C5aR and TLR crosstalk

*Regulatory effect of anaphylatoxin C5a on human dendritic cells*

Zaal, A.

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The anaphylatoxin C5a attenuates Fc-gamma receptor-mediated uptake of immune complexes by human dendritic cells

Manuscript in preparation

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Abstract

Objective. Dendritic cells (DCs) can take up and present immune complexes (ICs) to T cells, thereby modulating antigen-specific adaptive T cell immune responses. Recently, DCs were found to be susceptible to regulation by complement component 5a (C5a), which is formed upon complement activation. It is unclear, however, whether C5a affects IC uptake by DCs. This may be of relevance in diseases associated with both aberrant complement activation and presence of self-antigen-containing ICs, like observed in several autoimmune diseases. The present study investigates the effect of C5a on IC uptake by human monocyte-derived dendritic cells (moDCs).

Methods. Transcriptomics of human moDCs either or not exposed to C5a were analyzed for C5a-dependent effects on biological processes using Gene ontology (GO) term enrichment analysis. Functional effects of C5a on Fc-gamma receptor (FcγR) expression and uptake of ICs were investigated using (imaging) flow cytometry analysis.

Results. Stimulation of moDCs with C5a resulted in differential expression of 49 genes involved in Fc receptor signaling, phagocytosis and endosomal maturation. Functional analyses showed that C5a did not affect expression of FcγRs, but selectively reduced FcγR-mediated uptake of large ICs. Interestingly, C5a did not affect ingestion of small ICs.

Conclusion. Human dendritic cells can be selectively skewed away from uptake of large ICs in the presence of the activated complement component C5a.
Introduction

DCs can internalize and process antibody-coated antigens, also called ICs, and present their peptides to T cells. This mechanism helps fighting against invading pathogens by facilitating activation of antigen-specific T cells and production of antigen-specific antibodies through the induction of T cell-dependent B cell differentiation. On the negative side, presentation of ICs composed of self-antigens and autoantibodies in autoimmunity may support autoreactive T cells and further autoantibody formation. Indeed, several autoimmune diseases are associated with the presence of such ICs and autoantibodies, such as systemic lupus erythematosus\(^1\) and rheumatoid arthritis\(^2\).

C5a is the most potent chemoattractant produced during complement activation. Apart from recruitment of immune cells, C5a can directly modulate human DC function by regulating production of pro-inflammatory cytokines\(^3\)\(^-\)\(^5\). Furthermore, priming of human DC with C5a during LPS stimulation reduces IFN-\(\gamma\) production in DC-T cell co-cultures\(^5\). This shows that there is extensive crosstalk between complement and DC activation in inflammation and that complement activation products can indirectly contribute to adaptive immunity by modulating DC effector functions. Interestingly, complement activation and presence of complement activation products, such as C5a, are also associated with autoimmunity\(^6\)\(^-\)\(^10\). Whether C5a can affect IC uptake by DCs, and thereby antigen presentation to cells of the adaptive immune system, is still unclear. In the present study, we assessed involvement of C5a in antigen acquisition and specifically uptake of ICs by human moDCs.

Materials and Methods

RNA sequencing and GO term enrichment analysis

RNA sequencing analysis of human moDCs stimulated for 2 h with C5a followed by differential expression analysis was performed previously\(^11\). Sequencing data are publicly available at Gene Expression Omnibus series under accession number GSE101396. GO term enrichment analysis was performed on the 219 differentially expressed genes upon C5a exposure of human moDCs as previously described\(^11\), in which GO terms were filtered using the cut off >15 and <500 genes. For comparative analysis BioVenn was used (http://www.biovenn.nl/index.php)\(^12\).
Generation and stimulation of moDCs

Generation of moDCs was performed as described previously\(^3\). At day 6-7, moDCs were plated at 1 \(\times\) 10\(^6\) cells/well (24-wells plate; for qRT-PCR), 0.5 \(\times\) 10\(^6\) cells/well (24-wells plate; for Salmonella typhimurium and IC uptake experiments) or 1 \(\times\) 10\(^5\) cells/well (96-wells plate; for Luciferase yellow and Transferrin uptake experiments). moDCs were rested for 2 h in Cellgro DC serum-free medium (CellGenix, Freiburg, Germany) supplemented with penicillin/streptomycin (100 U/ml; Life Technologies, Gibco, Carlsbad, CA, USA) and 1% Fetal Calf Serum (FCS; Sigma-Aldrich, St Louis, MO, USA) at 37\(^\circ\)C, 5% CO\(_2\). Next, moDCs were stimulated with C5a (10 nM; Sigma-Aldrich or R&D Systems, Oxon, UK) and were incubated for 2 h or overnight at 37\(^\circ\)C, 5% CO\(_2\).

qRT-PCR

Lysis of moDCs, mRNA extraction, cDNA synthesis and analysis of mRNA expression was performed after 2 h of stimulation as described previously\(^5\). Primers were ordered at Eurogentec (Seraing, Belgium) and mRNA expression was normalized using 18S rRNA. Primer sequences can be found as in Supplemental Table 1.

Generation of ICs and HP-SEC

ICs between recombinant human monoclonal anti-infliximab and infliximab (Remicade, Centocor, PA, USA) labelled with DyLight488 (Invitrogen) (IFX-488) were made as described previously by van Schie et al. (manuscript in preparation). An equal amount of the anti-infliximab antibodies and IFX-488 was used, at a final concentration of 150 µg/ml for each antibody. The mixture was incubated for 1 h at room temperature to allow formation of ICs, which was confirmed by HP-SEC as described by van Schie et al. (manuscript in preparation). To obtain different sizes of ICs, two anti-infliximab clones were used. Both clones were expressed as IgG1.

Antigen uptake by moDCs

moDCs were stimulated overnight with C5a or left untreated followed by incubation with Transferrin Alexa Fluor 647 (40-400 µg/ml; Molecular Probes, Invitrogen, Carlsbad, CA, USA), Luciferase Yellow (25-250 µg/ml; Sigma-Aldrich) for 30 min or ICs (10 µg/ml total antibody) for 60 min. After incubation, moDCs were washed with PBS and fixated for 20 min in 4% paraformaldehyde. To block FcγRs, moDCs were pre-incubated for 20 min with 10 µg/ml anti-CD16 F(ab')\(_2\) (Ancell, MN, USA), 10 µg/ml anti-CD32 F(ab')\(_2\) (clone AT10, AbD Serotec,
Kidlington, UK and 10 μg/ml purified human IgG-Fc fragments (Bethyl, Montgomery, AL, USA).

To assess uptake of *S. typhimurium* (strain SL1344) by moDCs, dsRED-labelled carbenicillin resistant *S. typhimurium* (construct pMW211) were grown in Luria-Bertani (LB) medium supplemented with 50 μg/ml carbenicillin overnight at 37°C while shaking. Bacteria were diluted 1/33 and cultured for 2 h before they were washed twice with PBS and fixed with 4% paraformaldehyde. Aspecific binding sites were blocked with 0.02 M glycine. Bacteria were opsonized for 30 min at 37°C with 10% human serum or 10% heat inactivated human (h/i) serum in PBS supplemented with 10 mM CaCl₂, 2 mM MgCl₂. Bacteria were washed with PBS and resuspended in Cellgro DC serum-free medium, after which they were incubated with moDCs for 1 or 2 h. After incubation, moDCs were fixed as described above.

### Flow cytometry and Imagestream

To determine antigen uptake by moDCs, antigen-exposed moDCs were membrane stained using anti-HLA-DR APC or anti-DC-SIGN HorizonV450 (BD Biosciences, San Jose, CA, USA) and analyzed using flow cytometry (five-laser LSRII, BD Biosciences) or Amnis Imagestream Mark II (Millipore, Darmstadt, Germany). Data were processed using FlowJo v10 or IDEAS software version 6.1 (Merck Millipore), respectively. For flow cytometry analysis, moDCs were gated as single HLA-DR or DC-SIGN positive cells. For Imagestream analysis, moDCs were gated as single cells (using Aspect Ratio vs Area of both the bright field and the membrane stain), focused cells (using bright field Gradient RMS), and HLA-DR or DC-SIGN positive cells. In case of uptake of *S. typhimurium*, additional gating on moDCs with *S. typhimurium* in focus was performed. *S. typhimurium* or IFX-488 positive cells were selected (using Intensity in the dsRED channel or a combination of Intensity and max pixel of the IFX-488 channel, respectively). Internalization was determined using the Internalization Erode modus and gating of moDCs with intracellular *S. typhimurium* or IFX-488. Percentage of antigen- or IC-containing moDC refers to the percentage of moDCs containing intracellular *S. typhimurium* or IFX-488 of the total number of HLA-DR or DC-SIGN positive moDCs within the focal plane.

FCγR expression was determined using flow cytometry (five-laser LSRII; BD Biosciences). moDCs were stained with anti-CD16 PE (BD Biosciences), anti-CD32 APC (BD Biosciences) and anti-CD64 FITC (AbD Serotec) in PBS supplemented with 0.5% BSA, 0.01% sodium azide and 3 mg/ml human gamma globulin after overnight stimulation. Expression was analyzed in FlowJo v10.
**Statistical analysis**

Results were analyzed for statistical significance in GraphPad Prism Version 7.03 (La Jolla, CA, USA) using paired student t test (*P<0.05). For RNA sequencing data, a Wald test with a Benjamini-Hochberg (FDR-adjusted P-value) (*P<0.05, **P<0.001) was used as described previously\(^\text{11}\).

**Results**

*GO term enrichment analysis is suggestive for a role of C5a in phagocytosis, endosomal maturation and Fc receptor signaling*

Recently, we revealed that C5a induces an immune regulatory gene expression profile upon C5a receptor and Toll-like receptor crosstalk in human DCs\(^\text{11}\). In addition, whole transcriptome analysis on human moDCs revealed that exposure to C5a itself significantly affected expression of 219 genes in human moDCs\(^\text{11}\). Comparative analysis between the transcriptome of moDCs either or not exposed to C5a revealed that C5a reduced expression of 33 genes, while increasing expression of 186 genes\(^\text{11}\). It remains to be determined, however, whether C5a stimulation affects specific functional processes in DCs and whether these are relevant for to initiation or progression of autoimmune diseases.

GO term enrichment analysis on the 219 differentially expressed genes revealed that C5a affected the expression of genes involved in cell migration, immune response and cytokine production (Figure 1), confirming the well-known functions of C5a in these processes\(^\text{3, 13}\). Interestingly, C5a specifically induced differential expression of genes associated with Fc receptor signaling, phagocytosis and endosomal maturation (Figure 1 and Supplemental Table 2-3). In total, 22% of the genes (49 out of 219 in total) affected by C5a fell into these categories, and most genes were found to be upregulated upon C5a exposure (Figure 2A-B)\(^\text{11}\). The effect of C5a on gene expression upon transcriptome analysis was confirmed using qRT-PCR for three randomly chosen genes (Figure 2C). Comparative analysis between the genes involved in phagocytosis and endosomal maturation and the genes involved in Fc receptor signaling revealed that 18% (9 of 49) of the genes were involved in both processes (Figure 2B), whereas 82% of the genes were uniquely associated with phagocytosis and endosomal maturation (28 of 49) or Fc receptor signaling (12 of 49). C5a thus differentially regulates phagocytosis and endosomal regulation and Fc receptor signaling through a wide variety of genes and does not target only a few genes involved in these processes.
C5a attenuates FcγR-mediated uptake of immune complexes

C5a reduces uptake of immune complexes by moDCs

To assess if C5a indeed regulates antigen acquisition by moDCs, effects of C5a on uptake of antigen by moDCs via various potential routes of antigen acquisition was investigated. C5a did not modulate uptake of fluid phase antigen (Luciferase Yellow) (Supplemental Figure 1A), receptor-mediated cargo (Transferrin) (Supplemental Figure 1B) or uptake of large particles in the form of serum opsonized S. typhimurium (Supplemental Figure 1C and D).

Since Fc receptor signaling and Fc receptor-mediated internalization are essential for uptake of ICs, the effect of C5a on IC uptake by moDCs was

Figure 1. GO term enrichment analysis of differently expressed genes upon C5a exposure in moDCs. The 219 genes differentially expressed upon C5a exposure of moDCs were analyzed using GO term enrichment analysis. As described in Zaal et al.11, red nodes represent gene sets defined by a specific GO term, node size reflects the number of genes within specific GO terms and green lines indicate the degree of overlapping genes between the GO terms. Ellipses represent the different categories to which the GO terms were enlisted, in which the names reflect the GO terms within the ellipses.
Figure 2. C5a alters expression of 49 genes involved in phagocytosis, endosomal maturation and Fc receptor signaling. (A) Visualization of enrichment of significantly affected genes involved in phagocytosis, endosomal maturation and Fc receptor signaling (green; genes with selected GO term, number of reduced and induced genes are depicted in green in the lower left and right corner, respectively) upon C5a exposure of moDCs. Depicted are the fold change and FDR-adjusted $P$-value in a minus log 10 transformation (blue; all significantly affected genes, number of significantly reduced and induced genes are indicated in the lower left and right corner, respectively, orange; selected hits to confirm RNA sequencing results in C). (B) Comparative analysis of genes involved in phagocytosis and endosomal maturation and genes involved in Fc receptor signaling. Size and overlap of the circles is proportional. (C) Normalized counts of RNA sequencing analysis (upper graphs) and relative mRNA levels determined by qRT-PCR (lower graphs) of selected genes from A in moDCs either or not exposed to C5a. Error bars represent standard error of the mean of four or six independent experiments, respectively.
C5a attenuates FcγR-mediated uptake of immune complexes

addressed. ICs of different sizes were generated using DyLight488-labeled infliximab and two different human monoclonal anti-infliximab antibodies (clone 2.1 and clone 2.4; van Schie et al., manuscript in preparation). Infliximab with anti-infliximab clone 2.1 (IC 2.1) yielded mainly dimers and tetramers, whereas infliximab with anti-infliximab clone 2.4 (IC 2.4) gave mainly rise to tetramers, hexamers and ICs of larger sizes (Figure 3A; van Schie et al., manuscript in preparation).

Incubation of moDCs with ICs resulted in substantial fractions of IC 2.1-positive and IC 2.4-positive moDCs, although somewhat less pronounced for IC 2.1 (Figure 3B). Overnight exposure of moDCs to C5a before incubation with ICs specifically reduced the fraction of IC 2.4-positive moDCs (Figure 3B). To determine whether ICs were indeed internalized, moDCs were analyzed by imaging flow cytometry using Imagestream. Of the IC 2.1- and IC 2.4-positive moDCs, 98-100% displayed intracellular IC staining (Figure 3C and data not shown). Pretreatment with C5a significantly diminished uptake of IC 2.4, while not affecting uptake of IC 2.1 (Figure 3D), in line with our findings using flow cytometry (Figure 3B). IC uptake by moDCs was FcγR dependent, as uptake was completely abolished in presence of Fc block (Figure 3E). These data show that C5a specifically diminishes uptake of large ICs.

FcγR expression is not affected by C5a in moDC

Although C5a affected expression of 49 genes involved in phagocytosis, endosomal maturation and Fc receptor signaling (Figure 2B), expression of FcγRs itself was not affected by C5a in moDCs after 2 h of stimulation (Figure 3F). At the time of IC challenge, however, moDCs were pre-incubated with C5a for 24 h, instead of the 2 h used to analyze mRNA expression. To determine whether C5a affected FcγR expression by moDCs at the time of IC challenge, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) expression was assessed by flow cytometry after 24 h of exposure to C5a. Treatment with C5a did not alter surface expression of FcγRI, FcγRII or FcγRIII (Figure 3G). Also after 48 h of stimulation, no changes in FcγR expression were observed (data not show). In conclusion, C5a diminishes FcγR-mediated uptake of ICs by human moDCs without affecting expression of FcγRs.

Discussion

C5a can modulate human DC effector function by regulating production of pro-inflammatory cytokines by DCs and reducing the capacity of DCs to induce IFN-γ production by CD4+ and CD8+ T cells. In this study, transcriptome
Figure 3. C5a reduces FcγR-mediated uptake of ICs by moDCs without affecting FcγR expression. (A) HP-SEC analysis of immune complexes (ICs) generated with Dylight 488-labelled infliximab and anti-infliximab clone 2.1 (IC 2.1) or anti-infliximab clone 2.4 (IC 2.4). (B) moDCs were incubated with IC 2.1 or IC 2.4 after overnight incubation in absence (dotted line) or presence (solid line) of C5a. moDCs without ICs were used as negative control (grey). Positivity for ICs was determined by flow cytometry in 2 independent donors (upper and lower panels). (C) Example of IC 2.4 positive moDCs analyzed by Imagestream. (D-E) Percentage of moDCs that have taken up ICs analyzed using Imagestream. (D) moDCs were incubated overnight in presence or absence of C5a after which IC 2.1 or IC 2.4 was added for 60 min (n=6). (E) moDCs were incubated with IC 2.4 after overnight incubation in absence or presence of C5a. Fc block was added 20 min prior to incubation with ICs (n=2). (F) Normalized counts of RNA sequencing data are depicted for the 7 FcγR genes (n=4). (G) Cell surface expression of FcγRI, FcγRII and FcγRIII on moDCs after 24 h of incubation in absence or presence of C5a. Control depicts the relevant isotype control. Representative of 2 independent experiments is depicted.
analyses indicated a potential regulatory role of C5a on antigen acquisition. Functional analyses showed that C5a can diminish acquisition of larger, but not small-sized, ICs by DCs in a FcγR-dependent manner, without altering expression of FcγRs. Although the finding that C5a downmodulates uptake of large ICs by DCs is striking, the implications of our findings need further investigation. Acquisition of antigen-containing ICs by DCs is important for the induction of antigen-specific T and B cell responses. On one hand, these data indicate that C5a may downmodulate uptake of antibody-coated antigens and their subsequent antigen presentation by DCs to T cells during infection. This indicates that upon complement activation during infection, influx of antigen-specific antibodies and IC formation may effectively give rise to negative feedback signals; antigen uptake in the form of ICs will be limited through the action of C5a potentially contributing to return to homeostasis. In addition, C5a may have a protective role in autoimmune diseases by reducing activation of autoreactive T cells after DC acquisition of self-antigen containing ICs. Indeed, deficiencies in complement components of the classical complement pathway strongly increase the susceptibility to develop SLE\textsuperscript{7}. In contrast, however, increased complement activation and C5a levels have also been reported during disease activity in several autoimmune disease\textsuperscript{8-10}, indicating that high C5a levels are not by definition protective. Overall, the present study demonstrates that C5a has a regulatory role in FcγR-mediated IC acquisition by human DCs. Future research in this exciting new field of investigating the interactions between complement and DC activation, should address the full implications of our findings in autoimmunity and upon infections.

Acknowledgments

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Author Contributions

AZ, ATB, and SMVH designed the research and discussed data and content of the manuscript. BN performed the GO term enrichment analysis. MD performed the quantitative real-time PCR analysis. KVS performed the HP-SEC analysis. AZ performed all other experiments, analyzed the data, and wrote the manuscript. KVS and TR helped with the generation of immune complexes. ATB, SMVH, TR and KVS corrected the manuscript.
Conflict of interest

The authors declare that this study received an unrestricted grant from Viropharma. Viropharma was not involved in the study design or collection, analysis, or interpretation of the data.

References

C5a attenuates FcγR-mediated uptake of immune complexes

SUPPLEMENTARY MATERIAL

Figure S1. C5a does not affect uptake of Luciferase Yellow, Transferrin or Salmonella typhimurium by moDCs. (A-B) moDCs were incubated overnight in absence (medium; dotted lines) or presence of C5a (C5a; solid lines), followed by incubation for 30 or 60 min with (A) 25-250 µg/ml Luciferase Yellow or (B) 4-40 µg/ml Transferrin-AF647. moDCs not incubated with Luciferase Yellow or Transferrin are depicted as controls (filled histograms). Histograms are representatives of 2 independent experiments. (C) Example of S. typhimurium-positive moDCs analyzed by Imagestream. (D) Percentage of moDCs that have taken up serum opsonized S. typhimurium (n=2) or S. typhimurium opsonized with heat inactivated (h/i) serum (n=3).
### Table S1. Primers used for real time quantitative PCR

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### Table S2. GO terms in the category Fc receptor signaling

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FDR, false discovery rate adjusted p-value

### Table S3. GO terms in the category Phagocytosis and Endosomal maturation

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FDR, false discovery rate adjusted p-value