C5aR and TLR crosstalk

Regulatory effect of anaphylatoxin C5a on human dendritic cells

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Crosstalk between Toll-like receptors and C5a receptor in human monocyte-derived DCs suppresses inflammatory cytokine production

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Abstract

The complement anaphylatoxin C5a, has been implicated in regulation of adaptive immune responses through modulation of APC function, as shown mainly in studies in mice. C5a was shown to enhance cytokine production in immature dendritic cells (DCs), but the effect of C5a on DC function during DC activation has not been elucidated in human. In this study, we investigated the effect of C5a on human monocyte-derived DCs (moDCs) when simultaneously stimulated with TLR ligands. While C5a indeed enhanced cytokine production of immature DCs, the addition of C5a inhibited production of IL-12, IL-23 and TNF-α induced by various TLR ligands, such as LPS, R848 and Pam₃CSK₄. The inhibitory effect of C5a on LPS-induced IL-6 production was less pronounced and LPS-induced IL-10 was not affected at all. This indicates that C5a receptor (C5aR) signaling has a differential effect on human DC differentiation depending on the crosstalk with other receptors. Furthermore, we found that C5a affects the LPS induced cytokines in a small time frame, and requires almost concurrent signaling of C5aR and TLR4. These data emphasize the complexity of DC regulation by anaphylatoxins. While complement activation may provide pro-inflammatory signals to immature DCs in the absence of pathogens, the same products may serve to downmodulate or deviate immune responses upon combat against infections. These context depending effects of anaphylatoxins on immune responses may have important implications for the emerging use of complement inhibitors in clinical practice.
Introduction

During complement activation, the anaphylatoxins C3a and C5a are generated by the cleavage of complement proteins C3 and C5 by the C3 and C5 convertases. The anaphylatoxins have chemotactic and activating effects on innate immune cells, such as neutrophils and mast cells. More recently, the anaphylatoxins have also been implicated to play an important role in shaping the adaptive immune response\(^1\)\(^-\)\(^5\). In mice, local production and/or activation of C3 and C5 was shown to mediate graft rejection through increase in pro-inflammatory cytokine secretion\(^3\)\(^,\)\(^6\)\(^,\)\(^7\). These findings were supported by studies in mice, in which a disabled C3a-C3aR or C5a-C5aR interaction resulted in an impaired Th1 response and an increased time of graft survival\(^1\)\(^-\)\(^3\). C3a and C5a were shown to bind to their receptors (C3aR and C5aR, respectively) on DCs, hereby enhancing IL-12 production and upregulating co-stimulatory markers of these DCs. Subsequently, Th1 polarization of interacting T cells was enhanced\(^1\)\(^-\)\(^3\).

Most complement proteins are mainly synthesized by hepatocytes. Cells of the immune system, however, can also synthesize complement factors extrahepatically. Complement proteins produced by immune cells are thought to have local effects. Both APCs and T cells express several complement receptors and human DCs have also been described to produce complement factors\(^8\)\(^-\)\(^11\). The effect of C5a on cytokine production by human monocyte-derived DCs (moDCs) is still not fully cleared. While in the past no effects were observed\(^12\), a recent paper\(^13\) described an enhancing effect of C5a on immature human moDC activation and cytokine production, which is in agreement with observations in mice\(^1\)\(^-\)\(^3\). In contrast to the stimulatory effect assigned to C5a, negative effects of the anaphylatoxins on cytokine production by TLR-stimulated APCs in mice\(^1\)\(^,\)\(^14\)\(^,\)\(^15\) as well as in human monocytes have also been described\(^12\)\(^,\)\(^16\)\(^,\)\(^17\). Taken together, these data suggest that the impact of C5aR and C3aR signaling on production of inflammatory cytokine production by APCs is complex.

Until now most studies concerning the effect of anaphylatoxins on the adaptive immune response have been performed in mice and information on the effects of anaphylatoxins on human APCs is more restricted. Studies on the effects of complement on human APC function is necessary to better understand the contextual differential effects of the anaphylatoxins on immune modulation. This has recently become even more important, as several complement modulatory compounds (such as the C5 blocking monoclonal antibody eculizumab and low molecular weight complement inhibitors as compstatin) are now available for clinical practice or will enter the clinic soon\(^18\),\(^19\). In this study, we investigated the effect of C5a on human moDCs when simultaneously stimulated with TLR
ligands. Pro-inflammatory cytokine production, such as IL-12, IL-23 and TNF-α, induced by LPS-maturation of DCs was inhibited by C5a. In addition, also pro-inflammatory cytokines induced by other TLR ligands could be inhibited by C5a, indicating crosstalk between C5aR and TLR signaling. In contrast, C5a enhanced cytokine production of immature DCs. These results imply that C5a has a differential effect on human DCs depending on the absence or presence of pathogen-sensing signals by local DCs, thus emphasizing the complexity of DC regulation by anaphylatoxins.

Materials and Methods

Reagents, media and cell lines

Cellgro DC serum-free medium, IL-4 and GM-CSF were obtained from CellGenix (Freiburg, Germany). All monoclonal antibodies (mAb) used for flow cytometry were obtained from Becton Dickinson (BD, San Jose, USA), except the anti-C5aR which was obtained from Biolegend (San Diego, USA). C5a was obtained from Sigma-Aldrich (Steinheim, Germany). C5a receptor antagonist (C5aRA), W-54011, was from Calbiochem (Darmstadt, Germany). Fetal Calf Serum (FCS) was from Bodinco (Alkmaar, The Netherlands). LPS (E.coli 0111:B4), Pam3CSK4 and R848 were from InVivoGen (San Diego, USA). Salmonella typhimurium was grown as described before20. Bacteria were heat killed by incubation at 65°C for 20 min.

Dendritic cell generation and phenotyping

Monocytes were isolated from fresh aphaeresis material of healthy volunteers (Sanquin Blood Bank North West, Amsterdam, The Netherlands) upon informed consent by using the Elutra cell separation system (Gambro, Lakewood, USA). Monocytes were cultured at a concentration of 1 x 10^6 cells/ml in 20 ml of in Cellgro medium supplemented with GM-CSF (1000 IU/ml) and IL-4 (800 IU/ml) in a 80 cm² cell culture flask (Nunc, Roskilde Denmark). At day 6 the immature DCs were harvested and were cultured in 96-wells plates with 2 x 10^5 imDC/well or in 6-wells plates with 1 x 10^6 imDC/well in Cellgro medium containing 1% FCS. Routinely more than 85% of the harvested cells are within the ‘DC-gate’ and are alive. Previous studies showed that immature DCs generated in this manner are able to express CD83 upon maturation stimuli21, 22. Maturation was performed for 2 days at 37°C, 5% CO₂, unless indicated otherwise. To induce maturation we used different TLR stimuli, e.g. LPS (50 ng/ml), Pam3CSK4 (5 µg/ml), R848 (5 µg/ml) or 4 x 10^6 S.typhimurium. C5a (10 nM or stated otherwise) was added together with the TLR stimulus or at time points indicated in the text.
C5aRA (130 nM) was added 10 min prior to C5a addition. After maturation the supernatant was harvested and cells were analyzed. The adherent DCs were harvested after a few min' incubation with a 0.25% solution of trisodiumcitrate and washed. For phenotyping, the DCs were washed with PBS containing 0.5% bovine serum albumin (PBA) and incubated with mAb or appropriate isotype controls diluted in PBA with 3 mg/ml human gamma globulin for 30 min. DAPI was added to the cells before analysis to assess cell viability and exclude dead cells from analysis. Cells were analyzed on an LSRII flow cytometer (BD) and using FACS Diva software (BD).

Cytokine analysis

After maturation, supernatants were harvested. The production of IL-12p70, IL-23, IL-6 and IL-10 was determined by ELISA. For the detection of IL-10, IL-6 and TNF-α the PeliKine-compact ELISA kit was used (Sanquin Reagents). For the detection of IL-12p70 a combination of B-T21 mAb (Diaclone, Besançon, France) and C8.6 (BD) was used in an ELISA. For the detection of IL-23 a combination of a monoclonal IL-23p19 (eBioscience, San Diego, CA, USA) and C8.6 (BD) was used.

Statistics

Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was analyzed using either a paired t-test or an one-way Analysis Of Variance (ANOVA) with Bonferroni post test in Graphpad Prism 5.00 software (San Diego, USA).

Results

C5a effects cytokine production by human DCs dependent on the culture conditions

Extensive studies in mice showed that the anaphylatoxin C5a affects cytokine production by the APCs. Mainly enhancing effects of C5a, such as increased IL-12 production levels, were found, resulting in more Th1 polarized T cells[1-3]. In contrast, a few studies in mice showed inhibitory effects of C5a on cytokine production by APCs[1, 14, 15]. Recently Li et al[13] showed that C5a addition to human moDCs increased cytokine production. We investigated if C5a would regulate human moDCs function differently under different culture conditions. Immature DCs (imDCs) generated from monocytes expressed the C5a receptor (C5aR) (Figure 1A), confirming earlier findings[10, 13]. Exposure of these imDCs to C5a yielded increased IL-6 levels (Figure 1B), which is in
agreement with the study of Li et al.\textsuperscript{13}. Next, C5a modulation of DC function was investigated in the presence of pathogen-derived TLR ligands that induce DC maturation. Therefore, C5a was added simultaneously with LPS to imDCs. Interestingly, C5a reduced the LPS-induced production of IL-12, IL-23 and TNF-\(\alpha\) strongly and significantly to about 20-30\% of untreated cells (\textbf{Figure 1C, D}). IL-6 production was also significantly inhibited, but to a lesser extent (\textbf{Figure 1C, D}). IL-10 production induced by LPS was not affected by the addition of C5a (\textbf{Figure 1C, D}). To determine whether the inhibition of cytokine production by C5a is mediated via the C5aR, we investigated the effect of a C5aR antagonist (C5aRA). C5aRA by itself did not influence the LPS-induced cytokine production, but was able to counteract the suppression
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of IL-12, IL-23 and TNF-α production by C5a (Figure 2). Subsequently, we investigated if addition of C5a to a LPS stimulated DC culture also influenced the phenotypic maturation of the DCs. No effect however, was observed on the expression of CD40, CD80, CD83, CD86 and HLA-DR (Figure 3). These results indicate that C5a affects cytokine production by human DCs via C5aR either by stimulating or inhibiting the production depending on the presence or absence of LPS stimulation.

**Inhibitory effect of C5a on LPS-induced cytokine production is timing dependent**

In the above described experiments, C5a was added to the DCs simultaneously with LPS. To analyze if C5a would also influence LPS-induced cytokine production, C5a was added 10 min after LPS addition. After 48 h TNF-α, IL-12 and IL-23 levels were determined. Cytokine production levels are expressed as percentage of culture with LPS alone (TNF-α: 10.2-158 ng/ml, mean 91.2 ng/ml; IL-12: 110-2400 pg/ml, mean 820 pg/ml; IL-23: 635-7150 pg/ml, mean 2740 pg/ml) and are depicted as mean ± SEM of at least 3 different donors. Statistical analysis was performed by one-way ANOVA (*P<0.05, **P<0.01, ***P<0.001).

**Figure 2. Inhibitory effect of C5a on LPS-induced cytokine production by DCs is mediated via C5aR.** ImDCs were stimulated with LPS in presence or absence of C5aRA. C5a (1.25 nM) was added 10 min after LPS addition. After 48 h TNF-α, IL-12 and IL-23 levels were determined. Cytokine production levels are expressed as percentage of culture with LPS alone (TNF-α: 10.2-158 ng/ml, mean 91.2 ng/ml; IL-12: 110-2400 pg/ml, mean 820 pg/ml; IL-23: 635-7150 pg/ml, mean 2740 pg/ml) and are depicted as mean ± SEM of at least 3 different donors. Statistical analysis was performed by one-way ANOVA (*P<0.05, **P<0.01, ***P<0.001).

**Figure 3. LPS-induced phenotypic maturation of DCs was not affected by C5a.** ImDCs are stimulated with LPS in presence or absence of C5a. After 48 h expression levels of CD40, CD80, CD83, CD86 and HLA-DR were determined. Data are represented as percentage of the MFI of LPS-stimulated DCs. Data are mean ± SEM of 3 different donors.
production when present already before or some time after LPS stimulation, we added C5a to the culture at various time points relative to addition of LPS. C5a suppressed the LPS-induced TNF-α production efficiently up to 30 min after LPS addition. When C5a was added after 60 min, the inhibition of LPS-induced cytokine production was less prominent, while no effect was observed when C5a was added 90 min after LPS stimulation. C5a was much less effective in inhibiting TNF-α production when added before LPS stimulation. Even when added just 10 min before LPS stimulus it was less effective in inhibiting TNF-α production levels (Figure 4). The same pattern of inhibition by C5a was observed for LPS-induced IL-12 and IL-23 (data not shown). In conclusion, C5a regulates LPS-induced DC cytokine production within a small time frame and requires more or less concurrent signaling of C5aR and TLR4.

C5a inhibits cytokine production induced by several TLR ligands

In order to investigate if C5a also suppresses cytokines induced by other TLR ligands, we also stimulated imDCs with R848 (TLR7/8) or Pam³CSK₄ (TLR2) in the presence and absence of C5a. The TNF-α and IL-23 production induced by R848 and Pam³CSK₄ was significantly suppressed by C5a (Figure 5A, B). Finally, we investigated if C5a would still be modulatory in more physiological settings, where whole pathogens are likely to activate DCs via multiple pattern recognition receptors (PRRs). Therefore, we cultured imDCs in the presence of

![Figure 4. C5a affects the LPS-induced cytokines in a small time frame around TLR4 stimulation. ImDCs were stimulated with LPS. Moment of C5a addition in relation to the addition of LPS varied between 90 min before and 90 min after the LPS stimulus. After 48 h TNF-α production was determined in the culture supernatants. TNF-α levels are expressed as percentage of culture without C5a (32.9 ng/ml). A representative graph of 4 donors is depicted as mean ± SEM of triplicate samples.](image-url)
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the gram-negative bacterium *S. typhimurium*, which induced DC maturation and high levels of pro-inflammatory cytokines. Simultaneous addition of C5a could also inhibit the IL-12, IL-23 and TNF-α production induced by *S. typhimurium* (Figure 5C). Thus, C5a shows broad immune regulatory capacities, as it modulates DC function in the presence of multiple pathogen-derived compounds that activate DCs via different pathogen sensing receptors.

**Figure. 5.** Effect of C5a on cytokine production induced by various TLR stimuli and the gram-negative bacterium *S. typhimurium*. (A and B) ImDCs were stimulated with LPS, R848 or Pam₃CSK₄ in presence or absence of C5a. TNF-α (A) and IL-23 (B) levels were determined in culture supernatants after o/n culture. Cytokine production levels in presence of C5a are expressed as percentage of culture with only the TLR stimulus and are mean ± SEM of at least 6 (TNF-α) (LPS: 0.52-135 ng/ml, mean 3.07 ng/ml; R848: 1.1-346 ng/ml, mean 52.0 ng/ml; Pam₃CSK₄: 0.24-6.66 ng/ml, mean 3.26 ng/ml) or 5 (IL-23) (LPS: 40-15500 pg/ml, mean 2.8 ng/ml; R848: 45-24500 pg/ml, mean 4.3 ng/ml; Pam₃CSK₄: 15-2615 pg/ml, mean 1.7 pg/ml) donors. Statistical analysis was performed by student's t-test (*P*<0.05, **P*<0.01, ***P*<0.001). C. ImDCs were stimulated with *S. typhimurium*. TNF-α, IL-12 and IL-23 levels were determined after o/n culture. Representative graphs of 3 donors are depicted.
Discussion

Complement can be activated by pathogens and TLR ligands. In this paper we show that C5aR-TLR crosstalk inhibits TLR-induced cytokine production by human DCs. In addition, we showed that the extent of cytokine inhibition depends on the timing of C5aR stimulation in relation to TLR triggering. Although stimulatory effects of C5a on human immature DCs have been described\(^{13}\), to our knowledge we are the first to demonstrate inhibitory effects of C5a on human DCs upon DC activation. The observed suppressive effect of C5a on TLR-induced cytokine production is mediated via the C5aR and is timing dependent. Addition of C5a 30 or 60 min after the TLR trigger is less inhibitory, and when C5a was added 30 or 60 min before the LPS stimulus it also had minor effects on LPS-induced cytokine production. In a study performed by Braun \textit{et al}.\(^{12}\), no effect of C5a on stimulated human DCs was found. The timing dependency observed in our study might explain this lack of effect of C5a in that study, since C5a was added 1 h before the stimulation. Together, these data imply that signaling via C5aR can only affect TLR-induced signaling when initiated around the same time.

C5a also affected DC function when DCs were activated by other TLR ligands, R848 and Pam\(_3\)CSK\(_4\), or by the gram-negative bacterium \textit{S. typhimurium}, which simultaneously triggers multiple PPRs. This indicates that the inhibitory effect of C5a is not specific for TLR4-induced cytokines, but modulates the function of TLR-activated DCs in general. A different relative impact of C5a on the production of different types of cytokines was observed. IL-12, IL-23 and TNF-\(\alpha\) production was inhibited in a more pronounced manner than IL-6, while LPS-induced IL-10 levels were not affected by C5a. We cannot exclude that this is caused by different effectiveness in inducing the different cytokines. Interestingly, although C5a affected cytokine production, we did not observe an effect on DC maturation markers, HLA-DR and co-stimulatory markers induced by LPS. This indicates that C5a does not inhibit DC maturation in general, but specifically affects cytokine expression.

Data on the role of anaphylatoxins on APC function are slowly accumulating, but often show conflicting results. The effect of C5a that we observed on TLR-stimulated human DCs is in line with the effect of anaphylatoxins observed for APCs in mice and for monocytes in human\(^{1,12,14-17}\). In some studies however, TLR stimulation in conjunction with C5a triggering did not inhibit LPS-induced IL-6 production, but stimulated IL-6 instead\(^{23,24}\). In these studies the impact on other cytokines was not studied. The effect of C5a on LPS-induced cytokine production might be explained by sequestering of signal transduction components away
from the TLR-induced signaling pathway by C5aR induced signaling. In addition, the involvement of JNK and ERK activation have been implicated\textsuperscript{14, 16}. Moreover induction of cAMP by C5aR-TLR2 crosstalk induced by \textit{P. gingivalis} has been shown\textsuperscript{25}.

We could confirm that C5a addition to human immature DCs without presence of other stimulatory signals leads to an induction of IL-6 production\textsuperscript{13}. The opposite of the effect observed when C5a was present together with a TLR stimulus. It is evident that the anaphylatoxins modulate immune responses by DCs. The outcome, however, seems to be dictated by the environmental conditions, again pointing to the complex role of C5a in DC regulation. We hypothesize that complement-mediated signaling via the C5a receptor renders DCs competent for inflammatory cytokine production under non-infectious conditions, while exposure of DCs to C5a in combination with TLR stimulation leads to redirection of the immune system by downmodulation of the production of potentially harmful pro-inflammatory cytokines to prevent an overwhelming tissue-destructive response. Kildsgaard \textit{et al.}\textsuperscript{26} demonstrated by the targeted disruption of the C3a receptor gene that C3a has an anti-inflammatory role in endotoxin-shock \textit{in vivo}, which fits with this hypothesis. The context depending effects of anaphylatoxins on immune responses may have important implications for the emerging use of complement inhibitors in several diseases. Complement inhibition in plasma and by this the inhibition of anaphylatoxin formation might have differential effects on the APCs and the subsequent induced adaptive immune response depending on the diseases and environmental conditions. More research is necessary to delineate the fascinating crosstalk between the anaphylatoxin receptors and pathogen recognition receptors in APCs.

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**Author Contributions**

AZ, ATB, and SMVH designed the research. AZ, GVS, and SNLT performed the research and analyzed data. AZ and ATB made the figures and wrote the manuscript; and ATB, SMVH, and DW discussed and corrected the manuscript.
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