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Inhibition of osteoclast activity by complement regulation with DF3016A, a novel small-molecular-weight C5aR inhibitor

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ABSTRACT

Recent insights have indicated an active role of the complex complement system not only in immunity, but also in bone remodeling. Evidence from knockout mice and observations from skeletal diseases have drawn attention to the C5a/C5aR axis of the complement cascade in the modulation of osteoclast functions and as potential therapeutic targets for treatment of bone pathologies. With the aim to identify novel C5aR regulators, a medicinal chemistry program was initiated, driven by structural information on a minor pocket of C5aR that has been proposed to be a key motif for C5aR intracellular activation. The impact of the peptidomimetic orthosteric C5aR antagonist (PMX-53), of two newly synthesized allosteric C5aR antagonists (DF2593A, DF3016A), and of C5aR down-regulation by specific siRNAs, were examined for regulation of osteoclastogenesis, using a well-validated *in-vitro* model starting from RAW264.7 precursor cells. Both pharmacological and molecular approaches reduced osteoclast maturation of RAW264.7 cells induced by receptor-activator of nuclear factor kappa-B ligand (RANKL), which limited the transcription of several differentiation markers evaluated by real-time PCR, including nuclear factor of activated T-cell 1, matrix metalloproteinase-9, cathepsin-K, and tartrate-resistant acid phosphatase. These treatments were ineffective on the subsequent step of osteoclast syncytium formation, apparently as a consequence of reduction of C5aR mRNA levels in the course of osteoclastogenesis, as monitored by real-time PCR. Among the C5aR antagonists analyzed, DF3016A inhibited osteoclast degradation activity through inhibition of C5aR signal transduction and transcription. These data confirm the preclinical relevance of this novel therapeutic candidate.

1. Introduction

Recent studies have demonstrated the osteoimmunological pleiotropy of the complement system, with its participation in innate and adaptive immune responses, and its regulation of bone pathophysiology [1]. The complement system consists of fluid-phase and cell-surface-bound proteins, and activation of its cascade occurs as a chain reaction through three main pathways: the classic, alternative, and lectin pathways [2]. The terminal reactions consist of proteolytic cleavage of the C3 and C5 components, with production of anaphylatoxins C3a and C5a, which mainly function through interactions with their corresponding receptors, C3aR and C5aR [3]. C5 can also be activated through extrinsic pathways, which have relevant roles after injury [4].

In bone, the complement system is expressed and functions at different levels, during bone development [5], during osteogenic differentiation [6,7], and through modulation of osteoblast and osteoclast communication [1]. Osteoblasts can generate both C3 and C5, and they express C3aR and C5aR, which regulate osteoblast migration and release of pro-inflammatory cytokines [8,9]. On the other hand, osteoclasts can cleave C5 to the functional C5a, and C5a can directly regulate osteoclastogenesis [10]. Also, osteoclasts express C3aR and C5aR, as well as C5L2, another receptor for C5a [11,12]. In addition, C5a-

Abbreviations: α-MEM, Eagle's alpha-modified medium; DF2593A, 1-(4-oxo-4-{[(1R)-1-{4-[(trifluoromethanesulfonyl)oxy]phenyl}ethyl]amino}butyl)piperidin-1-ium chloride; DF3016A, sodium 5-[(1R)-1-(4-{[4-(trifluoromethyl)-1,3-thiazol-2-yl]amino}phenyl)ethyl]tetrazol-1-ide; DMEM, Dulbecco's modified Eagle's medium; $HBSS^{++}$, Hanks balanced salt solution with calcium and magnesium; PMX-53, Ac-Phe-cyclo(Orn-Pro-D-Cha-Trp-Arg); siRNA, small-interfering RNA

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regulated osteoblast secretion of IL-6 modulates osteoclast differentiation and bone resorption [13].

Mice lacking or deficient in specific complement components were instrumental in the definition of the involvement of the complement system in bone development and regeneration. Although C5-deficient mice did not show any severe bone phenotype, deeper analyses indicated a role for C5 in longitudinal growth of long bones and in fracture healing [14]. C3aR and C5aR regulate maturation of osteoclasts from stem cells of the macrophage-hematopoietic lineage, as demonstrated by the mouse knockout for these individual receptors [11,15]. Although, both $C5aR^{-/-}$ and $C5L2^{-/-}$ mice showed increased bone mass compared to wild-type mice, this phenotype was more evident for $C5aR^{-/-}$ mice, and was consequent to decreased numbers of osteoclasts; it was milder for $C5L2^{-/-}$ mice due to increased numbers of osteoblasts [15]. The role of C5aR in modulation of osteoclast formation was further demonstrated *in vitro* using human cells in primary culture [10].

Severe bone destruction can occur with autoimmune diseases, such as rheumatoid arthritis [16], and with abnormal or prolonged activation of the immune system, such as during periodontitis [17]. It can also occur in multiple myelomas [18] and metastatic bone tumors [19,20]. A pivotal role for complement is well established for all of these pathologies [1]. Interestingly, both C5-deficient mice and C5aR-knockout mice were protected against bone loss in experimental arthritis [21,22]. With septic inflammatory disorders, such as periodontitis, C5aR knockout [23] or its antagonism in different animal models [24,25] have shown decreased periodontal bone loss. The C5a-C5aR axis was demonstrated to promote cancer-cell invasion [26], and C5aR-knockout mice have shown decreased bone erosion consequent to establishment of bone metastasis [27]. All of these studies support complement–bone interplay, further pinpointing C5aR in the regulation of bone resorption.

Studies of bone biochemical metabolism are primarily performed *in vivo*. However, there has been increased interest for characterization of *in-vitro* models that allow reductions of the system complexity and that limit animal testing [28]. The strict interconnection of the immune system with bone pathophysiology, in conjunction with ubiquitous C5aR expression, make it difficult to define C5aR involvement in any specific cell function using animal models. In addition, the use of general knockout mice reported above are limited by potential indirect effects.

The aim of this study was to demonstrate that the C5a/C5aR axis represents a new target for regulation of osteoclast maturation using a simplified *in-vitro* model, starting from RAW264.7 precursor cells [29,30]. To understand better the relevance of C5aR modulation in this context, a medicinal chemistry program was activated, to identify novel allosteric inhibitors of C5aR. One of the most potent compounds, DF3016A, is shown to be an inhibitor of osteoclast differentiation and their resorptive activity.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose and GlutaMAX, Eagle's alpha-modified medium (α-MEM) without nucleosides and supplemented with GlutaMAX, fetal bovine serum, Hank's balanced salt solution supplemented with calcium and magnesium ($HBSS^{++}$), and phosphate-buffered saline were from Gibco (Life Technologies Italia, Monza, Italy). Ammonium chloride, bovine serum albumin, Hoechst, L-glutamine, penicillin–streptomycin, pyrogallol, saponin, silver nitrate, sodium azide (NaN_3), and Tween-20 were from Sigma-Aldrich (St. Louis, MO, USA). RNA isolation kits, QuantiTect Reverse Transcription kits, and QuantiTect SYBR Green PCR kits were from Qiagen (Hilden, Germany).

2.2. Cell culture

RAW264.7 cells (ATCC: TIB-7) were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in growth medium of DMEM with 10 % heat-inactivated fetal bovine serum (30 min, 55 °C), 100 U/mL penicillin, and 100 μg/mL streptomycin. RAW264.7 cells were used between passage 10 and 25.

For transient interference of C5aR, RAW264.7 cells were plated at 300,000 cells/well in 12-well plates in growth medium without antibiotics, and 24 h later they were transfected with 250 pmol small-interfering (si)RNAs/well, as the non-targeting siRNA pool #2 (si-NT) (Cat. N°. D-001206-14; siGENOME) or against mouse C5aR (si-C5aR) (gene ID. 12273; Cat. N°. M-043176-01; siGENOME), from Dharmacon (Chicago, IL, USA), using lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), according to the manufacturer instructions. Forty-eight hours later, the interfered cells were detached and plated for the *in-vitro* osteoclastogenesis assays or for RNA extraction to determine the C5aR knockdown efficiency by real-time PCR.

2.3. Osteoclastogenesis assay

For the *in-vitro* osteoclastogenesis assays, RAW264.7 cells were plated in differentiation medium of α-MEM supplemented with 10 % heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, at a density of 1,250 cells/well in 96-well plates (OsteoAssay), 5,000 cells/well in 24-well plates on coverslips for morphological analysis, or 10,000 cells/well in 12-well plates for RNA extraction. Twenty-four hours later, and after every 48 h, the medium was replaced, and the cells were treated without or with 15 − 30 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL), from Peprotech (Cat. N°. 310-01; London, UK), alone or with different agents diluted in the differentiation medium. When differentiation was complete, the cells were harvested in lysis buffer for RNA extraction, or were fixed.

2.4. C5a ELISA

Quantitative measurements of C5a in cell-culture supernatants were determined by ELISA using mouse C5a ELISA kits (Elabscience Biotechnology Inc., Houston, TX, USA), according to the manufacturer instructions. Briefly, RAW264.7 cells were plated in 7 mL differentiation medium at a density of 120,000 cells per 10-cm petri dish. Twenty-four hours later, and after every 48 h, the medium was replaced, and the cells were treated without or with 15 ng/mL RANKL. When differentiation was complete, the cell culture medium was harvested and centrifuged at 1,000*g* for 20 min at 4 °C, and the supernatants were used in the assays. All of the tests were performed in duplicate, across three independent experiments.

2.5. RNA extraction and real-time PCR

Total RNA was extracted using RNAeasy isolation kits, cDNAs were obtained using QuantiTect Reverse Transcription kits, and real-time PCR was performed with QuantiTect SYBR Green PCR kits, all according to the manufacturer instructions. The primers used for real-time PCR (LightCycler 480 Instrument II; Roche, Indianapolis, IN, USA) are listed in Table 1. β_2-Microglobulin was followed as the housekeeping gene. The real-time PCR program consisted of an initial 15 min at 95 °C, and then 45 cycles, as follows: 94 °C for 15 s, annealing temperature of each primer for 30 s, and 72 °C for 30 s.

2.6. Immunofluorescence microscopy

After the osteoclastogenesis assays, the cells were rinsed with $HBSS^{++}$, fixed in 4 % (w/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min, and washed three times with

Table 1
Sequences and annealing temperatures of the real-time PCR primers.

Gene	Annealing temperature (°C)	Primer sequence
NFATc1	60	FW: 5′-CATGCAGCCATCATCGA-3' RV: 5′-TGGGATGTGAACTCGGAAGAC-3'
Metalloproteinase-9	55	FW: 5′-CTGTCCAGACCAAGGGTACAGCCT-3' RV: 5′-GTGGTATAGTGGGACACATAGTGG-3'
Tartrate-resistant acid phosphatase	55	FW: 5′-AAATCACTCTTTAAGACCAG-3' RV: 5′-TTATTGAATAGCAGTGACAG-3'
Cathepsin-K	55	FW: 5′-CCTCTCTTGGTCTCCATACA-3' RV: 5′-ATCTCTCTGTACCCTCTGCA-3'
Calcitonin receptor	60	FW: 5′-ACCGACGAGCAACGCCTACGC-3' RV: 5′-GCCTTCACAGCCTTCAGGTAC-3'
β_2-Microglobulin	60	FW: 5''-TGGTGCTTGTCTCACTGACC-3' RV: 5′-GTATGTTGGCTTCCCATTC-3'
C5aR	60	FW: 5′-TCATCCTGCTCAACATGTACGCCA-3' RV: 5′-TCTGACACCAGATGGGCTTGAACA-3'
C5L2	55	FW: 5′-TTTGCTGGACCCCTTATCAC-3' RV: 5′-GATACCTTGGTCACCGCACT-3'

FW, forward; RV, reverse.

$HBSS^{++}$. Then, the cells were incubated for 1 h with blocking solution (50 mM ammonium chloride, 0.5 % bovine serum albumin, 0.1 % saponin, 0.02 % NaN_3, in phosphate-buffered saline), with 33 nM Alexa546-labeled phalloidin (Invitrogen, Carlsbad, CA, USA) for filamentous actin visualization, and 2 μg/mL Hoechst for nucleus staining. Finally, the cells were washed three times with phosphate-buffered saline plus 0.02 % Tween-20, and the coverslips were mounted with Mowiol 4–88 (Sigma-Aldrich, St. Louis, MO, USA) and examined by fluorescence microscopy. The nuclei of multinucleated cells were counted using a 63× objective, moving along the vertical and horizontal axes of the coverslips.

2.7. Degradation assay

To measure resorptive function, osteoclasts were grown on OsteoAssay plates (Corning, New York, USA) for 7 days. When differentiation was complete, the cells were removed (using 10 % bleach) and the wells were stained with 5 % silver nitrate for 1 h. The staining was stopped by addition of 1 % pyrogallol. Images were taken under a microscope (EVOS XL Core, Thermo Fisher Scientific) at a final magnification of 2×, and the total resorbed area was quantified using ImageJ (NIH).

2.8. FACS

The RAW264.7 cells were plated in 2 mL growth medium at a density of 20,000 cells/well in 6-well plates. Twenty-four hours later, and every 48 h, the medium was replaced without (Ctrl) or with addition of different compounds, for 8 days. At the end of the treatments, the cells were stained without or with propidium iodide and/or FITC-labeled annexin-V (Milteny Biotech GmbH, Bergisch Gladbach, Germany), according to the manufacturer instructions, immediately before the FACS analysis (Attune NxT Acoustic Focusing Flow Cytometer; Thermo Fisher Scientific, Waltham, MA, USA). Single-labeled control cells were used for gating of the other fluorescent probe, while all of the samples were double stained and analyzed as proportions of positive cells (%) for each marker.

3. Results

3.1. Activated C5aR stimulates osteoclast maturation

To unravel the C5aR involvement in osteoclastogenesis, a simplified *in-vitro* model was used that was based on the murine monocyte/macrophage RAW264.7 cell line. Upon treatment with RANKL, these cells form multinucleated and functionally active osteoclast-like cells [31].

Although C5aR (and C5L2) expression and functional activity in RAW264.7 cells are undisputed [32,33], its cell-autonomous activation is still questioned. Therefore, release of endogenous C5a by the RAW264.7 precursor cells and by mature osteoclasts was assessed by ELISA. Despite the trace amount of C5a detected in the culture medium of undifferentiated RAW264.7 cells (≤0.4 pg/μg cell lysate; equivalent to 15.0 ± 3.2 pg/mL; close to the assay detection limit), strongly up-regulated C5a release was seen in the supernatant of 72-h RANKL-differentiated cells (93.5 ± 31.5 pg/μg cell lysate; equivalent to 12.2 ± 1.5 ng/mL) (see Methods).

This increase in the extracellular levels of C5a was suggestive of modulation of RANKL-induced differentiation or mature osteoclast activity through C5aR signaling. To examine potential C5aR modulation of osteoclastogenesis, the impact of C5aR receptor antagonists was determined. The RAW264.7 cells were treated during the entire differentiation with RANKL alone and in combination with both orthosteric (PMX-53 [34]) and allosteric (DF2593A [35], DF3016A [36]) C5aR antagonists. The effects of these antagonists were initially determined for osteoclast maturation, with expression of differentiation markers followed by real-time PCR. To this end, a member of the nuclear factor of activated T-cell family (NFATc1) was monitored as an early differentiation marker, cathepsin-K protease and matrix metalloproteinase-9 (MMP-9) as intermediate differentiation markers, and tartrate-resistant acid phosphatase (TRAP) as a late differentiation marker. Treatment with 1 μM PMX-53 significantly inhibited RANKL-induced transcription of all of the differentiation markers analyzed, with about 60 % reduction for NFATc1 and MMP-9, 40 % for cathepsin-K, and 70 % for TRAP (Fig. 1). Among the allosteric antagonists, 1 μM DF2593A significantly inhibited RANKL-induced transcription of NFATc1 (40 %), cathepsin-K and MMP-9 (50 %), and TRAP (70 %) (Fig. 1). Finally, 1 μM DF3016A induced about 40 % inhibition of NFATc1, cathepsin-K, and MMP-9, and 50 % inhibition of TRAP (Fig. 1).

C5aR involvement was further defined by a molecular approach, with down-regulation of C5aR expression in precursor cells followed by osteoclast differentiation. The RAW264.7 cells were interfered using non-targeting (si-NT) and C5aR-specific (si-C5aR) siRNAs, and RANKL was then added after 72 h, when C5aR mRNA levels were halved by si-C5aR, as measured by real-time PCR. Under these conditions, as control, the si-C5aR-treated cells showed a small increase in C5L2 mRNA levels that did not reach significance (1.5-fold) compared to si-NT, while the low basal C5a release remained unchanged between these two set of cells (data not shown). This transient down-regulation of C5aR in the precursor cells was sufficient to decrease the subsequent RANKL-induced transcription of almost all of the differentiation

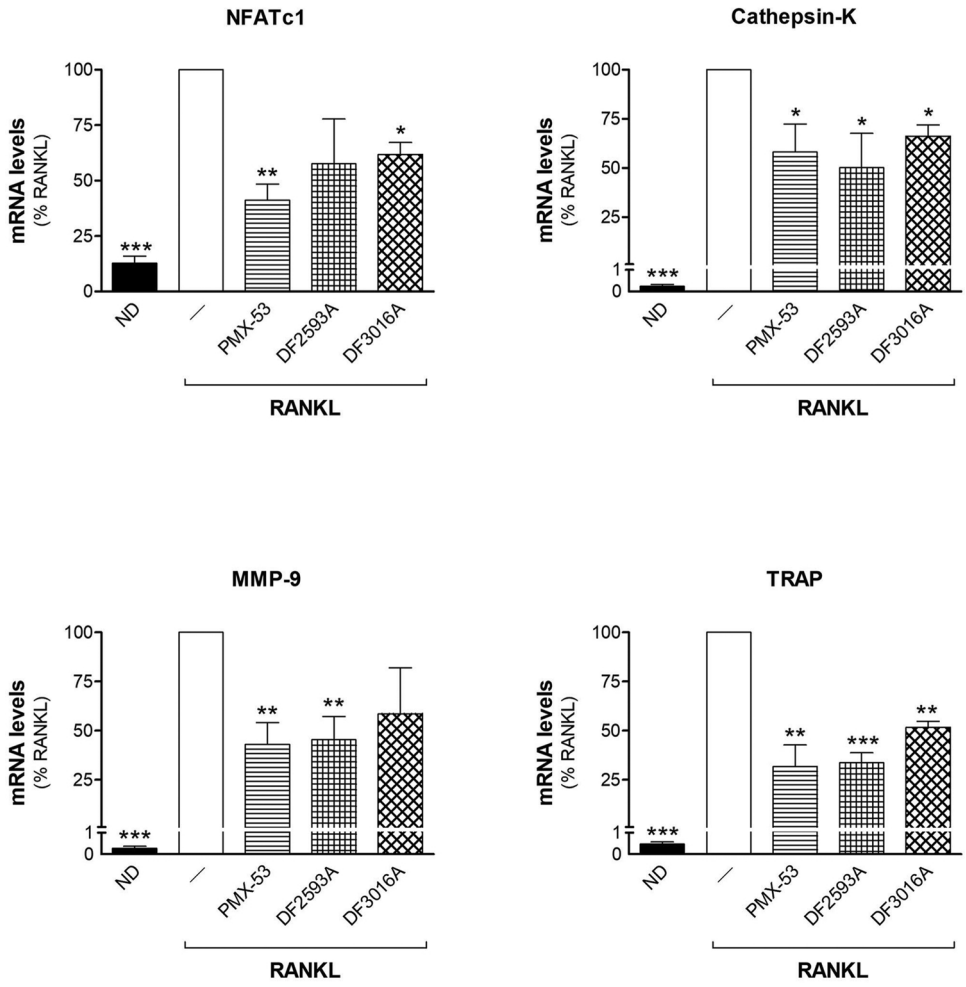

Fig. 1. C5aR antagonism decreases expression levels of the osteoclast-differentiation markers.
Real-time PCR analysis of the differentiation markers (as indicated) in RAW264.7 cells treated in the absence (ND) and presence of 15 − 30 ng/mL RANKL, without (−) or with 1 μM C5aR antagonists (PMX-53, DF2593A, DF3016A). The transcripts were quantified and normalized for β_2-microglobulin expression, as the housekeeping gene. Data are means ± SEM of at least three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* RANKL (paired Student's *t*-tests).

markers analysed, with inhibition by 30 % for NFATc1, 50 % for MMP-9 and cathepsin-K, and 20 % for TRAP (Fig. 2). Instead, their basal transcription rates remained unaltered (Fig. 2). Both of these pharmacological and molecular approaches indicated participation of C5aR activation in RANKL-induced osteoclast maturation of these RAW264.7 precursor cells.

3.2. DF3016A inhibits mature osteoclast activity

Upon RANKL-induced transcriptional remodeling, a series of adhesion molecules are up-regulated, and osteoclast differentiation culminates in cell-to-cell fusion, with the formation of giant osteoclast syncytia [37]. To determine how C5aR influences this late event of osteoclastogenesis, cell distribution based on the number of their nuclei was evaluated using fluorescence microscopy. In comparison to the undifferentiated cells that can undergo spontaneous differentiation to form a few multinucleated cells with up to 10 nuclei, the RANKL-treated RAW264.7 precursor cells differentiated into large syncytia, with sometimes > 50 nuclei (Fig. 3). None of these C5aR antagonists showed regulation of RANKL-triggered osteoclast cell-to-cell fusion (Fig. 3A). In agreement with this, C5aR down-regulation using the si-C5aR treatment did not significantly impact on multinuclear cell formation, in both the absence and presence of RANKL (Fig. 3B and C).

C5aR involvement in mature osteoclast functions was then directly assessed using *in-vitro* assays of bone resorbing activity. RAW264.7 cells were plated on OsteoAssay plates and RANKL-differentiated for 7 days in the absence and presence of the different C5aR antagonists. At the end of the differentiation, the cells were detached and the resorbing areas were analysed. Differently from the undifferentiated cells, RANKL-treated cells showed resorbing activity, with degradation of about 30 % of the entire plate surface. This activity was only slightly affected when 1 μM PMX-53 or 1 μM DF2593A were added throughout the differentiation (see Methods; Fig. 4). Interestingly, treatment with 1 μM DF3016A significantly reduced RANKL-promoted osteoclast degrading activity by 25 % (Fig. 4).

To ascertain that the reduced resorbing activity was not a consequence of toxicity of the 7-day treatment with 1 μM DF3016A, both cell death and induction of apoptosis were monitored in the RAW264.7 cells untreated and treated with up to 10 μM of these C5aR antagonists added for 8 days. For this FACS analysis, the RAW264.7 cells were serum deprived for 24 h for the positive control of induction of apoptosis, as already reported [38]. All of these C5aR antagonists showed comparable propidium iodide incorporation and annexin-V staining, compared to the untreated control cells, with the exception of 10 μM DF2593A and 10 μM DF3016A, which showed a small increase and a small decrease in toxicity, respectively (Table 2).

These data show that DF3016A directly inhibits the functional resorbing activity of mature osteoclasts without affecting RANKL-promoted cell fusion or RAW264.7 cell viability. The other two C5aR antagonists had similar effects, but were less potent for the reduction of

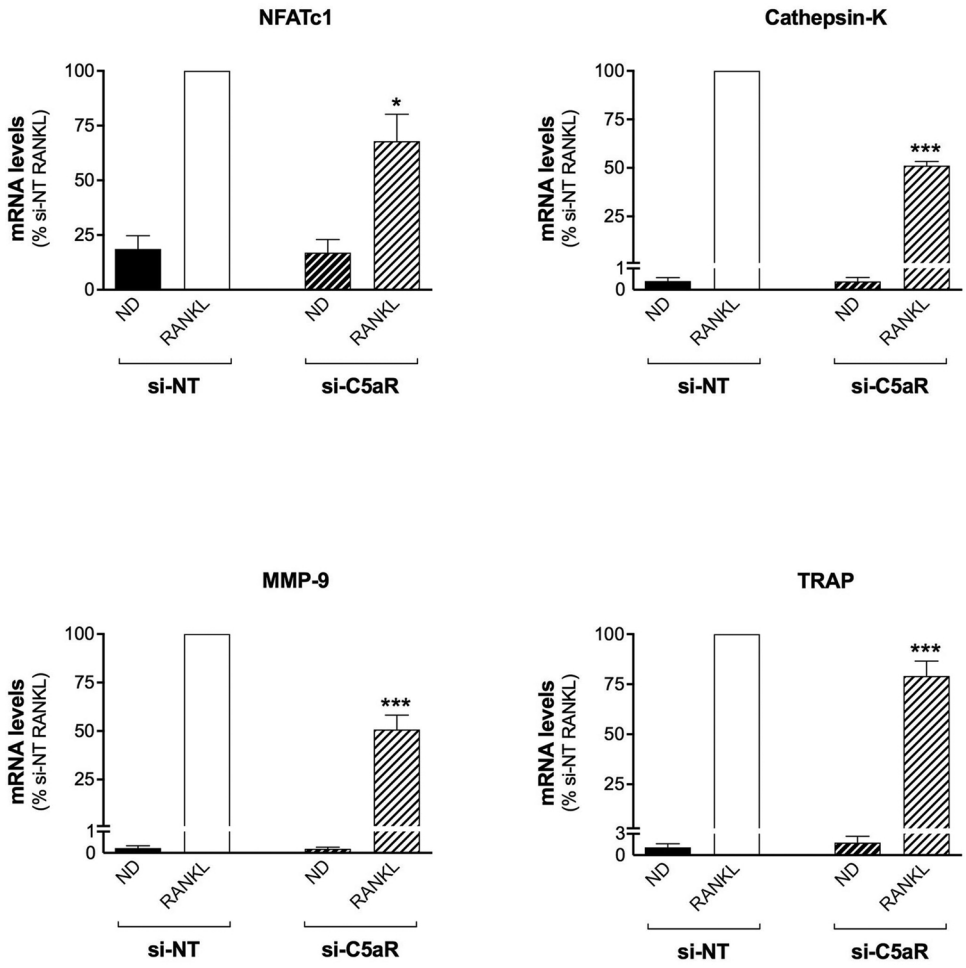

Fig. 2. C5aR interference in osteoclast precursors reduces osteoclast maturation.
Real-time PCR analysis of the osteoclastogenesis markers (as indicated) in RAW264.7 cells interfered with non-targeting (si-NT) or C5aR-targeting (si-C5aR) siRNAs, and subsequently treated without (ND) and with 15 ng/mL RANKL. The transcripts were quantified and normalized for β_2-microglobulin expression as the housekeeping gene. Data are means ± SEM from four independent experiments. $^*P < 0.05$, $^{***}P < 0.001$ (paired Student's *t*-tests), RANKL si-GPR55 *versus* RANKL si-NT.

osteoclast resorption.

3.3. Modulation of C5aR and C5L2 transcription during osteoclastogenesis

As prolonged treatment with these different pharmacological tools might affect osteoclast homeostasis well beyond direct C5aR antagonism, potential transcriptional regulation of C5aR was also considered here.

Time-courses of mRNA levels of both C5aR and C5L2 were thus monitored, which remained stable in the undifferentiated cells. Unexpectedly, these were both rapidly reduced (by 95 % over the initial 24 h), and remained down-regulated throughout RANKL-induced osteoclastogenesis, as monitored by real-time PCR (Fig. 5). The quantification of the mRNA levels at 72 h of RANKL treatment is shown in Table 3, along with the effects of these C5aR antagonists. For C5aR down-regulation during differentiation, the treatments with 1 μM PMX-53 and 1 μM DF2593A were significantly counteracting, while 1 μM DF3016A was slightly reinforcing. Instead, all of these compounds were ineffective in the regulation of C5L2 mRNA expression levels.

For comparison, the expression of another G-protein-coupled receptor (GPCR) was also monitored: the calcitonin receptor (CTR). This is osteoclast-specific and is a marker of the final osteoclast differentiation, as undifferentiated RAW264.7 cells completely lack its expression [30]. These three C5aR antagonists inhibited RANKL-induced transcription of CTR to similar extents, in agreement with what was previously reported for transcription of all of the other differentiation markers analysed (see above).

4. Discussion

Our findings indicate that C5aR has a role in the onset of osteoclast maturation through positive regulation of osteoclast-marker transcription. Indeed, in these RAW264.7 precursor cells, both C5aR antagonism and down-regulation reduced RANKL-triggered transcription of the main differentiation markers: NFATc1, MMP-9, cathepsin-K, and TRAP. As osteoclast differentiation progressed, the C5aR mRNA expression levels decreased, with a consequent loss of C5aR regulation of the later events of osteoclast fusion.

The different compounds used here show distinct antagonism mechanisms due to their specific binding sites on C5aR, which ensures high selectivity of this pharmacological approach. In addition to the natural ligand-binding site, the transmembrane region of GPCRs has a minor pocket that has been proposed as a 'triggering domain' that is crucial for fine tuning of global receptor activation [39]. The knowledge of the C5aR allosteric binding site and the skills that have been acquired through development of the technological platform based on the GPCRBase tool [40,41] allowed rational design of novel potent C5aR-selective inhibitors [42]. Among these small-molecular-weight compounds, DF2593A and DF3016A were derived from two different chemical structures and have been relatively extensively characterized

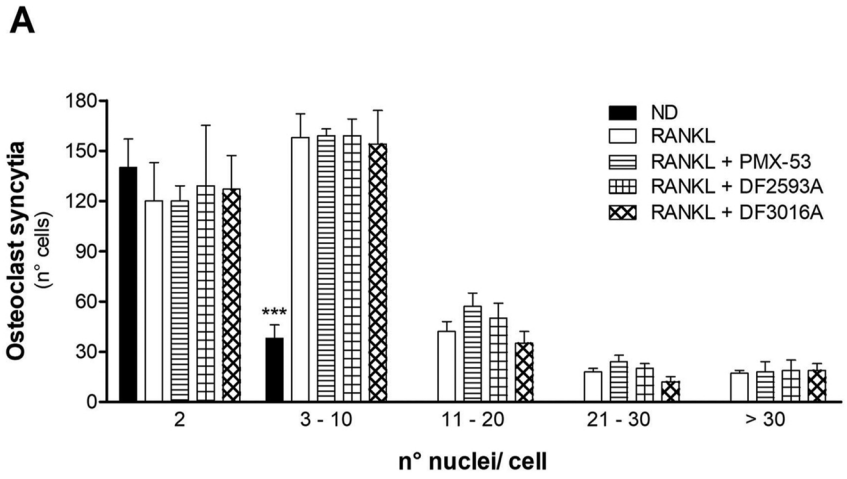

Fig. 3. C5aR antagonism and down-regulation do not modulate osteoclast fusion.
(A) RAW264.7 cells were treated in the absence (ND) and presence of 15 − 30 ng/mL RANKL, without and with the C5aR antagonists at 1 μM, and after 72 h osteoclast fusion was evaluated by quantification of the nuclei per cell, using fluorescence microscopy. Data are means ± SE of at least three independent experiments. (B, C) RAW264.7 cells were interfered using non-targeting (si-NT) and C5aR-targeting (si-C5aR) siRNAs, and then treated without (B) and with (C) 15 ng/mL RANKL, and after 72 h the osteoclast syncytia were analyzed. Data are means ± SE of three independent experiments. ND, undifferentiated cells. ***$P < 0.001$ *versus* RANKL (paired Student's *t*-tests).

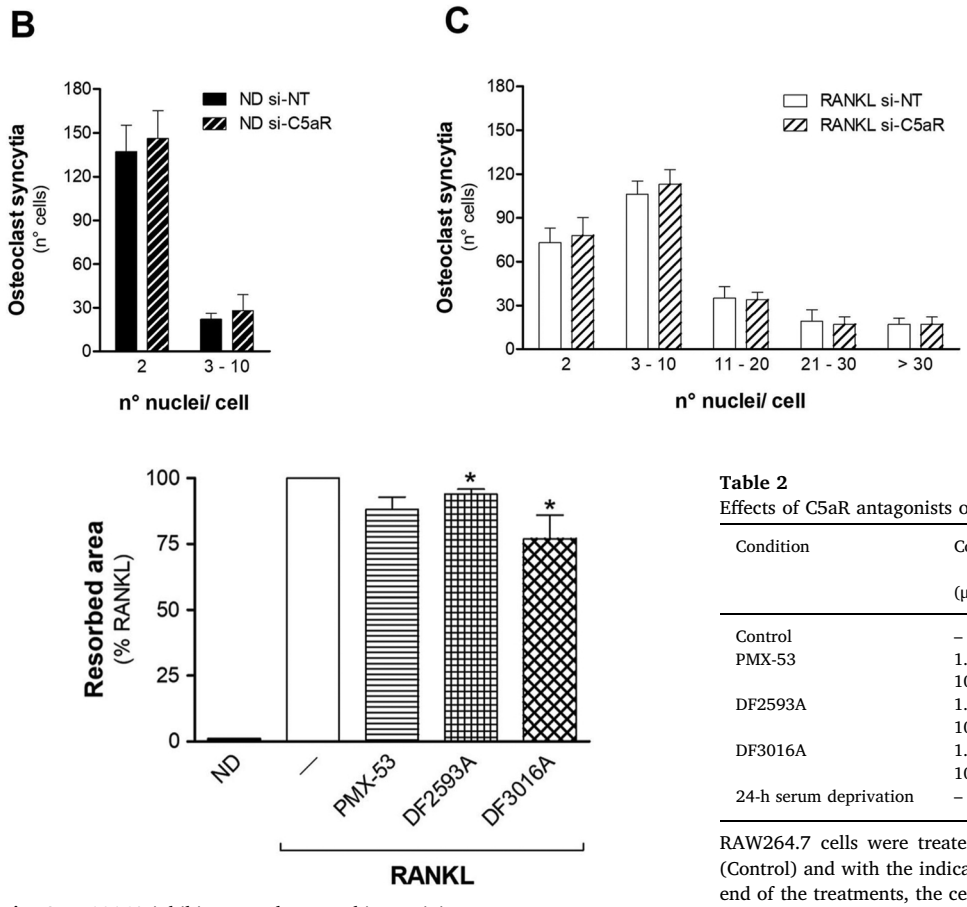

Fig. 4. DF3016A inhibits osteoclast-resorbing activity.
RAW264.7 cells were treated in the absence (ND) and presence of 15 ng/mL RANKL, without (−) and with the indicated compounds at 1 μM. Data are means ± SEM of at least three independent experiments performed in triplicate. *$P < 0.05$ *versus* RANKL (Student's *t*-tests).

in experimental models of inflammatory and neuropathic pain [35,36]. In contrast to the cyclic peptidomimetic PMX-53 that acts as a competitive C5a antagonist on C5aR [34,43], both DF2593A [35] and DF3016A (data not shown) do not compete with agonist binding to this receptor; rather, they stabilize the inactive receptor conformation. Despite these differences, all three of these compounds have similar actions, which includes inhibition of RANKL-induced transcription of the osteoclast markers (NFATc1, cathepsin-K, MMP-9, TRAP, and CTR), and which points to a common inhibitory pathway downstream of C5aR activation.

Accordingly, C5aR down-regulation affected osteoclast maturation, with a major effect on transcription of the intermediate markers, cathepsin-K and MMP-9. The reduced effect on TRAP transcription might be due to different transcription regulation of this marker, although this was more reasonably consequent to the loss of efficacy of the transient interference produced by si-C5aR, as TRAP is one of the final

Table 2
Effects of C5aR antagonists on RAW264.7 cell viability.

Condition	Concentration	Cell viability (% positive cells)	
	(μM)	Propidium iodide	Annexin-V
Control	–	32.6 ± 2.2	41.4 ± 2.1
PMX-53	1.0	26.4 ± 4.4	35.7 ± 5.9
	10.0	30.4 ± 2.3	39.4 ± 4.6
DF2593A	1.0	28.3 ± 2.7	39.7 ± 2.7
	10.0	35.6 ± 1.8*	47.0 ± 5.0
DF3016A	1.0	31.1 ± 7.5	39.5 ± 2.5
	10.0	26.7 ± 2.3*	38.0 ± 2.5
24-h serum deprivation	–	60.5 ± 1.2*	65.4 ± 9.4*

RAW264.7 cells were treated for 8 days in differentiation medium without (Control) and with the indicated compounds, freshly added every 48 h. At the end of the treatments, the cells were stained with FITC-labeled annexin-V and propidium iodide immediately before FACS analysis. Data are means ± SE of three independent experiments. *$P < 0.05$ *versus* Control (Student's *t*-tests).

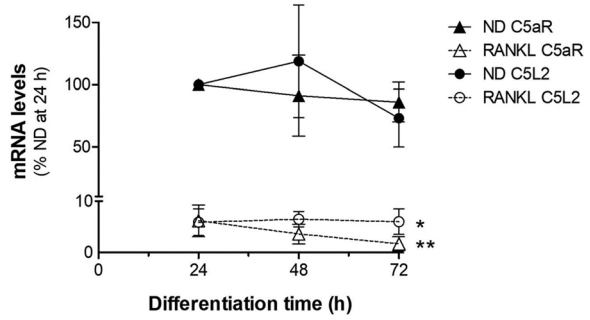

Fig. 5. Modulation of C5aR and C5L2 mRNA levels during osteoclastogenesis. RAW264.7 cells were treated in the absence (ND) and presence of 15 ng/mL RANKL. C5aR and C5L2 mRNA levels were analyzed using real-time PCR at 24, 48 and 72 h of differentiation, and here are expressed as percentage of the corresponding ND at 24 h. Data are means ± SEM of three independent experiments performed in duplicate. *$P < 0.05$ *versus* ND C5L2; **$P < 0.01$ *versus* ND C5aR.

Table 3
Modulation of C5aR, C5L2 and CTR mRNA levels by C5aR antagonists at 72 h of RANKL-induced osteoclastogenesis.

Condition		mRNA levels		
		C5aR (% ND)	C5L2 (% ND)	CTR (% RANKL)
ND	–	100.0	100.0	Not detectable
RANKL	–	3.4 ± 1.0***	16.3 ± 2.8***	100.0
	PMX-53	8.3 ± 2.5*	19.7 ± 8.1	56.0 ± 17.7*
	DF2593A	40.5 ± 21.0*	27.2 ± 18.0	59.7 ± 13.1*
	DF3016A	2.1 ± 0.7*	14.5 ± 4.6	47.6 ± 2.9**

RAW264.7 cells were treated in the absence (ND) and presence of 15 − 30 ng/mL RANKL, without and with the indicated compounds at 1 μM. Data are means ± SEM of at least three independent experiments performed in duplicates. *$P < 0.05$ and **$P < 0.01$ *versus* corresponding RANKL, ***$P < 0.001$ *vs* ND (Student's *t*-tests).

osteoclastogenesis markers to be transcribed. Altogether, these data demonstrate the involvement of C5aR signal transduction pathways in modulation of osteoclast maturation. Among the signaling cascades already described for C5aR in RAW264.7 cells [32,44; and references therein], activation of AKT and increases in intracellular calcium are the best candidates for modulation of RANKL-induced transcription remodeling. These signaling messengers are downstream of RANK activation by its ligand [37], and they represent hubs of pathways convergent with C5aR signal transduction.

C5a is a strong chemoattractant for many inflammatory cells, such as monocytes and macrophages [45]. In RAW264.7 cells, C5a induces cytoskeletal rearrangements by activating Cdc42 [46]. The subsequent chemotaxis through coupling with PTX-sensitive G proteins leads to differential stimulation of PLCβ_2, and ERK1/2 and p38 MAPK phosphorylation [33,46]. As actin remodeling and cell migration are essential for osteoclast fusion, the effects of C5aR on osteoclast syncytium formation were also evaluated. The loss of regulation of polykaryotic osteoclast formation through C5aR antagonism and interference can be attributed to reduced C5aR expression in the late stages of osteoclast differentiation of these RAW264.7 precursor cells.

The rapid large decreases in C5aR and C5L2 mRNA levels might be a consequence of RANKL-triggered transcriptional remodeling or of increased extracellular C5a levels. Both C5aR and C5L2 have been reported to undergo internalization and down-regulation upon sustained C5a activation [47]. For C5aR, this process is driven by homologous desensitization that is mediated by GPCR kinases and the arrestin proteins [48,49], and to avoid cell overstimulation, this can lead to receptor degradation in the lysosomal compartment [50]. Reduced C5aR neosynthesis might be another autoregulatory mechanism to maintain cell homeostasis in the presence of excessive agonist exposure. In line with this hypothesis, PMX-53 and DF2593A reversed the decrease in C5aR mRNA levels following 72 h of RANKL treatment. These compounds antagonize C5aR activation, and should also limit the consequent reduction in C5aR transcription. Instead, despite its C5aR antagonism, DF3016A further decreased C5aR levels through a regulatory process that has already been described in rat cortical neurons exposed to oxygen–glucose deprivation–reoxygenation [36]. Due to lack of sensitivity and specificity of the commercially available antibodies tested, information on C5aR protein expression levels and its cellular localization is still lacking. Nevertheless, these data highlight a fundamental difference among the C5aR antagonists that might explain the higher potency of DF3016A for inhibition of osteoclast functional activity. Indeed, DF3016A behaved not only as an antagonist of receptor signaling, but also as an inhibitor of C5aR transcription. This multiple inhibitory role of DF3016A might explain its high efficacy for inhibition of osteoclast bone resorbing activity, in comparison with the other antagonists analysed here. Instead, the finding that DF2593A and DF3016A assume different orientations in the binding site of the C5aR minor pocket (data not shown) might explain the distinct fine-tuned equilibrium of the downstream signaling events.

Although C5L2 is considered a decoy receptor, it has been reported to internalize in an arrestin-mediated process once it has bound the C5a ligand, to sequester and intracellularly degrade the excess C5a [51]. Also in this case, prolonged exposure to C5a might be the cause of the reduced C5L2 transcription. All three of these antagonists were ineffective on C5L2 levels, which suggests that they are selective towards C5aR. Indeed, although PMX-53 is a competitive antagonist for human C5aR, it does not interact with human C5L2 [52].

The presented data indicate a role for C5aR in transcription of the intermediate markers of differentiation that are essential in the cross-talk of osteoclasts with tumor cells. Several cancers have a predilection for skeletal tissue when they become invasive. Among these, breast and prostate cancers often metastasize to bone, where the cancer cells can find favorable factors related to bone resorption [53]. To metastasize, cancer cells must orchestrate diverse cellular functions to overcome the difficulties of the metastatic cascade, and so a way to block metastasis development might be through inhibition of bone resorption, which defines osteoclasts as the targets for such anti-metastatic treatments. Several lines of evidence have shown that C5a is present in the tumor microenvironment, as seen in preclinical animal models [54] and in tumor tissues from patients with breast or ovarian cancers [55,56], which indicates its pathogenic role. Aberrant expression of C5aR has been observed in several human cancer tissues and cell lines, and the C5a–C5aR axis has been demonstrated to promote cancer-cell invasion through motility activation and matrix metalloproteinase release [26]. Targeting this signaling pathway might represent a useful therapeutic option for cancer treatments.

Regardless of the precise mechanism of action, the finding that DF3016A diminishes osteoclast-resorbing activity uncovers a novel area of clinical interest. As a lead compound, DF3016A might represent a double-edged blade to fight bone metastases from several tumors, as it can decrease the osteoclast activity required for creation of metastatic niches and can act at the level of tumor cells by reducing their homing to bone.

Declaration of Competing Interest

The authors declare no potential competing interests.

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