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Mechanism of Neutrophil Dysfunction: Neutrophil Serine Proteases Cleave and Inactivate the C5a Receptor

Carmen W. van den Berg,* Denise V. Tambourgi,† Howard W. Clark,‡ S. Julie Hoong,* O. Brad Spiller,* and Eamon P. McGreal*

Neutrophil dysfunction, resulting in inefficient bacterial clearance, is a feature of several serious medical conditions, including cystic fibrosis (CF) and sepsis. Poorly controlled neutrophil serine protease (NSP) activity and complement activation have been implicated in this phenomenon. The capacity for excess NSP secretion and complement activation to influence the expression and function of the important neutrophil-activating receptor C5aR was investigated. Purified NSPs cathepsin G (CG), neutrophil elastase (NE), and proteinase 3 cleaved C5aR to a 26- to 27-kDa membrane-bound fragment, thereby inactivating its C5a-induced signaling ability. In a supernatant transfer assay, NSPs released from neutrophils in response to C5a induced the cleavage of the C5aR on unstimulated cells. Stimulation of myelomonocytic U937 cells and purified neutrophils with C5a resulted in downregulation of the C5aR on these cells, which, in the case of U937 cells, was largely caused by NSP-mediated cleavage of C5aR, but in the case of neutrophils, intracellular degradation was likely the main mediator in addition to a small role for NSPs. CG and NE in bronchoalveolar lavage fluid from CF patients both contributed to C5aR cleavage. We propose two converging models for C5a- and NSP-mediated neutrophil dysfunction whereby C5aR cleavage is induced by NSPs, secreted in response to: 1) excess C5a generation or other stimuli; or 2) necrosis. The consequent impairment of C5aR activity contributes to suboptimal local neutrophil priming and bacterial clearance. NSP inhibitors with specificity for both CG and NE may aid the treatment of pathologies associated with neutrophil dysfunction including sepsis and CF. The Journal of Immunology, 2014, 192: 1787–1795.
in whom relative expression of C5aR is suggested to be of prognostic significance (11).

The mechanisms of C5a-induced neutrophil dysfunction and regulation of C5aR expression have not yet been elucidated. NSPs have been shown to proteolytically reduce the expression of several cell-surface molecules involved in immunity, including CXCR1 (IL-8RA), CR1, CD16, CD43, and TNFRII, but their impact on C5aR expression has not been investigated. Proteolytic cleavage and inactivation of C5aR has been demonstrated by a variety of endogenous and exogenous proteases, including an endogenous metalloprotease, activated by the action of Loxosceles spider venom sphingomyelinase D, a serine protease from Porphyromonas gingivalis, and a metalloprotease from venom of the spider Plec- treurus tristes (22–24). Direct proteolysis and inactivation of C5aR by an endogenous protease has not been demonstrated so far; however, a reduced chemotactic response to C5a after NE and CG treatment has been reported (25). A recent study by Unnewehr et al. (21) suggested that the C5aR may be shed from the cell surface in response to C5a.

Considering the importance of NSPs and C5a in neutrophil dysfunction and the roles of NSPs and C5a in pathology of neutrophil-associated pathologies, we aimed to investigate the effects of NSPs on the expression and the function of the C5aR. We investigated the effects of purified NSPs, CF bronchoalveolar lavage fluid (BALF), and supernatants of activated neutrophils on C5aR expression and function and used Western blotting and measurement of intracellular calcium responses. We further investigated the mechanism of C5a-induced downregulation of endogenous C5aR expression. These data lead us to propose two interlinked models of NSP- and C5a-induced neutrophil dysfunction, which are relevant to neutrophil-mediated airway disease and sepsis.

Materials and Methods

Reagents

Abs and other reagents were from the following sources: mouse anti-C5aR (SS5/1) and rabbit anti-C5aR (SC-25774; H100) from Santa Cruz Biotechnology (Wembley, U.K.); rabbit anti-mouse IgG HRP and goat anti-rabbit IgG HRP from Stratech (Soham, U.K.); CR1 inhibitor I, elastase inhibitor IV, and PR3 from Merck Chemicals (Nottingham, U.K.); human recombinant C5a from Hycult Biotech (Cambridge Bioscience, Cambridge, U.K.); C5aR agonist (H-Phe-Lys-Pro-D-Cha-Cha-D-Arg-OH) and fura 2-AM from AnaSpec (Cambridge Bioscience, Cambridge, U.K.); and NE and CG from Athens Research & Technology (Athens, GA). All other agents including inhibitors were from Sigma-Aldrich (Poole, U.K.). Lysis buffer was composed of 25 mM N-acetyl-L-cysteine, 150 mM NaCl, and 1% Triton X-100 (pH 6.5) plus the following inhibitors: 5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 5 mM PMSE, 0.8 μM aprotinin, 10 μM bestatin, 14 μM E-64, 20 μM leupeptin, 15 μM pepstatin A, and 5 mM 1.10 phenanthroline. All cell incubations were carried out in Krebs/HEPES/BSA (KHB) buffer: 25 mM HEPES, 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 1.3 mM CaCl2 (pH 7.4), and 0.1% BSA.

Cell isolation, culture and treatments

Human neutrophils, isolated using dextran sedimentation and Ficoll gradient centrifugation, were incubated at 107 c/ml in KHB with the indicated stimuli for 1 h at 37˚C, for 3 min at 1800 × g, and supernatants were collected and used for incubations with U937 cells or analyzed by Western blotting. Cells were analyzed by flow cytometry or extracted with lysis buffer at 4˚C (30 min on ice followed by 5 min, 5000 × g at 4˚C). Native U937 cells were from Peter Monk (Sheffield, U.K.) and were differentiated for 3 d with 0.5 mM BetamAMP to induce C5aR expression. For C5aR cleavage experiments, cells at 107 c/ml in KHB were incubated with the indicated reagents for 1 h at 37˚C. Cells were spun for 3 minutes at 1800 × g, and cell pellets were analyzed by Western blotting or flow cytometry.

SDS-PAGE and Western blotting

Cell lysates or cell supernatants were run on 12% SDS-polyacrylamide gels under nonreducing conditions and in the presence of protease inhibitors and blotted onto Hybond Nitrocellulose (GE Healthcare UK. Little Chalfont, U.K.). C5aR was detected as described (22) using mAb SS5/1 or polyclonal anti-C5aR. Precision Plus All Blue standards (Bio-Rad, Hemel Hempstead, U.K.) were used to calculate the molecular weights of the bands.

Flow cytometry

Cell-surface C5aR expression was detected by flow cytometry as described (22) using mAb SS5/1, and fluorescence was measured on an Accuri flow cytometer (BD Biosciences). Results are expressed as average of mean of arbitrary fluorescent intensity ± SD of experiments carried out in triplicate.

Calcium flux measurements

Cells (at 107 c/ml) were loaded with 2 μM fura 2-AM for 30 min at room temperature. Cells were washed and resuspended in KHB buffer and incubated with NSPs for 1 h at 37˚C. After washing, 200 μl cells (5 × 10⁶/ml) was stimulated with 2.5 nM C5a or 250 nM C5aR agonist at 37˚C. Changes in intracellular calcium were measured as described (22, 26).

Patient recruitment and bronchoalveolar lavage

Four CF BALF samples were taken from a cohort recruited as previously described from children undergoing fiber optic bronchoscopy for diagnostic reasons at Southampton General Hospital (9). The study was approved by the Southampton and Wessex Local Research Ethics Committees, and written informed consent was obtained. BALF from cohort B was initially filtered through a 100-μm nylon cell strainer (BD Biosciences) prior to centrifugation at 500 × g for 10 min at 4˚C. Aspirated cell-free BALF was immediately stored at −80˚C, and elastase activity in these samples was assayed as previously reported (9).

NE activity

NE activity was measured as described (5).

Calculations

Calculations of potential cleavage site fragment sizes were carried out using C5aR sequence P21730.1 and calculated using the Sequence Manipulation Site m.w. calculation tool available at http://www.bioinformatics.org/sms2/protein_mw.html.

Statistics

Statistics were carried out using one-way ANOVA followed by Tukey multiple comparisons test. Differences were considered significant at p < 0.05. Experiments were carried out at least three times and representative results are shown.

Results

NSPs cleave and inactivate the C5aR

The effects of NSPs on C5aR expression and function were investigated using purified neutrophils and differentiated U937 myelomonocytic cells. Cells were exposed to CG, NE, and PR3 at concentrations commonly found in BALF of patients with CF (5, 27, 28). Using a mAb specific for the N terminus of C5aR, a large reduction in cell-surface C5aR expression, as detected by flow cytometry, was observed after incubation with all three NSPs (Fig. 1A, 1B). After incubation of both neutrophils and U937 cells with the NSPs, a reduction in C5a responsiveness, as measured by release of intracellular calcium, was observed (Fig. 1C, 1D). However, the calcium-release response to a small-molecule C5aR agonist was not affected (Fig. 1E, 1F).

Activation of C5aR requires C5a binding at two distinct sites: the main ligand binding site being the extracellular N terminus (aa 2–22), and the other signal-transducing site is formed by the extracellular loops formed by α-helices III, VI, and VII (12, 29–31). Thus, the reduction in responsiveness to C5a but not the C5aR agonist suggested that the loss of binding of the anti–N-terminal Ab and the loss of C5a-induced calcium response was not a consequence of total loss of the C5aR but suggested that only the C5a-binding
N terminus was lost but the calcium signaling C terminus was retained. Indeed, whereas after incubation with the NSPs, Western blots of the cell lysates, developed using the mAb recognizing the N terminus, showed a large reduction in the intensity of a 42-kDa band, typical for native C5aR (Fig. 1G), detection of the C5aR using the polyclonal Ab raised against the C terminus of the C5aR showed a reduction in intensity of the native 42-kDa band concomitant with the appearance of a band with a \( M_r \) of 27 kDa (after incubation with CG) or 26 kDa (after incubation with NE or PR), demonstrating that cleavage of the C5aR had taken place (Fig. 1H, 1I). Thus, NSPs can reduce C5a-induced cell activation by reducing expression of the N terminus by enzymatic cleavage.

**Neutrophils secrete C5aR-cleaving enzymes upon activation with C5a**

C5a is a well-known activator of neutrophils and known to cause neutrophil degranulation. To investigate if C5a, as a natural activator of neutrophils, can induce the secretion of NSPs, leading to C5aR cleavage on neighboring cells, neutrophils were stimulated with C5a, and cell supernatants were incubated with C5aR-expressing U937 cells as an indicator neighboring cell. Supernatants of C5a-activated neutrophils caused a small amount of cleavage of the C5aR, resulting in a 26-kDa fragment being retained in the membrane (Fig. 2A) and resulted in the downregulation of C5aR cell-surface expression (Fig. 2B). Cytochalasin D (CytoD), an actin inhibitor and enhancer of GPCR-induced granule release, increased fMLF and C5a-induced C5aR cleavage activity in the neutrophil supernatants (Fig. 2A, 2B). Supernatants of neutrophils stimulated with fMLF (± CytoD) or the phorbol ester PMA, a known inducer of neutrophil degranulation, had similar effects on the cleavage of the C5aR on U937 cells (data not shown). Analysis of neutrophil elastase activity in the polymorphonuclear neutrophil (PMN) supernatants demonstrated the C5a-induced secretion of NE mirrored the cleavage of the C5aR (Fig. 2C).

To identify the nature of the proteases involved in the C5aR cleavage, the supernatants of C5a-activated neutrophils were preincubated with the broad-spectrum metalloprotease inhibitor 1,10-phenanthroline, the broad-spectrum serine protease inhibitor PMSF, and the specific NE and CG inhibitors. The C5aR cleavage was inhibited by the NE inhibitor and the serine protease inhibitor PMSF, but not by the CG inhibitor or metalloprotease inhibitor, identifying the enzyme responsible for the cleavage as the serine protease NE (Fig. 2D). These results demonstrate that NSPs, secreted by neutrophils, can cleave C5aR on neighboring cells, which will result in downregulation and inactivation of this receptor on unstimulated bystander cells.
CF BALF contains enzymes that degrade C5aR

As shown above, NSPs released upon neutrophil stimulation can cleave the C5aR on neighboring cells. NSP-mediated cleavage and inactivation of C5aR has important consequences for conditions in which excess NSP release and defective protease/antiprotease balance are associated with pathology such as observed in CF. In CF, excess neutrophil influx results in apoptosis and necrosis of these neutrophils and release of excess NSPs. To investigate if lung fluids from CF patients have the ability to enzymatically cleave the C5aR, two BALFs with and two BALFs without detectable NE activity were tested for their ability to cleave the C5aR. Only the two BALFs containing detectable NE activity induced C5aR cleavage (Fig. 3A, 3B). The broad-spectrum serine protease inhibitor PMSF inhibited the C5aR cleavage by both BALFs, whereas the broad-spectrum metalloprotease inhibitor 1,10 phenanthroline had no effect, demonstrating that the cleavage was accomplished by a serine protease. Further analysis using specific protease inhibitors showed that in one BALF (Fig. 3A), the enzyme responsible for the cleavage was CG, as the only CG inhibitor prevented the cleavage. However, in the other BALF (Fig. 3B), a different pattern emerged. Although the CG inhibitor did not prevent the proteolysis, it led to a slight reduction in the M_r of the observed fragment, resulting in a size normally observed when cells are incubated with NE (Fig. 3B). Furthermore, the NE inhibitor caused partial inhibition of cleavage. This suggests that both CG and NE contributed to the cleavage. Two BALFs that did not contain any detectable NE activity did not cause cleavage of the C5aR (data not shown).

These data suggest a hierarchy exists between CG and NE toward C5aR, and inhibition of CG enabled NE to cleave the C5aR. Indeed, when purified CG and NE were coincubated with the U937 cells, a fragment size similar to that obtained with CG was observed, suggesting that CG is dominant over NE (Fig. 3C). These results demonstrate that NE and CG in BALF from CF patients have the ability to cleave the C5aR, which is likely to impair C5a-dependent functions such as phagocytosis and intracellular killing of pathogens.

C5a induces the cleavage of endogenously expressed C5aR on U937 cells

C5a itself has been implicated in the downregulation of its own receptor as measured by flow cytometry (20, 21, 32). It is generally acknowledged that ligand binding to GPCRs such as the C5aR results in the internalization and recycling of the receptor or possibly degradation. Thus, a reduced C5aR cell-surface expression could suggest internalization followed by lysosomal degradation. The effects of C5a on the C5aR expression on the stimulated cell was investigated, and when U937 cells were incubated with C5a, a reduction in the binding of the anti–N-terminal mAb was observed by flow cytometry (Fig. 4A); simultaneously, as detected by Western blotting using the anti–C terminus Ab, a 27-kDa band appeared, demonstrating that C5a can reduce the expression of its own receptor by inducing enzymatic cleavage (Fig. 4B). This cleavage was enhanced by the secretagogue and actin inhibitor CytoD, which enhances degranulation (Fig. 4A, 4B), thus demonstrating that this process is mediated by C5a-induced local secretion of enzymes, rather than internal lysosomal degradation. PMA, a potent inducer of cell activation and degranulation, also induced the cleavage of endogenously expressed C5aR (Fig. 4A, 4B).

FIGURE 2. Neutrophil elastase, secreted upon stimulation of PMN with C5a, cleaves the C5aR. U937 cells were incubated with dilutions of the supernatants of neutrophils (PMN) and cells were analyzed by Western blotting (A) and flow cytometry (B). PMN had been incubated with buffer only (control), PMA (10^{-7} M), C5a (100 nM) + CytoD (CD; 10 μM), or FMLF (10^{-6} M) + CD (10 μM) for 1 h. As an additional control, U937 cells were incubated with buffer, instead of neutrophil supernatant, for 1 h. (A) Cell pellets were Western blotted and developed with rabbit anti-human C5aR. (B) Cells were stained for flow cytometry using mouse anti C5aR (SS1). Results are presented as mean fluorescence intensity (MFI) ± SD. (C) NE activity of the PMN supernatants measured using a chromogenic substrate. (D) U937 cells were incubated with supernatants of C5a + CD-stimulated PMN, in the presence or absence of protease inhibitors. Cells were run on 12% SDS-PAGE gels, Western blotted, and developed with rabbit anti-human C5aR. Solid arrows indicate intact C5aR; dashed arrow indicates C5aR fragment. CG inhibitor (CGI): 20 μM; NE inhibitor (NEI): 20 μM; 1,10-phenantroline (Ph): 4 mM; PMSF (PF): 2 mM. *p < 0.001 compared with control cells. C, No inhibitor.

FIGURE 3. CF BALF contains C5aR-degrading enzymes. U937 cells were incubated with NE-containing BALFs from CF patients at a final NE concentration of 2 μg/ml in the presence or absence of protease inhibitors for 1 h. Cells were spun and run on 12% SDS-PAGE gels, Western blotted, and developed with rabbit anti-human C5aR. (A) BALF original NE concentration 4.2 μg/ml. (B) BALF original NE concentration 38 μg/ml. Solid arrows indicate intact C5aR; dashed arrow indicates C5aR fragment. —, no inhibitor; CG inhibitor (CGI): 20 μM; NE inhibitor (NEI): 20 μM; 1,10-phenantroline (Ph): 4 mM; PMSF (PF): 2 mM. (C) Cells were incubated with buffer, CG (5 μg/ml), NE (5 μg/ml), or CG + NE (5 μg/ml each).
The nature of the enzymes involved in the C5a- and PMA-induced cleavage of endogenously expressed C5aR on U937 cells was assessed using various protease inhibitors. Addition of inhibitors specific for the serine proteases CG or NE before stimulation with C5a or PMA showed that only the CG inhibitor prevented the induced cleavage (Fig. 4C, 4D), identifying the enzyme responsible for the C5a- and PMA-induced cleavage of endogenous C5aR in U937 cells as CG.

C5a, fMLF, and PMA induce reduction in neutrophil C5aR cell-surface expression, but reduction in response to C5a is only partially due to enzymatic degradation

In a recent study by Unnewehr et al. (21), it was suggested that the C5a-induced downregulation of the C5aR on neutrophils was caused by shedding of the receptor. In their study, an Ab recognizing the N terminus of C5aR was used, and thus possible cleavage would not have been detected. Our results described above demonstrated that NSPs can cleave the C5aR and that, on the promonocytic cell line U937, C5a induces CG-mediated cleavage of the endogenously expressed C5aR. To investigate if the reduction in cell-surface expression of endogenously expressed C5aR on neutrophils, in response to C5a, could be caused by NSPs-mediated enzymatic cleavage, neutrophils were assessed for C5aR expression by flow cytometry and Western blotting. Flow cytometry showed that C5a- and fMLF-induced downregulation of cell-surface expression (Fig. 5A). The C5a- and fMLF-induced C5aR downregulation was enhanced by CytoD, resulting in a near complete elimination of the N-terminal epitope from the cell surface.

Western blotting using the anti–N terminus mAb as the detecting Ab also showed a reduction in intensity of the 42-kDa band upon incubation with C5a or fMLF (Fig. 5B, top panel). Although addition of CytoD to fMLF caused the complete disappearance of the C5aR, it only had a small effect on the C5a-induced C5aR downregulation (Fig. 5B, top panel). Interestingly, C5a induced a slight increase in M₁ of the C5aR; this was not observed with fMLF. The slight increase in M₁ is likely caused by phosphorylation of the C5aR upon C5aR stimulation, which has previously been reported (33).

Probing of the Western blots with the anti–C terminus anti-C5aR Ab showed again a reduction in intensity of the 42-kDa native C5aR band; however, C5a on its own did not induce the cleavage of the C5aR (Fig. 5B, bottom panel); only when CytoD was coin incubated, in addition to the native C5aR 42-kDa band, a band of ~27 kDa was observed, consistent with NSP-mediated cleavage. In contrast, fMLF on its own induced the cleavage of the C5aR, and addition of CytoD resulted in complete elimination of the 42-kDa native C5aR band, with the appearance of an intense 27-kDa fragment (Fig. 5B, bottom panel). PMA had the same effect as fMLF combined with CytoD (data not shown).

These results suggested that the mechanism of downregulation of the C5aR in response to C5a on neutrophils was different from that of the promonocytic cell line U937. It is well known that GPCRs upon interaction with their ligand become internalized. This may explain why the C5aR did not become cleaved upon C5a induced activation as internalization would provide temporary protection against NSP-mediated cleavage. This C5aR internalization would not occur upon neutrophil activation by fMLF and PMA, and therefore, NSP secretion in response to these agents could thus directly cleave and inactivate the C5aR. A time-course experiment showed that only after 3-h incubation with C5a, a small amount of cleaved C5aR was observed, whereas the addition of CytoD greatly enhanced and accelerated this (Fig. 5C, bottom panel). The slight increase in M₁ of the native C5aR remained during this time period (Fig. 5C, top panel), which was only clearly observed using the anti–N terminus Ab. Unnewehr et al. (21) reported that the C5aR was released in the supernatant of the neutrophils; however, we were unable to detect any C5aR in the supernatants of C5a- or fMLF-stimulated neutrophils using the anti–N terminus or anti–C terminus Ab after loading of supernatants equivalent to the cell lysates (Fig. 5B), and our results suggest that the loss of C5aR may be caused by intracellular degradation after internalization. Prolonged and overexposure of the blots in Fig. 5B also did not reveal any sign of C5aR released in the supernatant.

Discussion

We have investigated the effects of NSPs on C5aR expression, integrity, and function and their role in downregulation of the C5aR.
after C5a-induced cell activation. Furthermore, we have investigated the action of BALF from CF patients, as a source of NSPs, on the integrity of the C5aR.

We show in this study that C5aR expressed on neutrophils and the differentiated monocytic cell line U937 is sensitive to cleavage by physiological concentrations of purified NSPs CG, NE, and PR3, resulting in loss of the C5a-binding N terminus and nonresponsiveness of the cells to C5a stimulation (Fig. 1). These C5aR-cleaving NSPs can be released from neutrophils and U937 cells upon stimulation with C5a itself. This can lead to cleavage of the C5aR expressed on neighboring, unstimulated cells (Fig. 2), in addition to the stimulated cells themselves (Figs. 4, 5). The enzymes responsible for this were identified as CG and NE. Furthermore, BALF from CF patients also contained this C5aR-cleaving activity, which could be prevented by CG and NE inhibitors (Fig. 3). Our data help to explain previously reported defects of neutrophil function under pathological conditions in which excess unregulated NSP activity or excess C5a generation are seen, such as CF lung disease and sepsis. Our data also show that loss of C5aR on neutrophils under such conditions can be caused by NSP-mediated degradation in response to both microbial (e.g., formylated peptides) and endogenous stimuli (e.g., excess C5a). We also show that excess C5a generation also leads to intracellular degradation of the C5aR, following ligand induced internalization, but this requires further investigation. Recently published findings from Umewehr et al. (21) suggest that shedding is one cause of reduced cells’ C5aR surface expression; however, we were unable to find evidence of shedding in this study. A possible reason for this may be a possible difference in methodology.

Considering the importance of C5a and C5aR in clearance of bacterial infections by potentiating phagocytosis and intracellular killing including of P. aeruginosa (10, 13, 14), our observation that the C5aR is cleaved and inactivated by both purified NSPs and NSPs found in CF BALF (Figs. 1, 3) may partly explain why chronic infection is such a recalcitrant aspect of CF airway disease. NE is the most widely studied NSP in CF and is a major therapeutic target. However, our data show that CG is also very effective at cleaving C5aR and may also be an important therapeutic target. This was exemplified by the observation that in one BALF, inhibition of CG effectively prevented cleavage, whereas in another BALF, when CG was inhibited, a fragment size typical of NE was observed, and when CG and NE were combined, a fragment size typical for CG cleavage was detected. Thus, dual inhibition of both CG and NE would be required to prevent C5aR cleavage and inactivation. The balance among NE, CG, and their respective natural inhibitors in BALF may determine the effective cleavage profile, and further analysis of a larger patient cohort is the subject of ongoing investigation. In the U937 cell line, C5a-induced cleavage was predominantly mediated by CG, whereas NE appeared to be the predominantly active NSP in supernatants of activated neutrophils. This difference may reflect cell-specific differences in enzyme as well as the relative abundance of their natural inhibitors.

Our results from the supernatant transfer experiments (Fig. 2) have important consequences for conditions in which large amounts of NSPs are secreted by neutrophils, as this has the potential to render cells nonresponsive to C5a. The manner in which NSPs are secreted could be in response to C5a but also other stimuli including the bacterial peptide fMLF [as we demonstrate in this study (Figs. 2, 5)]. Excess NSP secretion in diseases such as CF can also be a result of dysregulated clearance of apoptotic neutrophils with consequent large-scale secondary necrosis.

We also observed that C5aR can be cleaved after stimulation of cells by C5a; however, the extent of this cleavage was dependent on the cell type used. Although stimulation of differentiated U937 with C5a led to extensive CG-mediated cleavage of the endogenous C5aR (Fig. 4), on neutrophils, C5aR downregulation following C5a exposure was only partially explained by cleavage, although this was substantially enhanced by the secretagogue CytoD (Fig. 5). Neutrophils are very dynamic cells, and it was recently suggested that C5aR is shed from the cell surface in response to C5a (21). However, despite the inclusion of a mixture of protease inhibitors in the processing of the samples, we detected neither uncleaved nor cleaved C5aR in the supernatants of C5a-stimulated neutrophils in our experiments (Fig. 5). The reason we observed only
limited generation of the 27 kDa C5aR cleavage fragment on neutrophils is likely to be explained by internalization of the C5aR in response to C5a, which would have protected it from degradation by NSPs until recycling to the cell surface. However, the reduction in overall C5aR expression on neutrophils is likely due to degradation of internalised C5aR.

C5a- and PMA-induced downregulation of C5aR has previously been observed (20, 21, 32, 34). Our study is the first, to our knowledge, to show that enzymatic degradation is a significant contributor to this phenomenon. Our demonstration that NSPs are responsible for this cleavage and functional inactivation has important consequences for when considering pathologies associated with chronic unregulated NSP activity as discussed above. Our data complement and add to observations from several previous studies. Preincubation with PMA has previously been reported to reduce binding of C5a to neutrophils (34), which, on the basis of data presented in this study, is likely to be due to enzymatic cleavage of the C5aR in response to PMA-induced degradation (Fig. 5A), resulting in the generation of a membrane-bound 26- to 27-kDa C-terminal fragment (not shown). Other studies showed that preincubation of neutrophils with C5a reduced binding of an anti-C5aR Ab as measured by flow cytometry (20, 21, 32). This preincubation with C5a resulted in neutrophil dysfunction, characterized by reduced phagocytosis of zymosan and P. aeruginosa (20, 32). None of these studies investigated cleavage of the C5aR as a potential mechanism. C5a is known to induce the release of granule associated enzymes from neutrophils (35), and TNF- and GM-CSF–induced elastase release correlates with reduced C5a binding and inhibition of chemotaxis (36). Our novel observations of NSP-mediated cleavage of the C5aR and that C5a and fMLF–induced release of NSPs that subsequently cleave and inactivate the C5aR combine the above studies and explain findings of reduced C5a and anti-C5aR binding to the cells and the reduction in complement-dependent phagocytosis and intracellular killing (20, 32, 34). A study by Tralau et al. (25) is particularly interesting, as upon exposure to NSPs, neutrophils exhibited impaired responses to C5a but not to fMLF, IL-8, leukotriene B4, or platelet-activating factor. This is despite one other report of NE-mediated inactivation of the IL-8Ra/CXCR1, in which the authors suggested that inactivation of this receptor was the main cause of neutrophil dysfunction in CF (4). The results of Tralau et al. (25) may point to a difference in sensitivities of the C5aR, IL-8Ra/CXCR1, and the receptor for fMLF and leukotriene B4 to NSP-mediated inactivation.

Desensitization of neutrophils to C5a is thought to be caused by internalization of the C5aR following initial ligand binding. This becomes temporarily unavailable, before being recycled to the cell surface, at which point the cells can be restimulated. Our data using the monocytic cell line U937 support a model in which C5a stimulation leads to NSP secretion, which subsequently inactivates the C5aR. The cells only become responsive again after resynthesis of the C5aR. Desensitization to C5a after stimulation with fMLF (termed cross-desensitization) has been reported (37), and as we show, this is caused by fMLF-stimulated release of NSPs, resulting in C5aR cleavage. Desensitization and downregulation after C5a stimulation of neutrophils is likely mostly caused by internalization followed by intracellular degradation. However, C5a–induced NSP secretion can inactivate the C5aR on neighboring cells that have not been in contact with C5a. Internalization of the GPCRs followed by endosomal degradation and/or recycling to the cell surface is well documented and a very quick process that happens within minutes (38). Several studies investigated the fate of C5aR after ligand binding. Although some studies suggested that no internal degradation took place (39, 40, 41), another study (42) suggested that 50% of the C5aR was degraded after internalization. Our results suggest that in U937 cells no or minimal internal degradation may have taken place and that the degradation observed took place extracellularly, whereas in neutrophils, internal degradation may play a major role in reduction of C5aR expression. Van Epps et al. (39) showed that C5aR has a very long internalization/recycling time, and this may explain why only after 3 h, some of the 26- to 27-kDa C5aR-degradation fragment was observed. Kinetics of internalization and shedding of the C5aR expressed on U937 cells have not been investigated, which may explain the differences in C5aR cleavage observed after C5a exposure. Differences in cell types and experimental setup used in the above-mentioned studies and our study may account for the differences observed. As GPCR receptors are quickly internalized following ligand binding, a process that happens within minutes, they would be temporarily protected from extracellular degradation by NSPs, released from granules in response to GPCR activation, which also happens within minutes. This could be an autoprotective mechanism, as when the GPCR recycles to the cell surface, the NSPs may have diffused away or could have been inactivated. However, this would only be relevant if the GPCR is not degraded intracellularly, as observed for the C5aR in neutrophils.

The NE- and CG-induced cleavage described in this study resulted in the loss of the extracellular C5a-binding N terminus and retention of a 26- to 27-kDa C-terminal fragment in the membrane, reflected in a reduction in $M_i$ of 15 to 16 kDa. CG and NE have different enzymatic activities and would generate different fragments, which could be used as markers for NSP-mediated C5aR cleavage.

Model 1: Systemic C-activation (e.g. sepsis) results in excess C5a generation, resulting in C5aR mediated cell activation and NSP secretion. Formyl peptide release as in sepsis results in FPR-mediated NSP release.

Model 2: Excess neutrophil infiltration resulting in unregulated NSP secretion.

FIGURE 6. Proposed models for NSP-induced neutrophil dysfunction. Model 1, High levels of systemically activated C5a or formylated bacterial peptides as found in, for example, sepsis induces the release of NSPs, which subsequently cleave the C5aR on own cells and on neighboring bystander cells, resulting in neutrophil dysfunction. Model 2, Excess neutrophil infiltration, associated with excess unregulated secretion of NSPs by necrotic neutrophils, resulting in the cleavage of the C5aR on neighboring newly infiltrated bystander cells. Both mechanisms render the affected cells nonresponsive to further C5a stimulation, thereby impairing their ability to phagocytose and kill bacteria.
different amino acid preferences for cleavage of substrates. CG cleaves preferentially after L, H, and F, whereas NE cleaves after V, I, and A. Cleavage after H(100) by CG and cleavage after A (108) by NE would result in a predicted retained 27- to 28-kDa C-terminal fragment, similar to our experimental observations. These cleavage events are predicted to result in a loss of 11 and 12 kDa, respectively, whereas we observed a loss of 15 and 16 kDa, respectively. This is probably explained by differences in the calculated Mr (39 kDa) and our observed Mr (42 kDa) of native C5aR, likely due to N-linked glycosylation at N at position 5 (43).

A single cleavage event would still retain the N-terminal epitope in the membrane as a 15- to 16-kDa fragment. However, such a fragment was not observed using the anti–N terminal mAb, suggesting that a second cleavage event occurred most likely after L33 (CG) and V35 (NE), near the membrane, thereby releasing the N-terminal epitope. Although potential cleavage sites are present in the other extracellular loops, these sites could be protected due to steric hindrance. A C5aR agonist, which only requires the C-terminal C5a binding site, was still active, demonstrating that the C terminus had remained intact.

On the basis of the data presented in this study, we propose the following interlinked models for NSP-induced neutrophil dysfunction (Fig. 6). In the first model, C5a or other neutrophil stimulants such as fMLF are the initiating factor, and under conditions of excess systemic complement activation and C5a generation and bacteremia, as found in sepsis, these stimulants activate neutrophils, resulting in NSP secretion and cleavage and inactivation of the C5aR on the activated or unstimulated bystander cells, resulting in dysfunctional neutrophils and reduced bacterial clearance. In the second model, excess secretion of unregulated NSPs, as in the case of CF, is the initiating factor. In this case, circulating neutrophils are attracted to the lungs by locally produced chemokines such as IL-8 and also by C5a itself. Upon arrival in the lung, neutrophils are exposed to unopposed NSP activity due to the presence of large numbers of dead and dying neutrophils in the absence of appropriate phagocytic effectorcytosis (44) and the absence of appropriate antiprotease defense. These NSPs cleave the C5aR, resulting in these cells becoming impaired as in model 1. This results in a vicious cycle of newly arrived neutrophils becoming dysfunctional and releasing their contents, too, resulting in further neutrophil dysfunction and tissue damage.

In conclusion, C5a-induced NSP release or NSPs released through other mechanisms cleave and inactivate C5aR. Subsequent hypo- or responsiveness of cells to C5a may lead to defective phagocytosis and intracellular killing of opsonized bacteria. The use of NSP inhibitors in pathologies associated with excess NSP secretion but also with excess C5a and formyl peptide generation may be beneficial for patients with CF or sepsis and may prevent neutrophil dysfunction and aid the clearance of bacterial infections. Both CG and NE were found to cleave the C5aR in biological samples, and thus, therapeutically inhibition of both enzymes should be considered.

Disclosures

The authors have no financial conflicts of interest.

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