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In Human Macrophages the Complement Component C5a Induces the Expression of Oncostatin M via AP-1 Activation

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Objective—Macrophages produce the cytokine oncostatin M (OSM), which beside other functions is also involved in inflammation. The complement component C5a mobilizes and activates these cells at inflammatory sites. We examined the effect of C5a on OSM production in human monocytes and in human monocyte-derived macrophages.

Methods and Results—For macrophage transformation peripheral blood monocytes were cultivated for 8 to 10 days in the presence of human serum. C5a significantly increased in these cells OSM antigen as determined by specific ELISA and mRNA as quantitated by real-time polymerase chain reaction in these cells as well as in plaque macrophages. This effect was blocked by antibodies against the receptor C5aR/CD88 and by pertussis toxin. The C5a-induced phosphorylation of p38 and JNK and the C5a-induced increase in OSM production in macrophages was abolished by 2 p38 inhibitors and by a JNK inhibitor. Furthermore C5a increased the nuclear translocation of c-fos and c-jun. Using different OSM promoter deletion mutant constructs we show that the putative AP-1 element is responsible for activation of OSM promoter activity by C5a.

Conclusion—Our data establish a link between the complement system and the gp130 receptor cytokine family with possible implications for the pathology of inflammatory diseases. (*Arterioscler Thromb Vasc Biol.* 2008;28:498-503)

Key Words: atherosclerosis ■ macrophages ■ inflammation ■ complement ■ oncostatin M

Macrophages by producing a vast array of biomolecules play a key role in a variety of physiological and pathophysiological processes such as immunity, inflammation, and tissue remodeling.¹ Among these biomolecules are various inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, or IL-6.² Macrophages are also considered to be the major producers of oncostatin M (OSM), which is a multifunctional cytokine belonging to the glycoprotein 130 (gp130) receptor cytokine family.^{3–5} OSM was originally isolated from phorbol 12-myristate 13-acetate (PMA)-treated human histolytic lymphoma U937 cells and plays a critical role in numerous physiological and pathophysiological events including inflammation, hematopoiesis, tissue remodeling, development, and cell growth.^{6,7} Besides macrophages also T cells, neutrophils, osteoblasts, dendritic cells, Kaposi's sarcoma cells, and microglia produce OSM.^{8–13} Its expression is upregulated by granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, human chorionic gonadotropin (hCG), HIV-1, lipopolysaccharide (LPS), cisplatin, or prostaglandin (PG) E2.^{11,13–16}

The potent anaphylatoxin C5a is generated during complement activation through cleavage of C5 and is released at the

inflammatory site. There, C5a mediates immune and inflammatory processes such as increased vascular permeability, spasmogenesis, immune regulation, and the release of a variety of inflammatory cytokines and mediators.^{17,18} C5a is a strong chemoattractant and is involved in the recruitment of inflammatory cells such as T lymphocytes, eosinophils, neutrophils, and monocytes and is regarded as the most potent chemoattractant for the latter 2 cell types.^{19,20} It contributes to rapid mobilization of phagocytic cells to and activation of these cells at the site of inflammation where such activation induces the release of reactive oxygen species and inflammatory cytokines from these cells.²¹

It was the aim of our study to investigate a possible link between the complement system and the gp 130 receptor cytokine family in inflammatory cells.

Methods

Cell Culture

Peripheral blood monocytes (PBMs), macrophages, and plaque macrophages were isolated, cultivated, and characterized as de-

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scribed recently.^{22,23} For details please see the supplemental materials, available online at <http://atvb.ahajournals.org>.

Treatment of Cells With rhC5a

Before all experiments described below, monocytes, macrophages, and plaque macrophages were seeded into 48-well dishes at a density of 100 000 cells per well and were incubated for 24 hours in serum-free Ultraculture medium supplemented with antibiotics and 0.1% BSA. Thereafter 250 μ L fresh medium was added to the wells with or without rhC5a at concentrations indicated, and the cells were incubated at 37°C for the time periods indicated. At the end of the incubation period, conditioned media from these cultures were collected and stored at -80°C. Before the experiments rhC5a was tested for LPS contamination using the Coatest LPS kit from Kabi Diagnostica. No LPS could be detected in the rhC5a using this assay (detection limit 0.05 ng/mL).

Quantification of OSM

OSM antigen in conditioned media was determined by a specific enzyme-linked immunosorbent assay (human OSM ELISA; R&D) using monoclonal antibodies.

Analysis of Specific Binding of AP-1 to DNA

Preparation of nuclear extracts of monocytes derived macrophages (MDM) was performed using a nuclear extract kit (Active Motif) according to manufacturer's instructions. c-fos and c-jun binding to their consensus oligonucleotide was determined using the ELISA-based Trans-AM AP-1 kit (Active Motif) according to the manufacturer's instructions. Specificity of c-fos and c-jun binding, respectively, was confirmed by incubation of nuclear extracts with the immobilized AP-1 consensus-binding probe in the presence of excess wild-type or mutated oligonucleotide (data not shown).

mRNA Purification

Cells were stimulated as described above, the supernatant was removed, and mRNA was isolated using QuickPrep Micro mRNA Purification Kit (Amersham Biosciences) according to the manufacturer's instructions.

Real-Time PCR

Real-time PCR was performed using LightCycler-RNA Master SYBR Green I (Roche) according to the manufacturers instructions. For details please see supplemental materials.

Western Blotting

Western blotting was performed as described previously.²⁴ For details please see supplemental materials.

Transfection of MDM

MDM were transfected with chimeric OSM promoter-luciferase reporter constructs as described recently.²⁵ For details please see supplemental materials.

Statistical Analysis

Data were compared by ANOVA. $P < 0.05$ was considered significant.

Results

rhC5a Stimulates OSM Expression in Human Monocyte-Derived Macrophages, Human Peripheral Blood Monocytes, and in Human Plaque Macrophages

As can be seen from the Table rhC5a significantly increased OSM production after 24 hours in human monocyte-derived macrophages (MDMs) and in human PBMs isolated from 3 different donors up to 5-fold in MDMs and PBMs. As a control PBMs were also isolated by negative selection using

Table. Effect of rhC5a on OSM Production in Monocyte-Derived Macrophages and Peripheral Blood Monocytes Isolated From Different Donors

	MDM		PBM	
	Control	rhC5a	Control	rhC5a
Donor 1	68.86 \pm 24.41	244.34 \pm 19.66*	11.55 \pm 5.41	57.94 \pm 5.87*
Donor 2	22.76 \pm 2.51	112.79 \pm 14.93*	13.1 \pm 6.34	64.31 \pm 4.12*
Donor 3	35.09 \pm 4.77	131.08 \pm 10.88*	12.45 \pm 4.32	67.42 \pm 5.97*

MDM and PBM were incubated for 24 h with rhC5a (0.5 μ mol/L). OSM values are given in ng/ml and represent mean values \pm SD of 3 independent determinations. * $P < 0.0001$.

the Monocyte Isolation Kit (Miltenyi Biotec). This isolation kit is an indirect magnetic labeling system for negative isolation of untouched monocytes from human peripheral blood mononuclear cells. Nonmonocytes are magnetically labeled using a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and CD235a (Glycophorin A), and removed by magnetic separation using anti-biotin magnetic MicroBeads. A comparison of OSM protein production in such negatively selected monocytes with conventionally by CD14 positively selected cells showed virtually no differences, neither in the control cells nor in cells treated with C5a (negative selection, control: 15.21 \pm 2.68 ng/mL; negative selection, 0.5 μ mol/L rhC5a: 56.43 \pm 5.41 ng/mL; * $P < 0.0001$; for values of positively selected cells please refer to the Table). In MDMs the effect of rhC5a was dose-dependent with maximum stimulation seen at a concentration of 1 μ mol/L rhC5a (Figure 1A). When the cells were incubated in the absence and presence of rhC5a at a concentration of 0.5 μ mol/L for 2, 4, 8, 12, and 24 hours, a significant increase in OSM at 4, 8, 12, and 24 hours was observed compared with values determined in untreated cells (Figure 1B). mRNA levels specific for OSM were significantly increased after 2-hour treatment of the cells with 0.5 μ mol/L rhC5a as compared with untreated control cells (Figure 1C). rhC5a at the same concentration also significantly increased OSM mRNA expression after 2 hours in human plaque macrophages isolated from 3 different donors 2.4-fold, 1.5-fold, and 1.6-fold, respectively.

rhC5a-Induced OSM Production in Human Monocyte-Derived Macrophages Is Mediated by C5aR/CD88

To determine the specificity of the rhC5a-induced increase in OSM production, we studied the effect of a mAb against C5aR/CD88 and pertussis toxin (PTX) on OSM production in human MDMs treated with rhC5a. The stimulatory effect of rhC5a on OSM production in these cells was completely blocked by preincubation of the cells with 20 ng/mL anti C5aR/CD88 mAb or 0.5 mg/mL pertussis toxin for 60 minutes. Preincubation with mAb against CD163 used as a control did not affect OSM production induced by rhC5a. Treatment of the cells with the anti C5aR/CD88 antibody, CD163 antibody, or PTX in the absence of rhC5a did not affect basal OSM production in these cells (Figure 2).

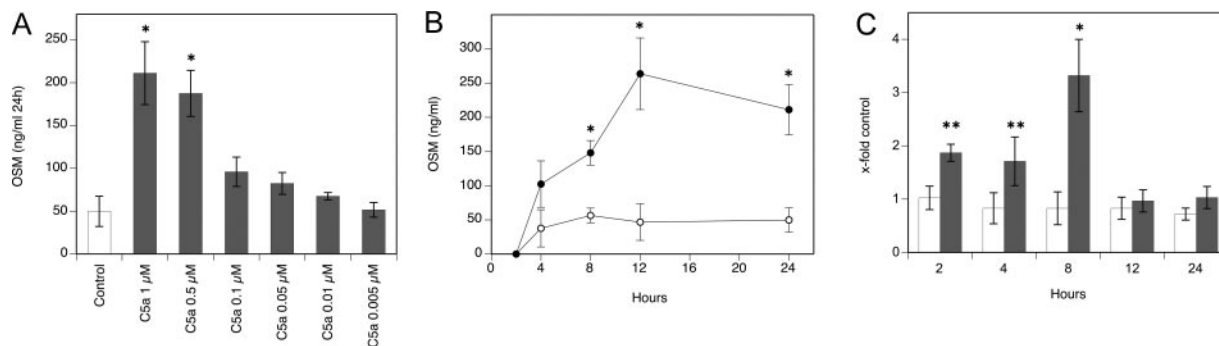


Figure 1. rhC5a increases OSM production and expression in monocyte-derived macrophages. MDM were incubated with rhC5a at indicated concentrations (A) or for indicated time periods in the absence or presence of rhC5a (0.5 μ mol/L; B). OSM mRNA levels were normalized according to the respective GAPDH mRNA levels (C). Values represent mean values \pm SD of 3 independent determinations performed for 3 different donors. ** P <0.05; * P <0.0001.

rhC5a Induces Phosphorylation of p38 Kinase and JNK in Human Monocyte-Derived Macrophages and rhC5a-Induced OSM Production in Human Monocyte-Derived Macrophages Is Mediated by p38 and AP-1

As can be seen from Figure 3A rhC5a time-dependently induced robust phosphorylation of p38 kinase and JNK. LPS was used as a positive control in these experiments at a concentration of 1 μ g/mL. This effect could be blocked by SB202190 used at 30 μ mol/L and 3 μ mol/L (Figure 3B), by BIRB0796 used at 1 μ mol/L and 0.1 μ mol/L (Figure 3C), and by JNK-Inhibitor used at 10 μ g/mL (Figure 3D), respectively. JNK-Inhibitor used at 1 μ g/mL, however, was not able to block C5a-induced phosphorylation of JNK (Figure 3D).

Pretreatment of MDMs with p38 inhibitors (SB202190 or BIRB0796) or with JNK inhibitor blocked the effect of rhC5a

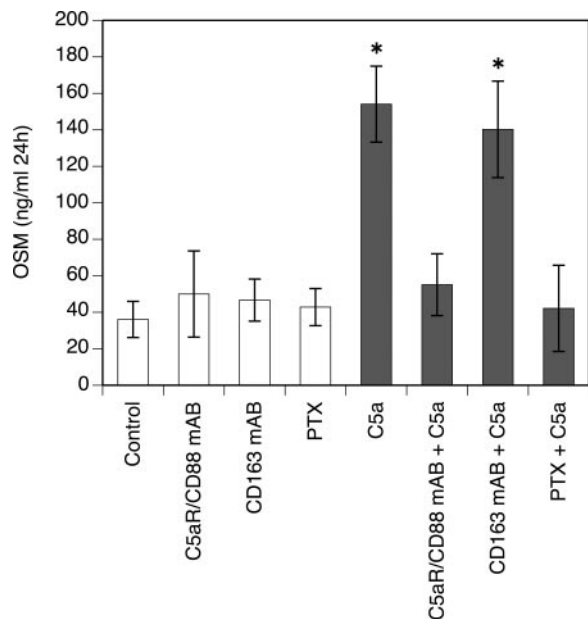


Figure 2. Effect of monoclonal anti-C5aR/CD88 and pertussis toxin on C5a-induced OSM production in monocyte-derived macrophages. MDMs were incubated for 60 minutes at 37°C with anti-C5aR/CD88 mABs (20 μ g/mL), with mABs against CD163 (20 μ g/mL) as a control, or with PTX (0.5 mg/mL), followed by incubation for 24 hours in the presence or absence of rhC5a (0.5 μ mol/L). OSM values represent mean values \pm SD of 3 determinations performed for 3 different donors. * P <0.0001.

on OSM production in these cells completely. For details please see supplemental materials. As can be seen from Figure 4A, rhC5a also induced translocation of the AP-1 subunits c-fos and c-jun to the nucleus of MDM 16-fold and 15-fold over control.

Transcriptional Activation of OSM Promoter by rhC5a Requires AP-1 Sequence

To identify functional *cis*-acting elements in the OSM promoter that are required for rhC5a induced gene expression, a -304bp wild-type, a -194bp wild-type, a -109bp wild-type, and a -304bp OSM-promoter construct with a mutated AP-1 binding site, respectively, were transfected into MDMs.²⁵ Luciferase analysis demonstrated that the minimum promoter sequence required for rhC5a-induced OSM expression in MDMs resides within -194bp. Stimulation with rhC5a at a concentration of 0.5 μ mol/L resulted in significant increase of luciferase activity when cells were transfected with -304bp and the -194 bp constructs. Further deletion to -109bp decreased luciferase activity to the control level of unstimulated cells. Similarly, when cells were transfected with the -304bp construct containing a mutated AP-1 binding site, luciferase activity in rhC5a stimulated cells was reduced to values seen in cells transfected with the same construct but incubated in the absence of rhC5a (Figure 4B), demonstrating that a functional AP-1 binding site in the OSM promoter is required for its transcriptional activation by rhC5a.

Discussion

As providers of innate immune surveillance macrophages are ubiquitously present in the human body. Besides their role in host defense and immunology, macrophages are key regulators of other physiological and pathophysiological processes such as inflammation and tissue remodeling by producing a host of cytokines, growth factors, and proteases.² The multi-functional cytokine OSM is one of these macrophage-derived modulators of such processes. OSM is also produced by T cells, eosinophils, and neutrophils, but besides the latter cell type macrophages are thought to be its main source. OSM, which was originally described as a regulator of cell growth, has been more recently shown to be also implicated in the regulation of inflammation and in the remodeling of extra-

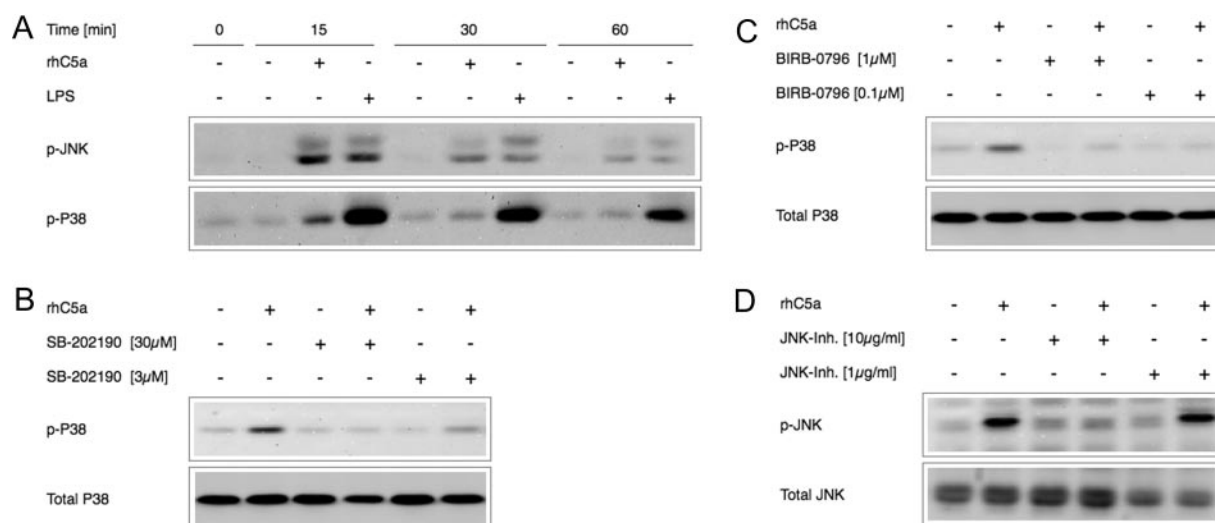


Figure 3. rhC5a induces phosphorylation of p38-kinase and JNK (A–D). MDMs were incubated with rhC5a (0.5 $\mu\text{mol/L}$) or LPS as a positive control (1 $\mu\text{g/mL}$); A). MDMs, preincubated for 30 minutes with SB202190 (B), BIRB0796 (C), and JNK Inhibitor (D), were treated with rhC5a (0.5 $\mu\text{mol/L}$) for 15 minutes.

cellular matrix.^{3–5} Here we present evidence that the expression of OSM in human peripheral blood monocytes and in human monocyte-derived macrophages is upregulated by the anaphylatoxin C5a. C5a generated through complement activation at sites of inflammation is a strong chemoattractant for monocytes and macrophages and activates these cells to release reactive oxygen species and inflammatory cytokines.^{19–21}

We found that in human monocyte-derived macrophages the stimulating effect of rhC5a on OSM synthesis was dose- and time-dependent. Maximum effects were observed with 0.5 $\mu\text{mol/L}$ rhC5a, and a significant increase in OSM production by human monocyte-derived macrophages was seen after 4, 8, 12, and 24 hours of treatment with rhC5a. It should be noted that the lowest effective concentration in our experiments was 0.1 $\mu\text{mol/L}$. Because this concentration is somewhat higher than the highest plasma concentrations of C5a found in humans, caution should be used in extrapolating results of our experimental study to the clinical setting.²⁶ One could speculate, however, that local concentrations of the anaphylatoxin at the site of complement activation in vivo might be higher than plasma levels and thus might be sufficiently high to induce the expression of OSM in monocytes or macrophages present at these sites. Furthermore it should be noted that the concentrations used here are similar to concentrations of C5a used in other in vitro studies.^{27–29} Macrophages treated with the anaphylatoxin expressed significantly higher levels of OSM specific mRNA as compared with mRNA levels in untreated macrophages. rhC5a also increased OSM mRNA levels in human macrophages isolated from atherosclerotic plaques.

Boiling destroyed the capacity of C5a to induce OSM production in macrophages, whereas pretreatment of cells with polymyxin-B had no effect on C5a-induced upregulation of OSM production. Based on these results a possible contribution of LPS to the observed increase in OSM can be ruled out. Pretreatment of cells with an anti-C5aR/CD88 antibody blocked the rhC5a-induced expression of OSM

whereas a control antibody against CD163 showed no effect. Thus we conclude that the interaction of rhC5a with its receptor C5aR/CD88, which belongs to the 7 transmembrane receptor subfamily of G_i -coupled receptors, is necessary to induce OSM production. We could show that the rhC5a-induced upregulation of OSM expression in human macrophages is PTX-sensitive, indicating that this effect—like other C5a-induced leukocyte-reactions such as degranulation or chemotaxis—is also modulated through G_i -coupled receptors.³⁰

Furthermore, we provide evidence that the rhC5a-induced increase in OSM production in macrophages involves AP-1 activation. We could show that rhC5a caused phosphorylation of JNK and p38 kinase in these cells and that such phosphorylation was blocked by p38 inhibitors and by a JNK inhibitor. Furthermore we showed that in macrophages these inhibitors also blocked C5a-induced upregulation of OSM indicating that this effect could be dependent on activation of c-jun and c-fos. It should be noted that p38 is not necessarily involved in activation of c-jun and c-fos, but our results are in agreement with other findings in macrophages and other cell types.^{31,32} In support of these results we could show that rhC5a induced translocation of the AP-1 subunits c-fos and c-jun to the nucleus of macrophages. These findings are in line with a recent study showing that C5a activates AP-1 in macrophages.³³ Furthermore it should be emphasized that an AP-1 site has been identified in the human OSM promoter.²⁵ By transfection with a series of different OSM promoter deletion mutant constructs and a promoter construct with a mutated AP-1 binding motif we could show that the putative AP-1 element is responsible for activation of OSM promoter activity by rhC5a.

It is also known that NF- κ B plays a role in spontaneous release of OSM in osteoarthritic synovial cells.³⁴ Our results showing that an NF- κ B inhibitor did not affect C5a-induced upregulation of OSM in human macrophages suggests that nondirect effects of C5a-induced NF- κ B activation are not involved in this upregulation.

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