the results of RT-PCR demonstrating increased IL-6 mRNA expression under these conditions (Fig. 5B).

INFLUENCE OF C3a AND C5a ON OSTEOGENIC DIFFERENTIATION

The influence of C3a and C5a on the osteogenic differentiation of MSC was investigated after 21 days of culture in differentiation medium supplemented with 1 μg/ml C3a or 0.1 μg/ml C5a, respectively. The anaphylatoxins did not influence osteogenic
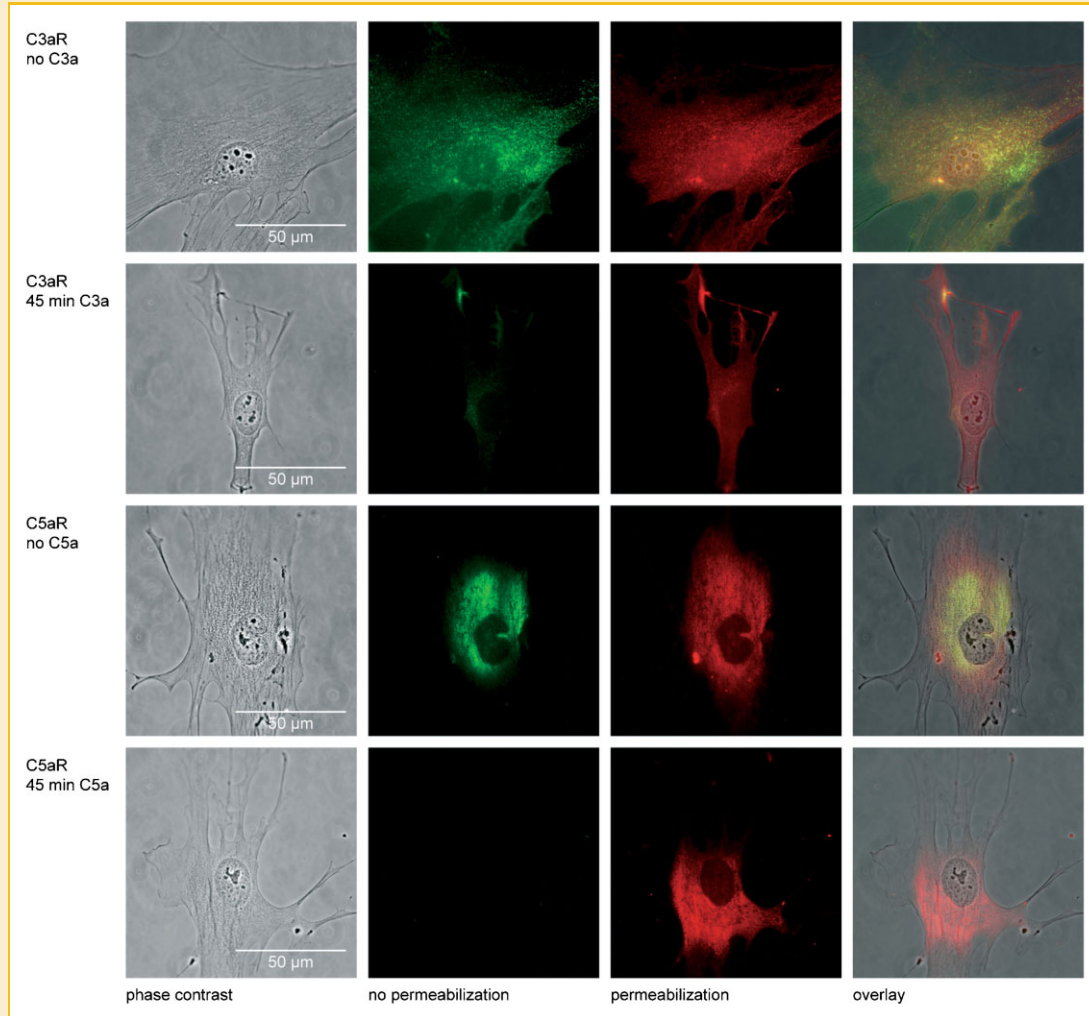
Fig. 3. Phase contrast and fluorescence images of immunostained C3aR and C5aR in osteoblasts without and after 45 min stimulation with 1 μg/ml C3a or 0.1 μg/ml C5a; first column: phase contrast images; second column: for the detection of membrane-bound receptors cells were fixed and the respective receptors were detected by a green fluorescent antibody as described in the Materials and Methods Section; third column: for the detection of membrane-bound as well as internalized receptors cells were permeabilized before labeling with a red fluorescent antibody; fourth column: in the overlay the yellow color indicates the membrane-bound receptors, whereas the internalized receptors are stained red.

differentiation demonstrated by positive von Kossa and AP staining (Fig. 6). The mRNA expression of the osteogenic marker genes AP, bone sialoprotein (BSP), and osteopontin did not differ in the presence or absence of C3a and C5a after 28 days of osteogenic differentiation (data not shown).

DIRECT AND INDIRECT EFFECTS OF C3a AND C5a ON OSTEOCLAST FORMATION

C3a or C5a were added to PBMNC cultures to investigate their effects on osteoclast formation. As a positive control, parallel cultures were treated with 20 ng/ml RANKL and 10 ng/ml M-CSF, which is known to induce osteoclast formation [Boyle et al., 2003]. As a negative control no supplements were added. In the controls the absolute number of multinucleated TRAP-positive cells ranged between 23 and 94 per well (six independent experiments). As expected the treatment with RANKL/M-CSF significantly increased the number of

TRAP-positive multinucleated cells in comparison to untreated cultures (Fig. 7A). Photomicrographs of TRAP-positive multinucleated cell formation in cultures without supplementation and after addition of C3a, C5a, or RANKL/M-CSF are presented in Figure 7B. In the absence of RANKL/M-CSF C3a as well as C5a significantly increased formation of TRAP-positive multinucleated cells in the same range as the RANKL/M-CSF treatment. If RANKL and M-CSF were present C3a and C5a had no additional effect on formation of TRAP-positive multinucleated cells (data not shown). The dissolution of a calcium matrix revealed that the cells resorbed the calcium phosphate-coated cell culture surface only after treatment with RANKL/M-CSF (Fig. 7B). In the absence of RANKL/M-CSF C3a and C5a did not induce resorption activity. If RANKL and M-CSF were present C3a and C5a had no additional effect on osteoclast resorption activity (imaging results not shown).
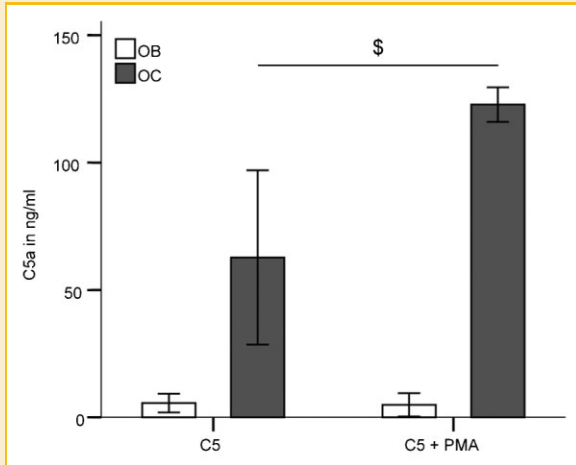
Fig. 4. C5a induction in supernatants of OB and OC after incubation with C5 for 4 h. $^\$$Significant difference between osteoclasts treated with C5 alone and with C5 and phorbol 12–myristate 13–acetate (PMA); $^\$P > 0.05$; n = 3 independent experiments.
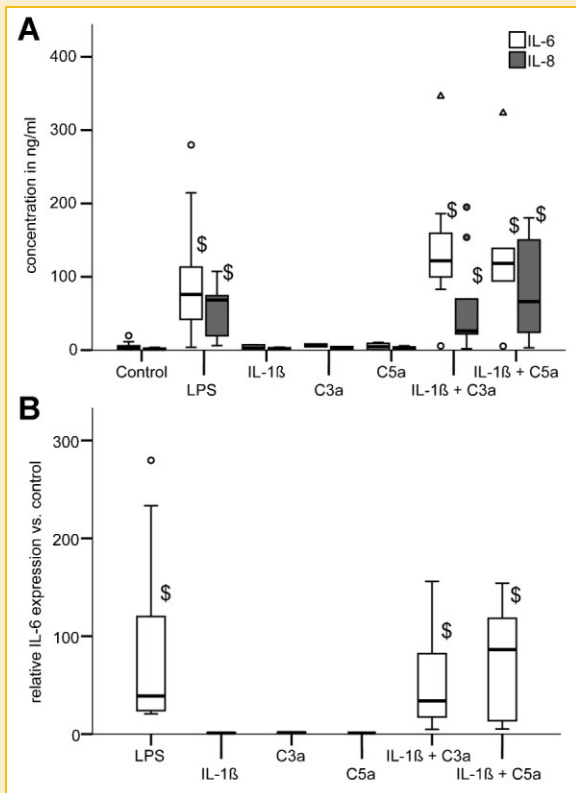

Fig. 5. Stimulation of osteoblast with lipopolysaccharide (LPS; 0.1 μg/ml), C3a (1 μg/ml), C5a (0.1 μg/ml), IL–1β (0.1 ng/ml), or combinations of IL–1β/C3a and IL–1β/C5a for 24 h. The controls received medium without supplements. A: IL–6 and IL–8 concentration in the supernatants of osteoblasts measured by ELISA (enzyme–linked immunosorbent assay); (B) IL–6 mRNA expression in stimulated osteoblasts relative to the untreated controls. Experiments were performed independently 3–7 times in triplicate or quadruplicate cultures; $^\$P < 0.05$.

To investigate whether C3a or C5a could influence osteoclast formation indirectly via the expression of osteoclast related genes in osteoblasts we investigated the mRNA expression of RANKL and OPG after the cells were stimulated with C3a, C5a, and IL-1β or with a combination of IL-1β/C3a and IL-1β/C5a for 24 h. LPS was used as a positive control. LPS treatment significantly enhanced RANKL and OPG expression in comparison to untreated osteoblasts (Fig. 7C). An isolated treatment with C3a, C5a or IL-1β had no significant effect on RANKL and OPG expression. However, co-stimulation with IL-1β/C3a or IL-1β/C5a significantly increased the mRNA expression of RANKL and OPG (Fig. 7C). In non-stimulated osteoblasts OPG expression was about 100-fold higher compared to RANKL. The RANKL/OPG ratio was not significantly altered.

DISCUSSION

There is evidence that complement plays a role in bone formation and regeneration, but the exact mechanism is poorly understood [Ehrnthaller et al., 2011]. Based on our previous work showing C5aR-mediated osteoblast migration and expression during bone healing [Ignatius et al., 2011], this study demonstrated the presence of complement receptors (C3aR, C5aR), complement regulatory proteins (CD46, CD55, CD59), as well as of C3 and C5 in bone cells. We demonstrated that osteoblasts and especially osteoclasts were able to activate complement by cleaving C5 to its active form C5a. Both C3a and C5a alone were unable to trigger the production of inflammatory cytokines in osteoblasts. In contrast, co-stimulation with IL-1β significantly induced the release of IL-6 and IL-8, indicating that complement may modulate the inflammatory response in osteoblastic cells in a pro-inflammatory environment. While C3a and C5a did not affect osteogenic differentiation of MSC, osteoclastogenesis was significantly induced even in the absence of RANKL/M-CSF suggesting that complement is involved in osteoclast formation.

Our data revealed that C3aR and C5aR were present in human MSC, primary osteoblasts, and multinucleated osteoclasts confirming our previous results [Ignatius et al., 2011]. Immunostaining of osteoblasts demonstrated that C3aR was located mainly on the cell surface and to a lesser extent in the cytoplasm and the nucleus, whereas C5aR was found both extra- and intracellularly but not in the nucleus. After stimulation with their respective ligands both receptors were completely internalized. Ligand-induced internalization of C3aR and C5aR into the cytoplasm was reported for immune cells [Van Epps et al., 1990; Settmacher et al., 1999] and also for MSC [Schraufstatter et al., 2009]. Our results of nuclear translocation of C3aR are consistent with observations in MSC by Schraufstatter et al. [2009]; these authors discussed whether this intriguing result may be restricted to these cells because of their stem cell nature. The data of the present study suggest that nuclear translocation of C3aR could also occur in differentiated cells of mesenchymal origin; its role in intracellular signaling has to be further elucidated.

Our results showed that C5aR mRNA was significantly up-regulated during osteogenic differentiation, indicating an important
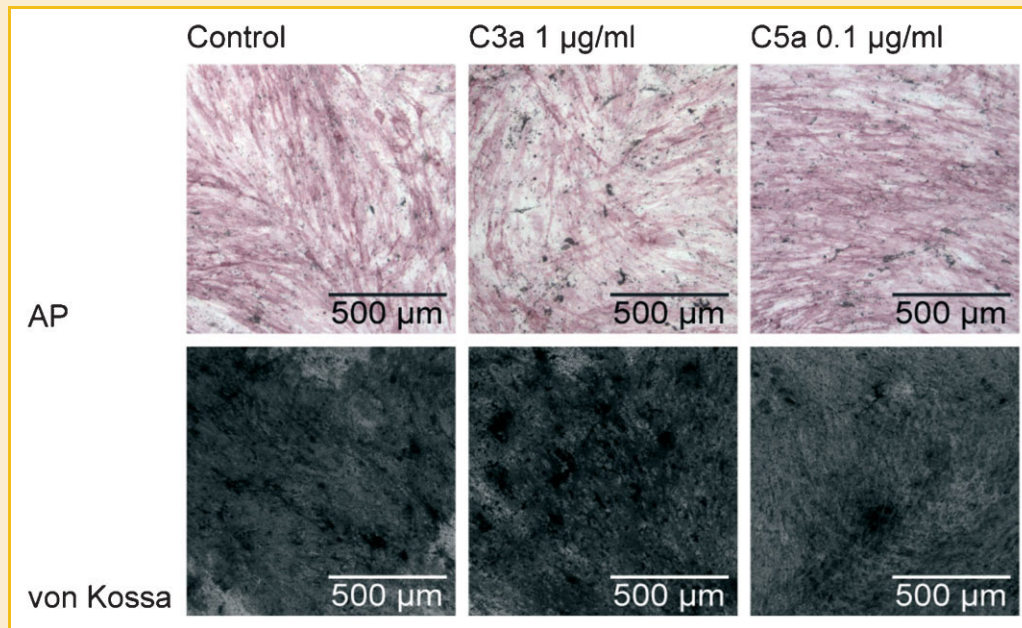
Fig. 6. Osteogenic differentiation of hMSC after 28 days in differentiation medium containing 1 μg/ml C3a or 0.1 μg/ml C5a. First row: positive staining of alkaline phosphatase; second row: positive von Kossa staining indicating matrix mineralization.

role in osteoblast function. C3aR and C5aR modulate many cell activities including cell migration, cytokine release, and apoptosis [Fernandez et al., 1978; Scholz et al., 1990; Haas and van Strijp, 2007; Klos et al., 2009]. Currently little is known about their function in bone cells. Our results revealed that the presence of anaphylatoxins did not disturb osteoblast function as demonstrated by successful matrix deposition and mineralization. We previously reported that C5a induced the migration of osteoblasts and their precursors via C5aR [Ignatius et al., 2011]. C3a could also act as a chemoattractant for MSC [Schraufstatter et al., 2009]. Because C3a and C5a are locally generated in injured and inflamed tissues it may be suggested that complement contributes to the recruitment of osteoblast precursor cells during bone regeneration. In addition to migration, complement receptors could potentially modulate the inflammatory response of osteoblasts. Other authors reported that C5a in synergy with IL-1β induced the release of the inflammatory cytokine IL-6 from osteoblast-like MG-63 cells [Pobanz et al., 2000]. In the present study we found similar effects in human primary osteoblasts for C5a, and furthermore, also for C3a. Whereas the individual factors were unable to induce a considerable release of IL-6 and IL-8, cytokine secretion was significantly increased after co-stimulation with IL-1β/C3a or IL1β/C5a. The inflammatory response of osteoblasts after co-stimulation was as high as after LPS treatment. These data were confirmed by the results of RT-PCR demonstrating transcriptional IL-6 induction after IL-1β/C3a and IL-1β/C5a treatment. For other cell types it was previously reported that anaphylatoxin effects are complexly interrelated with pro-inflammatory stimuli. IL-1β was shown to increase C5aR expression on mononuclear cells [Takabayashi et al., 2004] while C5a treatment stimulated IL-1β release from PBMNC [Okusawa et al., 1987]. Furthermore C5a and LPS synergistically enhanced IL-6 release

from Kupfer cells [Mack et al., 2001]. The mechanism of the synergism of IL-1β and anaphylatoxins might be due to crosstalk between the involved intracellular signaling cascades. It is known that IL-1β effects are mediated by MEKK1 activation and proteins of the interleukin receptor-associated kinase (IRAK) family, leading to activation of C-JUN or NF-κB [Flannery and Bowie, 2010]. C5aR signaling involves activation of ERK1/2, AKT, and MAPK p38 [Rousseau et al., 2006]. C3aR activation was shown to induce ERK1/2 and NF-κB [Li et al., 2008]. It was speculated that the synergistic effects of C5a and IL-1β on IL-6 production in MG63 cells may possibly occur through co-stimulation of NF-κB bindings sites in the IL-6 promoter [Pobanz et al., 2000]. However, experimental data about the underlining mechanisms are presently lacking and should be the object of further studies. In conclusion it might be suggested that C3a and C5a could enhance the inflammatory response of osteoblasts in a pro-inflammatory environment, for example, during bone healing or in inflammatory bone disorders.

It was reported that mouse bone marrow stromal cells produced C3 in response to $1\alpha,25$-dihydroxyvitamin D_3 and that C3-induced osteoclast formation mediated by C3 receptors (MCR1, MCR2, CR3) on osteoclast precursor cells [Sato et al., 1991, 1993]. These findings were strengthened by a recent paper demonstrating that C3 is activated via the alternative pathway in mouse bone marrow cells and that in addition to C3 receptors, C3aR and C5aR are required for osteoclasts differentiation [Tu et al., 2010]. Whereas these studies used mixed bone marrow cell cultures hampering the clear differentiation between the role of stromal cells and cells of hematopoietic origin, we investigated osteoclast formation from human PBMNC. Our data demonstrated that the interesting results previously obtained in mouse bone marrow cells [Sato et al., 1993; Tu et al., 2010] could be at least partly transferred to human cells.
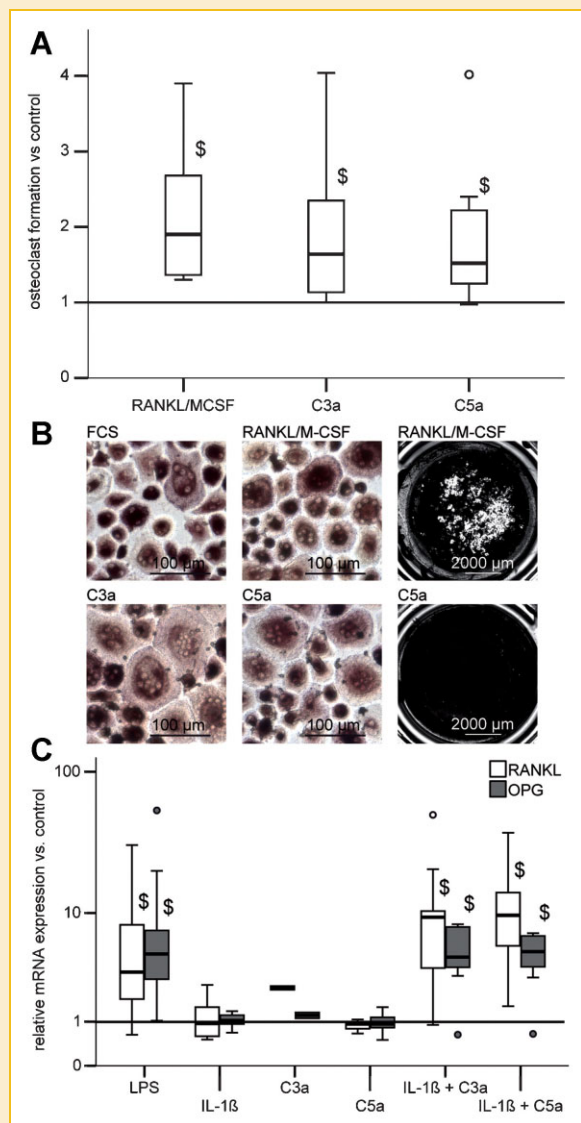
Fig. 7. A: Formation of multinucleated TRAP-positive cells from PBMNC after stimulation with RANKL/M-CSF (20/10 ng/ml), C3a (1 μg/ml), or C5a (0.1 μg/ml). The number of multinucleated TRAP-positive cells in stimulated cultures were related to control cultures without supplements (bar at 1). Experiments were performed independently six times in triplicate cultures; $P < 0.05. B: Photomicrographs of TRAP-stained cells (left and middle column) after 21 days cultivation of PBMNC on plastic and von Kossa-stained 28 days cultures (right column) on calcium phosphate-coated culture vessels. TRAP-positive multinucleated cells containing at least three nuclei were generated in presence of medium with 10% FCS without supplementation with osteoclastogenic factors (FCS), in presence of 20 ng/ml RANKL and 10 ng/ml M-CSF (RANKL/M-CSF), in presence of 0.1 μg/ml C5a (C5a) or 0.1 ng/ml C3a (C3a). Osteoclast resorption lacunae in calcium phosphate-coated cell culture surface (gray and white) were observed in presence of 40 ng/ml RANKL and 20 ng/ml M-CSF. After stimulation with 0.1 μg/ml C5a calcium phosphate coating was not resorbed, culture surface was black after von Kossa-staining. C: RANKL and OPG mRNA expression in osteoblasts relative to the untreated controls after stimulation with LPS (0.1 μg/ml), C3a (1 μg/ml), C5a (0.1 μg/ml), IL-1β (0.1 ng/ml), or combinations of IL-1β/C3a and IL-1β/C5a for 24 h. The controls received medium without supplements. Experiments were performed independently 3–7 times in triplicate or quadruplicate cultures; $P < 0.05. FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; M-CSF, macrophage-colony stimulating factor; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor–kappaB ligand.

C3 and C5 were strongly expressed in MSC during osteogenic differentiation as well as in primary osteoblasts, whereas osteoclasts generated from PBMNC, unlike mixed mouse bone marrow cultures, only expressed C3. Furthermore, both osteoblasts and osteoclasts could actively cleave C5 to C5a, with osteoclasts being much more effective. C3 was cleaved neither by osteoblasts nor by osteoclasts. The catalytically active center of systemic C3 and C5 convertase complexes is known to reside in serine protease domains [Huber-Lang et al., 2002]. Although several other proteases including various coagulation factors appear to be capable of cleaving both, C3 and C5, present findings revealed a rather distinctive cell-type dependent effect with the lack of a specific C3 cleaving protease in osteoblasts and osteoclasts.

The most intriguing finding is that both anaphylatoxins could significantly enhance the formation of multinucleated TRAP-positive cells from PBMNC even in the absence of RANKL/M-CSF, which are crucial factors for osteoclastogenesis and are needed to be supplemented for efficient osteoclast formation in PBMNC cultures [Boyle et al., 2003; Kreja et al., 2007]. C3a and C5a were as effective as RANKL/M-CSF supplementation. These data suggest that osteoblasts and their precursors as well as osteoclasts may locally produce and activate complement, which could directly increase osteoclast differentiation. However, C3a or C5a were not able to induce resorption activity in PBMNC-derived TRAP-positive multinucleated cells in the absence of RANKL/M-CSF indicating that the anaphylatoxins might predominantly influence osteoclastogenesis. In vivo, the induction of osteoclast formation might be enhanced by a increased recruitment of osteoclast precursor cells to the site of inflammation due to the chemotactic effects of the anaphylatoxins [Flierl et al., 2006]. We also investigated whether anaphylatoxins could influence osteoclasts indirectly by modulating RANKL and OPG expression in osteoblasts. Neither RANKL nor OPG expression was significantly altered by C3a or C5a treatment. However, co-stimulation with IL-1β/C3a or IL-1β/C5a synergistically increased RANKL and OPG expression. This effect could potentially be mediated by the observed release of IL-6, which is known to influence osteoclastogenesis by modulation of RANKL/OPG expression [Liu et al., 2005; Suzuki et al., 2011]. These results suggest that C3a and C5a might not only influence osteoclast formation directly but also modulate osteoblast/osteoclast interaction via RANKL/OPG.

MSC and osteoblasts as well as osteoclasts expressed membrane-associated complement regulatory proteins CD46 (membrane cofactor protein), CD55 (decay accelerating factor), and CD59 (protectin). These molecules are widely expressed and protect cells against a complement attack [Miwa and Song, 2001; Kim and Song, 2006]. CD46 regulates C3 activation as a cofactor for factor I-mediated cleavage of C3b. CD59 prevents the formation of the terminal lytic membrane attack complex. CD55 inhibits the activation of C3 and C5 by preventing the formation and accelerating the decay of C3/C5 convertases [Miwa and Song, 2001; Kim and Song, 2006]. Confirming our results it was recently reported that MSC express CD46, CD55, and CD59 and were largely resistant to complement-mediated cytotoxicity [Komoda et al., 2010]. We found that CD46 and CD59 were significantly up-regulated during osteogenic differentiation possibly indicating that

more mature osteoblastic cells may be more efficiently protected against complement attack.

In conclusion this study demonstrated that bone cells express complement components such as C3 and C5, related receptors C3aR and C5aR, and regulatory proteins. Osteoblasts as well as osteoclasts could generate C5a. Both anaphylatoxins directly induced osteoclast formation from PBMNC. In synergism with IL-1β, C3a, and C5a induced the release of IL-6 and IL-8, and up-regulated RANKL/OPG expression, suggesting that complement may enhance the inflammatory response of osteoblasts and modulate their interaction with osteoclasts, especially in a pro-inflammatory environment, for example, during bone healing or in inflammatory bone disorders.

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