C5aR and TLR crosstalk

Regulatory effect of anaphylatoxin C5a on human dendritic cells

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Summarizing discussion
The very potent chemoattractant C5a recently has been implicated in modulation of mouse MØ and DC function\textsuperscript{1-8}. Studies on the modulating effect of C5a on APC function, however, often show conflicting results, and the number of studies on the effect of C5a on human DCs is limited (reviewed in chapter 1)\textsuperscript{9,10}. DCs play a profound role in T cell activation and polarization\textsuperscript{11,12}, thereby forming an important link between innate and adaptive immunity both upon pathogen invasion, and in autoimmune diseases. Since both the complement system and DCs are activated upon infection, this emphasizes the importance of unraveling the effect of C5a on human DC activity. In addition, the increased interest in the use and development of C5/C5a interfering compounds for the treatment of various diseases\textsuperscript{13-15} further stresses the need to increase our knowledge on overall effects of C5a on the different arms of the immune system.

**Consequences of C5aR and TLR crosstalk on DC-mediated T cell activation**

_C5a dampens TLR-induced pro-inflammatory cytokine production by human DCs_

DCs are activated by various PAMPs, including TLR4 ligand LPS\textsuperscript{16}. C5a diminishes LPS-induced production of the pro-inflammatory cytokines IL-6, IL-12, IL-23 and TNF-α in human moDCs (chapter 2), in line with previous observations in MØs\textsuperscript{3, 6, 7, 17}. Similar results were found using other TLR ligands, namely R848 (TLR7/8), and Pam\textsubscript{3}CSK\textsubscript{4} (TLR2), or during exposure to the gram-negative bacterium _S. typhimurium_ (chapter 2). Further analysis of human blood DC subsets indicated that especially slanDCs are prone to regulation by C5a (chapter 3)\textsuperscript{18, 19}, and revealed that C5a also inhibits R848-induced pro-inflammatory cytokine production by human slanDCs. Expression of co-stimulatory molecules and hallmark genes involved in antigen presentation, together responsible for the first essential steps for T cell activation, are not affected upon C5aR and TLR crosstalk (chapter 2 and 4). Taken together, the inhibitory effect of C5a on human DCs seems to be more restricted to DC cytokine production, but is not restricted to either the subset of moDCs or to TLR4 signaling alone, as C5aR and TLR crosstalk also affects slanDC activity and pro-inflammatory cytokine production induced by other TLR receptors. The effect of C5a on DC activation via other pattern recognition receptors (PRRs), such as C-type lectin receptors and NOD-like receptors, however, has not been investigated and remains to be elucidated.
C5a hampers DC-mediated Th1 polarization and pathogen clearance in vivo

Since cytokine production by DCs dictates T cell polarization, changes in the production profile of DC cytokines can strongly affect adaptive immune responses. The inhibitory effect of C5a on DC cytokine production can, therefore, effect the modulating capacity of DCs on T cells. This fits with the observation that IFN-γ production by Th1 and cytotoxic T cells is reduced upon stimulation with C5a-primed human DCs (chapter 3) and mouse MØs3. In mice, the inhibitory effect of C5a on TLR-induced MØ activation and subsequent Th1 differentiation is, furthermore, associated with inefficient pathogen clearance3-5. C5aR deficiency increased the resistance to L. major infections in vivo3. In addition, the clearance of P. gingivalis is increased in C5aR deficient mice, using in vivo studies as well as in vitro MØ cultures4, 5. P. gingivalis can even exploit C5a-mediated protection via the expression of its own C5 convertase5, which results in increased C5a production and pathogen survival. These findings indicate that C5a can strongly dampen DC-mediated Th1 immune responses as well as MØ-mediated pathogen clearance, thus negatively regulating Th1 dependent pathogen elimination.

C5aR and TLR crosstalk induces a more regulatory DC phenotype

C5a decreases production of IL-6, IL-23 and TNF-α upon C5aR and TLR crosstalk in human moDCs (chapter 2), indicating that C5a might in addition dampen DC-mediated Th17, Tfh or Th22 polarization12, 20. The modulating effect of C5a on DC-mediated Th17, Tfh and Th22 polarization has not yet been investigated in humans. More is known from studies in mice, in which C5aR deficiency has been associated with increased production of Th17 polarizing cytokines IL-6 and TNF-α, and induction of Th17 polarization by spleen-derived DCs and BMDCs21, 22. This suggests that C5aR and TLR crosstalk might indeed dampen DC-mediated Th17 polarization in human moDCs and slanDCs.

Although Th1 and Th17 immune responses are required to induce efficient pathogen clearance, immune regulation of these most pro-inflammatory Th subsets is crucial to prevent overwhelming and uncontrolled immune activation and tissue damage. IL-10 production has been associated with formation of regulatory T cells12, 23, which are essential in control of immune responses and in prevention of immunopathology23, 24. Strikingly, C5aR and TLR crosstalk in DC increases IL-10 production (chapter 3), indicating that C5a skews towards a more regulatory DC phenotype and most likely towards Treg polarization. This is further supported by whole transcriptome analyses we have performed (chapter 4), in which a core regulatory network was identified upon C5aR and TLR crosstalk. Central in this regulatory network is the inhibition of the
pro-inflammatory transcription factor FOXO1\textsuperscript{25} and induction of the anti-inflammatory transcription factor FOXO3\textsuperscript{26}. Although also some potential pro-inflammatory genes outside the core regulatory network were identified, these findings suggest that C5aR and TLR crosstalk preferentially skew towards increased immune cell regulation (Figure 1). Further research should reveal whether C5a indeed affects DC-mediated induction of regulatory T cells.

Signal transduction pathways involved in the regulatory effect of C5a in human DCs

The past decade, the use of C5/C5a modulating compounds for the treatment of various diseases is gaining more interest, focusing mainly on the chemoattractant effector function of C5a in innate immunity. This underscore the importance of understanding the underlying mechanism of C5aR and TLR crosstalk in human DCs, especially because of the existing contradictions regarding the effect of C5aR and TLR crosstalk among different APCs (reviewed in chapter 1). In addition, increased knowledge on the involved signal transduction pathways may provide interesting new targets for future investigations with respect to pharmaceutical interference.

Key role for CREB1 in C5a-mediated inhibition of TLR-induced pro-inflammatory cytokine production

In contrast to previous observations in human MØs and monocytes\textsuperscript{2, 3, 6, 9, 17, 27}, LPS-induced phosphorylation of JNK or NF-κB nuclear translocation in human moDCs is not affected by C5a (chapter 3). Instead, C5a rapidly induces phosphorylation of ERK1/2 and p38 in human moDCs (chapter 3), resulting in phosphorylation of the downstream transcription factor CREB1. Accelerated TLR-induced CREB1 phosphorylation plays a key role during C5aR and TLR crosstalk, as it leads to the early induction of IL-10 in maturing DCs (Figure 1). Negative feedback signaling by IL-10 inhibits production of the pro-inflammatory cytokines TNF-α and IL-12 by human DCs (chapter 3). Similar results are observed in human slanDCs, in which C5a-induced IL-10 production inhibits TNF-α production. The identification of CREB1 phosphorylation upon C5aR and TLR crosstalk is novel, and was not investigated before. ERK1/2 induction, however, has been observed before upon C5aR and TLR crosstalk in mouse MØs\textsuperscript{3, 6, 17}, indicating that CREB1 might also be involved here. Overall, accelerated CREB1 phosphorylation and the induction of IL-10-mediated negative feedback signaling thus play a key role in the inhibitory effect of C5a on TLR-induced pro-inflammatory cytokine production in human moDCs.
**IRF4 and bZIP activation upon C5aR and TLR crosstalk may contribute to the inhibition of IL-12 and induction of the regulatory phenotype in human DCs**

In contrast to our observations in human moDCs, the inhibitory effect of C5a on TLR-induced pro-inflammatory cytokines by mouse and human MØs, and human monocytes, is mainly IL-10 independent. Although IL-10 production by MØs is increased upon C5aR and TLR crosstalk, blockage of IL-10 signaling using IL-10 and IL-10R blocking antibodies, or IL-10 deficient MØs, did not diminish the effect of C5a on TLR-induced pro-inflammatory cytokine production. Of note, the role for IL-10 in inhibition of pro-inflammatory cytokines by C5a has been based only on the effect on IL-12 production. In line with this, we also observed that the inhibitory effect of C5a on IL-12p40 mRNA expression in human moDCs is not completely IL-10 or CREB1 dependent (chapter 3). Together, these findings are suggestive for the existence of additional regulatory mechanisms besides CREB1 that affect IL-12 production upon C5aR and TLR crosstalk.

Further assessment of CREB1-mediated signaling in C5aR and TLR crosstalk...
has been performed by whole transcriptome analysis, comparing the
differential expressed genes between human moDCs stimulated with LPS or
LPS and C5a in the absence or presence of MSK inhibitor (which prevents
CREB1 phosphorylation) (chapter 4). This revealed that the final outcome
of C5aR and TLR crosstalk is mainly CREB1 independent. So, although CREB1
signaling plays a key role in regulating IL-23 and TNF-α production by DCs
via the induction of IL-10 and IL-10 negative feedback signaling (chapter 3),
whole transcriptome analysis in presence and absence of MSK inhibitor further
provides evidence for the existence of additional regulatory mechanism(s)
involved in C5aR and TLR crosstalk.
Motif enrichment analysis on the differentially expressed genes upon C5a
stimulation in both immature moDCs, and LPS-stimulated moDCs revealed a
prominent role for bZIP transcription factors (ATF3, BATF and AP-1), both upon
C5aR and TLR4 crosstalk, as well as during C5a stimulation alone. Furthermore,
IRF4 motifs are specifically enriched upon C5aR and TLR crosstalk and among
synergistically induced genes upon C5aR and TLR4 stimulation. Both IRF4 and
ATF3 activity have been associated with dampening of TLR4-induced immune
activation29-33, indicating that they may be involved in the regulatory effect of
C5aR and TLR crosstalk in human DCs. In addition, IRF4 interferes with TLR-
induced IL-12 production by inhibiting IRF5 signaling in mouse MØs and
DCs29, 34. We therefore like to propose that IL-12 production upon C5aR and
TLR crosstalk is inhibited through the induction of IRF4 activation as well as
via CREB1-mediated IL-10 production (Figure 1). Further investigations are
needed to determine the involvement of bZIP transcription factors and IRF4 in
C5aR and TLR crosstalk and IL-12 inhibition.

Presence or absence of additional environmental stimuli determines outcome of
C5a stimulation on pro-inflammatory cytokine production by moDCs
The inhibitory effect of C5a on human moDC cytokine production depends on
the presence of other (environmental) triggers (Figure 2), as C5a promotes
pro-inflammatory cytokine production in the absence of a TLR stimulus
(chapter 2)9. C5aR signaling induces ERK1/2 and p38 phosphorylation both
in the absence and presence of additional stimuli, such as LPS (chapter 3). Strikingly, C5a-induced ERK1/2 and p38 activation does not lead to IL-10
induction without TLR stimulation, most probably due to the absence of
prolonged CREB1 phosphorylation in absence of TLR stimulation (chapter 3).
ERK1/2 and p38 activation upon C5a stimulation in the absence of CREB1-
induced IL-10-mediated negative feedback signaling, therefore, leads to the
induction of several pro-inflammatory cytokines in human moDCs. The fact that C5aR signaling is different in the presence of a TLR ligand, is supported by our finding that only 23% of the 173 genes regulated by C5a during LPS stimulation overlapped with the 219 differentially expressed genes in moDC stimulated with C5a alone (chapter 4). Although motif enrichment analysis both in the presence and absence of LPS leads to enrichment for bZIP transcription factors, no enrichment for IRF4 was observed in the absence of LPS. This further highlights the context dependent difference in C5aR-mediated signaling in human DCs.

Differences in C5aR1 and C5aR2 expression might explain contradicting effects of C5a among different APCs

C5aR2 is mainly expressed by MØs, and not by mouse BMDCs

C5a can bind to two different receptors, namely C5aR1 and C5aR2. Both C5aR1 and C5aR2 are expressed on human monocyte-derived MØs (moMØs) and immature moDCs, whereas in human slanDCs only C5aR1 expression has been investigated and detected (chapter 3). Although no C5aR1 expression is observed on circulating conventional DC subsets, some reports state that tissue residing DCs do express C5aR1. Data regarding C5aR1 and C5aR2 expression in human DC and MØ subsets is very limited, and should be included in future studies as will become clear below.

In mice, both C5aR1 and C5aR2 are strongly expressed on various MØ subsets, including peritoneal macrophages, whereas expression of both receptors varies among mouse DC subsets. Modest C5aR2 expression is observed in lung moDCs (60-65%) and lung CD11b+CD103- DCs (15-20%), and low C5aR2 expression is observed in intestinal CD11b+ and CD103+ DC subsets. C5aR2

![C5a](Figure 2. The balance between pro-inflammatory and anti-inflammatory effects of C5a on DCs. Whereas C5a promotes DC recruitment to the site of inflammation and is suggested to promote DC development, the effect of C5a on DC activation depends both environmental conditions and C5aR expression by DCs.)
expression is absent on all other DC subsets investigated, including pDC, splenic DCs, and BMDCs. C5aR1 is expressed on BMDCs, lung resident CD11b+, lung moDCs, but not on intestinal and pulmonary CD103+ DCs.

**C5aR1 and C5aR2 heterodimerisation as important step in the regulatory effect of C5a on TLR-induced cytokine production**

The inhibitory effect of C5a on IL-12, TNF-α and IL-23 production by human moDCs was almost completely abolished by blocking C5aR1 (chapter 2), suggesting that C5a mainly signals via C5aR1. The inhibitory effect of C5aR and TLR crosstalk was also C5aR1 dependent in peritoneal MØs. However, involvement of C5aR2 has not been investigated specifically. For a long time C5aR2 has been postulated as a non-signaling decoy receptor that limits C5a availability to C5aR1, thereby regulating C5aR1-mediated pro-inflammatory immune responses. More recent publications, however, state that C5aR1-C5aR2 heterodimerisation is crucial for the induction of ERK1/2 phosphorylation in human monocyte-derived MØ, resulting in increased IL-10 production. C5aR and TLR crosstalk in human moDCs inhibits TLR-induced pro-inflammatory cytokine production via ERK/p38-induced CREB1 phosphorylation and subsequent IL-10 induction (chapter 3), and in addition induces a more regulatory phenotype in human moDCs (chapter 4). Together, these observations suggest that C5aR2 might be involved in the inhibitory effect of C5a on human moDCs. The receptor antagonist we used to determine the involvement of C5aR1 has been reported as specific for C5aR1, however, blockage of C5aR1 may also interfere with the functionality of the C5aR1-C5aR2 heterodimer complex. In this way, interference with C5aR1 indirectly affects C5aR2 signaling. The inhibitory effect of C5a is, thus, likely to occur via C5aR1-C5aR2 heterodimerisation. However, we have to keep in mind that heterodimerisation of C5aR1 and C5aR2 has only been investigated until now in MØs by two groups, and thus needs to be confirmed in other human APC subsets.

**C5a may differently regulate TLR-induced pro-inflammatory cytokine production in mouse BMDCs due to the absence of C5aR2**

Based on C5aR1 and C5aR2 expression profiles and the proposed involvement of C5aR1-C5aR2 heterodimerization during C5a-mediated inhibition of TLR-induced pro-inflammatory cytokine production, we could speculate that specific APC subsets are more sensitive to the inhibitory effect of C5a compared to others. MØ subsets, lung resident CD11b+ DC and moDC, for example, are more likely to be subject to the regulatory effect of C5a because of their high
expression of both C5aR1 and C5aR2\textsuperscript{41, 42}. This fits with the observed inhibitory effect of C5aR and TLR crosstalk on pro-inflammatory cytokine production by human moDCs (chapter 2 and 3) and peritoneal MØs\textsuperscript{3-8}. In line with this, BMDCs might be much less sensible to C5a-mediated inhibition because they lack C5aR2 expression. This might explain why findings regarding the effect of C5aR and TLR crosstalk on cytokine production in human DCs (chapter 2 and 3) do not so much resemble previous observations in mouse DCs (reviewed in chapter 1)\textsuperscript{1, 2}. So, we propose that the inhibitory capacity of C5a on TLR-induced pro-inflammatory cytokine production depends on C5aR2 expression, which explains why C5a does not inhibit pro-inflammatory cytokine production in BMDCs, which do not express C5aR2 (Figure 2). Of note, the involvement of C5aR and TLR crosstalk in BMDCs is mostly investigated by the use of complete C5aR1 deficient mouse\textsuperscript{1, 2, 21, 22, 51}, in which co-stimulatory marker expression and cytokine production was already reduced during steady state\textsuperscript{2, 52}. This may indicate that C5aR1 signaling is important during DC development (Figure 2). Interference with C5aR signaling already during DC development can, therefore, affect the responsiveness of APC prior to TLR stimulation, providing an additional explanation for the differences observed between C5aR and TLR crosstalk in human and mouse DCs.

**The ongoing debate on APC nomenclature**

The classification of monocyte, DC and MØ subsets is still a point of debate, and has changed to a great extent during the last decade. Traditionally, classification of APC subsets is based on the expression of a wide variety of cell surface proteins as well as APC-specific functional properties\textsuperscript{53, 54}. MØs have been defined as the APC subsets mainly involved in pathogen clearance, whereas DCs are considered to be unique by their capacity to activate naive T cells\textsuperscript{53, 54}. Based on this classification, slanDCs have been characterized as a novel DC subset, because they induce strong T cell responses\textsuperscript{19, 55}. However, others state they are a specific inflammatory monocyte subset that induce only weak T cell activation, and that they are part of the non-classical CD16\textsuperscript{+} monocyte population\textsuperscript{38, 56}. Discriminating between monocyte-derived cells, DC and MØ subsets can be difficult using traditional classification, especially with regard to inflammatory DC and MØ subsets, and it is not always clear to which subset a population of APCs belongs\textsuperscript{53, 57-59}. Guilliams et al.\textsuperscript{53} proposed that monocyte-derived cells, DCs and MØs should therefore be classified based on ontogeny\textsuperscript{53}, resulting in a more robust classification of different APC subsets. This nomenclature permits for overlapping functions or phenotypic properties among APCs, and is already widely accepted in the scientific community.
Based on this nomenclature, monocyte-derived cells are a distinct lineage of APCs, which exists next to the DC and MØ lineages. Inflammatory DCs, such as moDCs and monocyte-derived MØs (moMØs), thus belong to the lineage of monocyte-derived APCs. In addition, dermal CD14+ DCs, intestinal CD103+CD172+ DCs, and tissue residing CD16+ cells (including slanDC), are suggested to be of monocytic origin, and would therefore be part of this lineage. Although monocyte-derived cells are a different class of APCs next to MØ and DC subsets, they are of great importance upon infection and in several diseases. Inflammatory DCs, such as slanDCs, moDCs and moMØ, strongly contribute to local immune activation. In addition, slanDCs and moDCs are found in many autoimmune diseases and induce T cell immune responses. With regard to C5aR and TLR crosstalk, both slanDCs and moDCs can produce complement components and express C5aRs. Taken together, this underscores the possible impact of C5aR and TLR crosstalk in monocyte-derived APCs in vivo.

Studies on “traditionally classified” peritoneal MØs revealed that they actually comprise of two subsets. One of these subsets is of embryonic origin, which is classify as MØ subset based on ontogeny. However, the other subset is of monocytic origin, which is classified as monocyte-derived APC lineage based on ontogeny. Interestingly, the procedure to collect peritoneal MØ for in vitro studies includes the injection of thioglycollate several days before isolation of MØs. This injection increases the recruitment of monocytes to the peritoneum, and promotes the development of monocyte-derived peritoneal MØ. This suggests that in vitro experiments on peritoneal MØs are actually performed using monocyte-derived APCs. Of note, the inhibitory effect of C5aR and TLR crosstalk on pro-inflammatory cytokine production by human moDCs and slanDCs are in line with previous findings in in vitro cultured mouse peritoneal MØs and human monocyte-derived MØs (chapter 2 and 3). Since all of these APC subsets are thus part of the monocyte-derived lineage, the inhibitory effect of C5a during C5aR and TLR crosstalk seems to be more general among monocyte-derived APC subsets. Whether the inhibitory effect of C5aR and TLR crosstalk on APC activation is restricted to monocyte-derived DCs remains to be determined and will largely depend on their capacity to express C5aRs.

C5a affects FcγR-mediated IC uptake by human moDCs

Antigen uptake and processing by DCs is important for the induction of antigen-specific adaptive immune responses, as it leads to antigen presentation in MHC molecules required to activate antigen-specific T cells. Although the expression of MHC molecules increases upon LPS stimulation, C5a has no effect on
expression of MHC molecules by human moDCs, both in presence and absence of TLR stimulation (chapter 2 and 4). Stimulation of moDCs with C5a, however, does alter the expression of genes associated with Fc-receptor mediated phagocytosis and endosomal maturation (chapter 5).

Preliminary data indicate that C5a inhibits Fc-gamma receptor mediated ICs uptake (chapter 5). Since uptake of antigen-containing ICs by DCs results in antigen presentation to T cells, reduced IC uptake is expected to reduce activation of the adaptive immune system. Although striking, the implications of these findings are hard to predict and need further investigation. Especially the context (autoimmunity or infection), and composition of the ICs (the antigen they contain) strongly affect whether reduced IC uptake by C5a will increase or dampen disease severity. In autoimmune diseases, for example, an decrease in the uptake of ICs containing self-antigens might decrease activation of autoreactive T cells, thereby decreasing disease progression. Since PAMPs or DAMPs are also present during IC encounter by moDCs, activation of DCs via C5aR alone is unlikely to occur in vivo. The effect of C5a on IC uptake should, therefore, be investigated in more detail in the presence of additional stimuli, such as DAMPs or PAMPs. The effect of multidimensional crosstalk between C5aRs, TLRs and FcγRs on DC activation has not been investigated, and will further reveal the importance of C5a during IC uptake.

Crosstalk between FcγRs and C5aRs was shown to be complex in mouse MØs (70). C5a modulated the expression of FcγRs, increasing expression of inhibitory FcγRIIb, while decreasing expression of stimulatory FcγRIIa. In turn, FcγR signaling regulated C5aR signaling (70). The balance between expression of inhibitory FcγRIIb and the stimulatory FcγRIIa demonstrated to be important in immune regulation (71). Although C5a affects FcγR expression in mouse MØs, no effect on FcγR expression was observed in human moDCs stimulated with C5a (chapter 5). This suggests that C5a affects FcγR expression differently in moDCs and MØs. Of note, however, no discrimination was made between the inhibitory FcγRIIb and the stimulatory FcγRIIa in human moDCs. Further analysis dissecting these two FcγRs may therefore be interesting in the future.

Most of the 49 genes designated to the GO terms phagocytosis, endosomal maturation and Fc receptor signaling are induced upon C5a stimulation. Interestingly, we observed that C5a decreased IC uptake, which was opposite of our initial expectation. The 49 differentially expressed genes include building blocks of the actin cytoskeleton and microtubule network as well as genes that promote actin polymerization (chapter 5) (72). To facilitate IC uptake, dynamic cytoskeleton remodeling is required to allow increased receptor mobility and internalization. Increased expression of genes involved in actin polymerization
may interfere with dynamic cytoskeleton remodeling required for Fc receptor-mediated uptake. Overall, it is yet unclear how the effect of C5a on the expression of these 49 genes specifically reduced IC uptake and which genes may be more essential in this process compared to others.

Implications of C5aR and TLR crosstalk in autoimmune diseases

Autoimmune diseases are associated with excessive complement activation\(^{73-79}\), increased slanDC recruitment to the site of inflammation\(^{19,62-67}\), and undesirable Th1 and Th17 responses\(^{80,81}\). In addition, DAMPs are present at the site of inflammation which can stimulate PRRs. C5aR and TLR crosstalk in slanDCs is, therefore, likely to occur during autoimmunity, and would have desirable affects by inhibiting inflammatory T cell responses and increasing immune regulation. Pro-inflammatory immune responses are, however, not well regulated in these diseases, as illustrated by the presence of chronic inflammation and undesirable Th1 and Th17 responses\(^{80,81}\).

*C5aR and TLR crosstalk may be dysregulated in autoimmune diseases*

C5aR and TLR crosstalk is not the only process that should be considered with regard to autoimmune diseases, and we do not want to suggest that autoimmunity can be explained solely by unraveling C5aR and TLR crosstalk. Still, dysregulation of C5aR and TLR crosstalk may contribute to the development of autoimmune diseases. This idea is supported by several observations in autoimmune diseases (Box 1). Polymorphisms in the IL-10 gene and a specific SNP in IRF4 have been associated with increased susceptibility to development of several autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus (SLE)\(^{82-85}\). Since CREB1-mediated IL-10 negative feedback signaling and IRF4 activity are involved in the regulatory effect of C5aR and TLR crosstalk (chapter 3 and 4), alterations in IL-10 or IRF4 activity provide a direct link between dysregulated C5aR and TLR crosstalk and autoimmune diseases. In addition, C5aR and TLR crosstalk may become insufficient due to presence of (too) many other pro-inflammatory signals and other immune cells active in autoimmunity. Furthermore, the complement system is suggested to become exhausted due to excessive activation in SLE\(^{86,87}\), which could result in the absence of active C5a for interference with TLR-mediated signaling. Future research should focus on providing more insights in the importance of C5aR and TLR crosstalk in autoimmune diseases.
Considerations for future research on C5aR and TLR crosstalk in autoimmunity

Autoimmunity is a well-represented field of research. C5aR and TLR crosstalk, however, has not been addressed in these studies. The emerging interest in the use of C5/C5a modulating drugs for the treatment of several autoimmune diseases, emphasizes the need to clarify the involvement of C5aR and TLR crosstalk in these diseases. The involvement of IL-10 polymorphisms and a SNP in IRF4, for example, should definitely be further explored, as both IL-10 and IRF4 activity is involved in the regulatory effect of C5a on DC activation in human DCs (chapter 3 and 4). We listed several additional points that should be considered during further investigations on C5aR and TLR crosstalk in autoimmune diseases in box 1, which are also further explained below.

<table>
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<tr>
<th>Box 1. Considerations with regard to C5aR and TLR crosstalk in autoimmune diseases</th>
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<tr>
<td>Possible explanations for dysregulated C5aR and TLR crosstalk in autoimmune diseases:</td>
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<tr>
<td>1. Downstream signal transduction pathways of C5aR and TLR crosstalk may be affected in autoimmune disease</td>
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<tr>
<td>i. a SNP in IRF4 has recently been identified as risk locus for the development of rheumatoid arthritis^{82}</td>
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<tr>
<td>ii. Polymorphisms in the IL-10 gene are correlated with susceptibility for rheumatoid arthritis^{83}, IBD^{84}, SLE^{85} and many autoimmune diseases</td>
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<tr>
<td>2. Immune regulation by C5aR and TLR crosstalk may be insufficient due to the presence of many other pro-inflammatory signals and other immune cells</td>
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<tr>
<td>3. Due to strong complement activation, the complement system may become exhausted during chronic inflammation^{86, 87}</td>
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Considerations for future experiments:

1. Possible differences in C5aR1 and C5aR2 expression on APCs at the site of inflammation should be investigated
2. Further research should focus on APC subset(s) that are involved in a specific disease
3. The complement system has other important functions apart from DC activation^{88}
4. ICs induce complement activation
5. Some autoimmune diseases, such as SLE, are strongly associated with complement deficiencies^{89}
6. *In vivo*, DCs will be subject to multidimensional crosstalk by the activation and regulation of many different receptors, such as other PRRs, cytokine receptors, and Fc receptors, and is thus not limited to C5aR and TLR4 signaling^{90}
First, C5aR1 and C5aR2 expression appears to be of relevance for the modulating effect of C5aR and TLR crosstalk on human DC function. Much is still unclear on C5aR1 and C5aR2 expression by human APCs, especially at the site of inflammation. Expression of C5aRs by APCs might be altered during chronic inflammation, directly affecting the outcome of C5aR and TLR crosstalk in autoimmune disease. Second, further research should focus on the role of slanDCs in autoimmunity, for they represent an important DC subset in several autoimmune diseases and induce undesirable Th1 and Th17 immune responses. Other APC subsets may also be relevant for further investigations, and should be selected based on their involvement the disease of interest.

One should not forget that the complement system has many important functions apart from modulation DC activation which may also be involved or altered in disease development. This is nicely illustrated by findings in SLE, in which complement deficiencies of the classical pathway (C1q, C1r, C1s, C4 and C2) are strongly associated with the susceptibility to develop SLE. This emphasizes the importance of considering also other complement components during future studies on complement and how these may affect APC activation. In addition, ICs can strongly activate the complement system, which can lead to chronic complement activation in autoimmune disease in which increased IC deposits are found. This may affect DC activation differently compared to observations in in vitro studies. Last, DCs are probably subject to multidimensional crosstalk in vivo, by activation of both PRRs, cytokine receptors, and Fc receptors, indicating that crosstalk will not be limited to C5aR and TLR4 signaling. This should therefore be a point of attention in future research where possible.
Concluding remarks

In this thesis, we described and discussed our discoveries on C5aR and TLR crosstalk on the activation of human DCs. C5a reduced the production of TLR-induced pro-inflammatory cytokines both by human moDCs and slanDCs, and reduced the induction of Th1 and cytotoxic T cell responses by DCs. Furthermore, C5a skewed DC activation towards immune regulation, as shown by the finding of a core regulatory network upon C5aR and TLR crosstalk. We postulate that the regulatory effect of C5a on DC activation is a mechanism to prevent overwhelming and prolonged DC activation. This is important to prevent uncontrolled immune activation, which can lead to tissue damage and the development of autoimmune diseases. The induction of IL-10 upon C5a stimulation might further contribute to regulation of uncontrolled immune activation by the induction of regulatory T cells.

Although this thesis focused on the effects of C5aR and TLR crosstalk on DC activation, it is important to realize that C5a is also a very potent chemoattractant. Although C5a can regulate DC activation, the recruitment of more immune cells, including neutrophils, monocytes and slanDCs, to the site of infection has not been taken into account in these studies, and might compensate for the inhibitory effect of C5a on pro-inflammatory cytokine production by individual DCs. In addition, produced C5a might be involved in DC development prior to DC activation. We therefore suggest that the balance between immune cell recruitment, C5aR and TLR crosstalk in APCs (the presence of environmental triggers), and the effect of C5a on other immune cells is crucial for the overall effect of C5a during inflammation. Nevertheless, we discovered a potential regulatory role of C5a in human DC activation, which may especially be of importance to prevent the development of autoimmunity and to regulate inflammation during bacterial infections. The use of C5/C5a interfering drugs for the treatment of complement-mediated diseases or diseases associated with altered complement activity should, therefore, be considered with extreme care.

More knowledge on the interplay between all the different effector functions of C5a is required to fully understand the implications of C5aR and TLR crosstalk in human DCs.


43. la Sala, A., Gadina, M. & Kelsall, B.L. G(i)-protein-dependent inhibition of IL-12 production is mediated by activation of the phosphatidylinositol 3-kinase-protein 3 kinase B/Akt pathway and JNK. J Immunol. 175, 2994-2999 (2005).


