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Cholesterol Crystals Induce Complement-Dependent Inflammasome Activation and Cytokine Release

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Inflammation is associated with development of atherosclerosis, and cholesterol crystals (CC) have long been recognized as a hallmark of atherosclerotic lesions. CC appear early in the atheroma development and trigger inflammation by NLRP3 inflammasome activation. In this study we hypothesized whether CC employ the complement system to activate inflammasome/caspase-1, leading to release of mature IL-1β, and whether complement activation regulates CC-induced cytokine production. In this study we describe that CC activated both the classical and alternative complement pathways, and C1q was found to be crucial for the activation. CC employed C5a in the release of a number of cytokines in whole blood, including IL-1β and TNF. CC induced minimal amounts of cytokines in C5-deficient whole blood, until reconstituted with C5. Furthermore, C5a and TNF in combination acted as a potent primer for CC-induced IL-1β release by increasing IL-1β transcripts. CC-induced complement activation resulted in upregulation of complement receptor 3 (CD11b/CD18), leading to phagocytosis of CC. Also, CC mounted a complement-dependent production of reactive oxygen species and active caspase-1. We conclude that CC employ the complement system to induce cytokines and activate the inflammasome/caspase-1 by regulating several cellular responses in human monocytes. In light of this, complement inhibition might be an interesting therapeutic approach for treatment of atherosclerosis. The Journal of Immunology, 2014, 192: 2837–2845.

Cholesterol crystals (CC) have long been recognized as a hallmark of atherosclerotic lesions (1, 2). Identified as “cholesterol crystal clefts,” these crystalline structures were thought to arise late in the course of the disease (3). Using hyperlipidemic apolipoprotein E-deficient mice, we previously reported that CC are associated with early atheroma development (4). Oxidized low-density lipoprotein is endocytosed by CD36 that coordinates the intracellular conversion of this ligand to CC (5, 6).

However, the phagocytosis receptor for CC has yet to be discovered. Phagocytosis of CC induces lysosomal damage that results in the activation of the NLRP3 inflammasome, with subsequent activation of caspase-1 and secretion of IL-1β (4, 7), suggesting that the interaction between CC and NLRP3 inflammasomes could be linking lipids and inflammation, the two fundamental hallmarks of atherosclerosis.

IL-1β has long been described as a potent inflammatory cytokine, and its activation is associated with the severity of atherosclerosis (8). Release of the mature form of IL-1β is controlled by two signals in macrophages. The transcription of pro–IL-1β and NLRP3 are NF-κB-dependent and induced by a priming signal that either is provided by activation of pattern recognition receptors or via the presence of proinflammatory cytokines (9). Once activated, NLRP3, its adaptor ASC, and procaspase-1 form an inflammasome complex, which activates caspase-1 that leads to cleavage of the proforms of IL-1β and IL-18 to their mature forms (10). Although CC have been shown to activate NLRP3 inflammasomes, the endogenous primers for this activation are not well understood. One candidate is the complement system; however, this has so far not been explored in detail.

The complement system is an integral component of the innate immunity and has been shown to contribute to the pathology of several inflammatory diseases (11, 12). Complement can be activated by the classical, lectin, and the alternative pathways. All three pathways converge at the central C3 molecule, generating convertases that catalyze the conversion of C3 into its active fragments C3a and C3b. C3b is the amplification step that leads to downstream complement events with the generation of C5a, a highly potent inflammatory mediator, and the terminal complement complex (TCC) (12). Activation of the classical pathway starts with C1q that binds to IgG, C-reactive protein, and distinct structures on microbial or apoptotic cells. The lectin pathway is initiated through mannose-binding lectin and the ficolins, whereas

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Abbreviations used in this article: CC, cholesterol crystal(s); CR3, complement receptor 3; HSA, human serum albumin; IL-1Ra, IL-1R antagonist; ROS, reactive oxygen species; TCC, terminal complement complex.

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the alternative pathway is spontaneously activated by hydrolysis of the internal C3 thioester and acts to substantially amplify activation induced by the classical and lectin pathways. The molecular mechanisms by which a damage-associated molecular pattern, similar to CC, employs the complement system to activate inflammasome/caspase-1 is currently not known.

In this study we report that CC activate both the classical and alternative complement pathways that result in cytokine release. Our data demonstrate that the complement system controls several cellular processes involved in CC-induced inflammasome/caspase-1 activation. Moreover, we propose that C5a in combination with TNF may act as an endogenous priming signal for the CC-induced inflammasome, and we identify complement receptor 3 (CR3) as a candidate receptor for phagocytosis of CC.

**Materials and Methods**

**Reagents**

Cells were isolated by Lymphoprep (Axis-Shield, PBMCs) or Polymorphprep (Axis-Shield, granulocytes or PBMCs). Anticoagulant in whole-blood experiments was lepirudin (Celsite). C1q-depleted serum and purified C1q were from Complement Technology. TNF was from Genentech. Purified C5a and recombinant C5a, ultrapure cholesterol, ATP, cytochalasin D, and zymosan were from Sigma-Aldrich. LPS from Escherichia coli was from InvivoGen (0111:B4), recombinant C3a was from R&D Systems, and human serum albumin (HSA) was from Octapharma. The following reagents were used for quantitative PCR analyses: RNasey Mini Kit (Qiagen), DNase and high-capacity RNA-to-cDNA kit (Applied Biosystems), and Perfecta qPCR FastMix (Quanta Biosciences). Probes and primers were from Applied Biosystems: GAPDH (Hs99999905_m1), NLRP3 (Hs0018082_m1), and IL-1β (Hs01555410_m1). The following Abs were used: anti-CD11b-PE (D12; BD Biosciences), anti-CD14-FTTC (Mep9; BD Biosciences), anti-CD14-PE (Mep9; BD Biosciences), CD45-PerCP (2D1; BD Biosciences), anti-IGG detector, rabbit, PE (C01-359; BD Biosciences), anti-human C1q (A0136; Dako), cleaved IL-1β (2021; Cell Signaling Technology), polyclonal goat anti-mouse HRP (P0447; Dako), normal rabbit IgG (Ab105-C; R&D Systems), infliximab (Janssen Biosciences), ecuclizumab (Alexion), rituximab (Roche), and anti-C7 (Quidel). For complement inhibition, the C3 inhibitor compstatin analog 22 CP40 (AcF[OPdChaWR]) was synthesized as previously described (14). Compstatin (20 μg/ml) was incubated with CC (1.5–6 × 10^7 particles/ml) for 30 min at 37°C in the presence of PBS/HSA. Positive control was a mixture of zymosan (10 μg/ml) and heat-aggregated human IgG (10 μg/ml; Octapharma). The following complement activation products were measured by ELISA as described elsewhere (16, 17): soluble TCC, C1rs-C1-INH, C3bBbP, C3bc, and C4bc. These assays are based on monoclonal capture Abs specific for neoepitopes expressed after a component is activated, and thus only the activated form is detected. TCC is detected by an Ab to a neoepitope expressed in C9. The C3b fragment is composed of C3c and C3d and is rapidly split into these two fragments upon activation. For C3bc measurements, the Ab detects a neoepitope that appears on the C3c part of C3b when C3 is cleaved. This neoepitope is preserved on the C3c fragment after cleavage of C3b and is therefore designated anti-C3bc and measures the total amount of this neoepitope expressed after C3 activation. The principle also works for detection of C5 activation, which is denoted C4bc. C1q-depleted serum (diluted 1:2) with or without reconstitution with purified C1q (5 μg/ml) was incubated with CC (1.5 × 10^7 particles/ml) or PBS/HSA and TCC was measured as above. C1q deposition on CC was determined in plasma that had been incubated with CC (1.5 × 10^7 particles/ml) for 30 min and stained with anti-human C1q (5 μg/ml) and anti-IgG detector, rabbit, PE as described by the manufacturer. Measurements were performed on a Beckman Coulter Epics XL-MCL.

**Quantitative real-time PCR**

Total RNA was extracted from PBMCs or monocytes with an RNasey Mini kit and QIAcube robotic work station (Qiagen) following the manufacturer’s instructions. Cell extracts were DNase treated before being reverse transcribed using a high-capacity RNA-to-cDNA kit. mRNA was analyzed by a StepOnePlus real-time PCR system and its software (Applied Biosystems), TaqMan gene expression assays, and TaqMan Universal Master Mix (Applied Biosystems) with 20 μl reaction volume in triplicate wells. All data were normalized to GAPDH and expressed as fold change over controls.

**Western blot**

Cleaved IL-1β was detected in serum-free supernatants from monocytes. Samples were immunoprecipitated by methanol/chloroform, loaded into 10–12% Bis-Tris gel (Invitrogen), and subjected to SDS-PAGE. Gels were blotted onto nitrocellulose membranes using an iBlot 7-min blotting system (Invitrogen) and stained with cleaved IL-1β Ab (2 μg/ml) overnight at 4°C. Membranes were washed three times with 0.1% Tween 20 containing PBS, incubated with HRP-conjugated goat anti-mouse (1:25,000) for 1 h at RT, washed three times, and scanned using an Odyssey infrared imaging system (LI-COR Biosciences).

**Caspase-1 detection**

Whole blood was stimulated with CC for 4 h and incubated for 2 h with probes for caspase-1 detection (FAM FLICA caspase-1 assay kit; ImmuNoChemistry Technologies). Blood was stained with anti-CD11b-PE (50 μg/ml) before RBC lysis with FACS lysing solution (BD Biosciences). Analyses were performed on a BD FACSCanto II (BD Biosciences).

**CD11b and reactive oxygen species detection**

For CD11b detection, whole blood was fixed with 1% paraformaldehyde for 4 min at 37°C and stained with anti-CD11b-PE (50 μg/ml) and anti-CD14-FTTC (25 μg/ml). Identification of nucleated cells was done with LDS-751 (8 μg/ml; Sigma-Aldrich). Oxidative burst was determined using the Burstest (Phagoburst) kit from BD Biosciences with some modifications. Whole blood was incubated with complement inhibitors or PBS control and stimulated with CC or PBS/HSA. After 10 min of incubation, DHR 123 was added according to kit procedure, incubated for 10 min prior to lysis of RBCs with FACS lysing solution, washed, and stained with anti-CD11b-PE and anti-CD14-PerCP for 15 min on ice. Samples were washed once and ran on a FACSCalibur flow cytometer (BD Biosciences). Data analyses were performed with FlowJo (version 10, Tree Star).
Phagocytosis of CC

Whole blood was preincubated with compstatin Cp40 (20 μM) and other inhibitors as described above. Samples were stimulated with CC (3 × 10^7 particles/ml) or PBS/HSA for 20 min at 37°C. Cells were fixed with 0.5% paraformaldehyde for 4 min and stained with anti–CD45-PerCP, anti–CD14-FITC, and anti–CD11b-PE. After staining, RBCs were lysed using FACS lysing solution, washed, run on an LSR II flow cytometer (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences). Granulocytes and monocytes were selected as CD45+ cells and gated based on CD14 expression. Phagocytosis was determined based on shift in side scatter induced by CC ingestion, gated such that not >4% of negative cells were defined as positive for phagocytosis.

Statistical analysis

SPSS version 21 (IBM) was used for the nonparametric two-way ANOVA, used in Fig. 3A. GraphPad Prism version 5 (Graphpad Software) was used for the remaining analyzes, and p < 0.05 was considered statistically significant. Statistical analysis was performed on at least six independent experiments from different donors, unless otherwise indicated. Fig. 1A–E were analyzed using two-way ANOVA on log-transformed data with Bonferroni posttests. Figs. 1G, 2, and 3 were analyzed with Wilcoxon matched-pairs signed rank test, whereas Fig. 5C and 5D were analyzed using one-way ANOVA and Dunn’s multiple comparison test. The statistical significance of the decrease compared with the control peptide group was determined using one-way ANOVA and Dunnett’s multiple comparisons test on log-transformed data.

Study approval

Approval no. S-04114 from the Regional Ethics Committee, South-East Regional Health Authority and approval no. 32-2004 from the Regional Ethics Committee, Northern Norway Regional Health Authority were received for the use of whole blood. Approval no. 2009/224 was received from the Regional Ethical Committee, Norwegian University of Science and Technology for the use of human buffy coats and leukocytes. Blood from the C5-deficient individual was obtained and used in accordance with a protocol approved by the Regional Ethics Committee (REK Nord no. 2010/1141). The study conforms with the principles outlined in the Declaration of Helsinki for use of human tissue or subjects. Signed informed consent was obtained from all participants.

Results

CC activate the classical and alternative complement pathway

In the first set of experiments CC were added to serum for 30, 60, and 120 min and the complement activation products were measured. This led to a strong, time-dependent, and significant (p < 0.001) increase in both TCC and C3bc (Fig. 1A, 1B). Moreover, marked depletion of C3bc and TCC was observed on the crystal surface, demonstrating the strong opsonization and complement activation potential of CC (Fig. 1H, 1I). The alternative pathway convertase C3b-Bb-properdin complex was also markedly and significantly (p < 0.001) increased by CC at all time points (Fig. 1C). The modest increase in C4b/c, a marker of both classical and lectin pathway activation, did not reach statistical significance (Fig. 1D). No increase in C1r-C1-INH in the soluble phase was observed (Fig. 1E), consistent with attachment of C1r-C1-INH complexes to the CC surface, which were not liberated into the liquid phase. Supporting this, C1q bound strongly to the surface of the CC incubated in plasma for 30 min (Fig. 1F). To further investigate the involvement of the classical pathway, we compared the formation of TCC in C1q-depleted serum in the absence and presence of purified C1q. CC were unable to activate complement in C1q-depleted serum, whereas C1q reconstitution significantly (p < 0.05) restored the complement activation potential (Fig. 1G). Collectively, these data demonstrate the robust complement activation potential of CC, where the classical pathway plays an instrumental role in the initial phase of the activation, propagated by the alternative amplification loop.

CC induce complement-dependent cytokine release

The ability of CC to induce cytokine responses in the whole blood system was then examined. Addition of CC for 6 h resulted in a marked induction of proinflammatory cytokines (TNF, IL-1β, IL-6), chemokines (IL-8, MIP-1α, MIP-1β, MCP-1) and the inhibitor IL-1Ra (Fig. 2). LPS contamination of the CC was eliminated as a cause for these responses, as the amount of LPS present in the CC was below the detection limit in the Limulus amebocyte lysate assay. Furthermore, anti-CD14 Abs or lipid IVa did not reduce the CC-induced cytokines, whereas the LPS response was completely inhibited with the anti-CD14 Ab (data not shown). By separation of blood leukocytes into peripheral mononuclear cells (PBMCs) and granulocytes, we found that the PBMC fractions were responsible for the CC-induced IL-1β, TNF, IL-6, MCP-1, and MIP-1α, as significantly fewer (p < 0.05) of these cytokines were detected from CC-stimulated granulocytes (Fig. 3). The granulocytes responded to CC with a minor IL-8 release compared with PBMCs, whereas a vigorous secretion of myeloperoxidase occurred in granulocytes, but not in PBMCs (p < 0.05).

The cytokine responses induced with the CC in whole blood were complement dependent, as the C3 convertase inhibitor compstatin significantly reduced (p < 0.05) release of all cytokines measured (Fig. 2). For TNF, IL-6, IL-8, MIP-1α, and MCP-1, the inhibition with compstatin was >90%. In general, cytokine responses to LPS were greater than to CC. In contrast to CC, compstatin did not inhibit LPS-induced cytokine responses, except for IL-8, which was reduced by 50%, but overall did not reach statistical significance (Fig. 2D). LPS has in earlier studies been shown to have a weak complement-activating potential that can contribute to the IL-8 response in whole blood (18). Because compstatin inhibits generation of C3a, we also included a C5-blocking Ab (eculizumab), which blocks the cleavage of C5 to C5a and C5b. In general, the results obtained with eculizumab showed the same inhibition profile as for compstatin (Supplemental Fig. 1).

Addition of CC to whole blood resulted in a 5- to 10-fold increase in the TNF release compared with nonstimulated controls. Because TNF represents an early cytokine response, we addressed whether TNF induced by CC could act in a feedback loop and affect the production of other cytokines in the whole blood model. Thus, we added the TNF-blocking Ab infliximab together with CC and measured cytokine release and observed significant inhibition (p < 0.05) of MIP-1α and MCP-1 as compared with the negative control Ab rituximab, but no effect was found on IL-6 release (Fig. 2). Infliximab also reduced CC-stimulated release of IL-1β, IL-8, MIP-1β, and IL-1Ra; however, these effects did not reach statistical significance. These results suggest that TNF is involved in the release of cytokines that are induced by CC in whole blood.

CC-induced CD11b/CD18 (CR3) expression and cytokine release are reconstituted by C5 in whole blood from a C5-deficient person

In the next set of experiments the role of C5 in the inflammatory response induced by CC was investigated in whole blood from a C5-deficient person. C5 deficiency is a rare condition and is previously described in ~40 cases worldwide. The case report for the C5-deficient person used in this study has previously been published (19). No formation of TCC occurred in whole blood from the C5-deficient person incubated with CC, whereas reconstitution with purified C5 completely restored the TCC formation to that of healthy individuals (Fig. 4A). Furthermore, CC did not result in granulocyte or monocyte CR3 expression in the C5-deficient person, whereas addition of C5 completely...
restored the CR3 expression to levels seen in healthy individuals (Fig. 4B, 4C). Some CR3 expression was induced by LPS in the C5-deficient person, confirming earlier reports of a complement-dependent and -independent CR3 expression by LPS (18).

The CC-induced cytokine response was then investigated in whole blood from the C5-deficient person. In C5-deficient blood, CC induced low secretion of IL-1β, TNF, IL-6, and IL-8 that was comparable with levels in nonstimulated blood from healthy individuals (Fig. 4D–G). Reconstitution with purified C5 resulted in a 2.5- to 5-fold increase in the CC-induced cytokine secretion. Addition of C5 to C5-deficient whole blood led to a slight increase in TCC and cytokines in the nonstimulated samples, consistent with purified C5 being slightly activated spontaneously as seen previously (20). Because we only have one available case of C5 deficiency, proper statistical analysis of these data is not feasible. However, this case supports that C5 contributes to the CC-induced cytokine response in whole blood.

C5a activates the C5a receptor (CD88) on immune cells, resulting in several types of inflammatory responses (21). Of interest, we observed that addition of a C5a receptor antagonist to whole blood reduced the CC-induced IL-1β by 81 ± 8% (mean ± SEM, n = 3, p < 0.01) and CC-induced TNF by 60 ± 3% (mean ± SEM, n = 3, p < 0.01) compared with untreated control. Taken together, these data support a role of C5 in the CC-induced cytokine responses in whole blood and point to C5a as a mediator of these responses.

Combining C5a and TNF prime PBMCs and monocytes for CC-induced IL-1β

We have previously reported that CC activate the NLRP3 inflammasome in phagocytes in vitro (4). Before CC can activate NLRP3, the phagocytes must be primed in an NF-κB–dependent manner so that the pro–IL-1β and NLRP3 are induced and present in sufficient amounts (9). Low doses of exogenously added LPS can act as a potent primer for CC-induced IL-1β processing (4); however, endogenous primers for NLRP3 by CC have not yet been fully addressed. Because we observed involvement of C5 on the CC-induced cytokine responses as well as markedly enhanced TNF release, C5a and TNF were examined alone or in combina-

![FIGURE 1.](https://www.jimmunol.org/Download/2840_ComplementContolsCC-inducedInflammation/fig1.png)

**FIGURE 1.** CC activate the alternative and classical complement pathways. Human serum was incubated at 37°C, for indicated times, in the presence of CC (3 × 10^7 particles/ml), zymosan, and heat-aggregated IgG (Zym-IgG) or PBS. (A) The end product in complement activation, TCC, showed a significant increase (***p < 0.001) in response to CC compared with PBS at every time point; (B) the activation product C3bc, from the common complement component C3 for all three initial pathways, showed a significant increase (***p < 0.001) at all time points in response to CC compared with PBS; and (C) the alternative pathway convertase, C3bBbP, showed a significant increase (***p < 0.001) in response to CC compared with PBS at every time point. The increase in (D) the common activation product for the classical and lectin pathways, C4bc, and (E) the activation product for the classical pathway, C1rs-C1-INH, did not reach statistical significance. Data plotted are means ± SEM from six independent experiments with serum from healthy donors. (F) C1q binding to CC, when incubated in plasma for 30 min, measured by flow cytometry. (G) TCC in C1q-depleted serum (C1q dep.) with or without reconstitution (C1q rec.) (p < 0.05) with purified C1q upon CC (1.5 × 10^7 particles/ml) stimulation, measured by ELISA. One of six independently performed experiments is shown. In the lower panel, human whole blood was incubated with CC (1.5 × 10^7 particles/ml) for 30 min. (H) Binding of TCC to the crystals was detected using anti-C5b-9 and anti-mouse IgG conjugated with Alexa 488, and for C3bc (I) anti-C3bc Ab directly conjugated to FITC. (J) Control IgG2a conjugated to FITC. Scale bars, 10 μm. Data are representative of two independent experiments. AU, arbitrary units; DIC, differential interference contrast.
tion for their priming properties. C5a and TNF were found to synergize in providing a potent priming signal in CC-activated human PBMCs (p < 0.001) (Fig. 5A) whereas a minimal priming effect was observed with the C3a and TNF combination (Supplemental Fig. 2A).

To verify that the released IL-1β represented the mature form and was a result of inflammasome activation, human monocytes were primed and activated. Supernatants were precipitated and subjected to Western blot analysis (Fig. 5B), showing the cleaved and mature 17-kDa form of IL-1β. Priming of monocytes with a combination of C5a and TNF followed by addition of CC resulted in a robust release of the mature form of IL-1β. Also, the NLRP3 activator ATP gave a strong release of mature IL-1β from monocytes treated with the C5a/TNF combination (Fig. 5B). We observed that the C5a/TNF combination increased the IL-1β transcripts in monocytes (p < 0.05) (Fig. 5C). TNF alone also increased the IL-1β mRNA, whereas C5a alone had no or minimal effect. The NLRP3 mRNA was hardly increased by the C5a/TNF combination (Fig. 5D), suggesting that the priming effect of C5a/TNF is predominantly to induce increased amounts of pro–IL-1β.

**FIGURE 2.** CC induce complement-dependent cytokine release. Human whole blood was incubated with CC (3 x 10⁷ particles/ml), PBS, or LPS for 6 h after preincubation with PBS, control peptide, compstatin, anti-TNF infliximab, or control anti-CD20 rituximab at 37°C. Cytokines and chemokines were quantified in plasma by multiplex analysis (A–H). T0 represents the start of the experiment. Dataset on the left of the dividing line (T0, CC, PBS) is plotted on the left y-axis, and dataset on the right of the dividing line (LPS) is plotted on the right y-axis. Data plotted are means ± SD from triplicate determinations in one out of at least six independently performed experiments from healthy donors (*p < 0.05, **p < 0.001).

**FIGURE 3.** CC-induced release of cytokines from PBMCs and granulocytes. Human PBMCs and granulocytes were isolated from whole blood and resuspended in plasma/PBS before incubation with CC (3 x 10⁷ particles/ml) for 5.5 h. CC-induced production of the cytokines (A) IL-1β, (B) TNF, (C) IL-6, (D) IL-8, (E) MIP-1α, and (F) MCP-1 from PBMCs and granulocytes were quantified by multiplex analysis and compared (*p < 0.05). (G) Myeloperoxidase (MPO) from PBMCs and granulocytes was detected by ELISA (*p < 0.05). Data plotted are means ± SD in triplicate determinations in one of six independent experiments.
CC-induced upregulation of CR3 and phagocytosis of CC are complement-dependent

Phagocytosis of CC is required for NLRP3 activation (4). Thus, the role of complement activation in the phagocytosis of the crystals was investigated. Compstatin inhibited phagocytosis of CC in monocytes by 50–60% after 20 min of CC incubation, with minimal inhibition after 60 min (Supplemental Fig. 2B). This result indicates that complement activation contributes to the phagocytosis of CC in isolated human monocytes at early time points, which may be crucial for the initial inflammasome activation and subsequent IL-1β release.

To further address the involvement of complement activation in the phagocytosis of CC by leukocytes in whole blood, the effect of complement inhibition was examined. Compstatin, eculizumab, and the C5a receptor antagonist reduced the CC-induced CR3 upregulation down to baseline levels ($p < 0.05$) (Fig. 6A, 6B). No inhibitory effect was seen with the anti-C7 Ab that inhibits TCC generation without affecting C3 and C5. Granulocytes were more effective than monocytes to phagocyte CC (Fig. 6C, 6D). Compstatin, eculizumab, and the C5a receptor antagonist reduced granulocyte phagocytosis by >80% ($p < 0.05$) and monocyte phagocytosis by >60% ($p < 0.05$). In line with these findings, granulocytes, and to lesser extent monocytes, from the C5-deficient person had markedly reduced phagocytosis of CC, and reconstitution with C5 restored the phagocytosis to control levels (Fig. 6E, 6F). Taken together, these data suggest that CR3 might be an important phagocytosis receptor for CC in monocytes and, in particular, in granulocytes. Furthermore, the clear inhibitory effect by the C5a receptor antagonist demonstrates that C5a is a mediator of CC phagocytosis in leukocytes, consistent with its potential to upregulate expression of CR3.

CC induce production of reactive oxygen species and activation of inflammasome/caspase-1 in a complement-dependent manner

A consequence of complement dependent phagocytosis is the induction of reactive oxygen species (ROS), which has been implicated in activation of inflammasomes (10). Addition of CC to human whole blood resulted in a dose-dependent relationship between CC and phagocytosis as well as CC and ROS production in both granulocytes and monocytes (Fig. 7A, 7B). The CC-induced ROS production was reduced by compstatin, eculizumab, and C5aR antagonist ($p < 0.05$) (Fig. 7C, 7D). Of interest, eculizumab and the C5aR antagonist were equally effective in reducing the CC-induced oxidative burst, suggesting that C5a, and not the sublytic TCC, is an important mediator in this response. Moreover, granulocytes and monocytes from the C5-deficient person had attenuated CC-induced ROS responses that were restored to control levels by C5 reconstitution (Fig. 7E, 7F). Importantly, the CC-induced ROS production was accompanied by...
a marked caspase-1 activation in the monocytes, whereas a lower caspase-1 activity was detected in granulocytes (Fig. 7G, 7H). Compstatin was particularly effective in reducing caspase-1 activity; however, also eculizumab and the C5aR antagonist significantly (*p, 0.05) inhibited caspase-1 in monocytes, suggesting that C5a is a mediator also for CC-induced caspase-1 activation.

Discussion

Despite early identifications of their physical properties, CC have, until recently, been considered as relatively inert (1). However, we and others have shown that CC-induced inflammation is a critical contributor to atheroma development and progression in experimental atherosclerosis (4, 7). In the present study we have identified new and important principles of CC-induced inflammatory responses and, to our knowledge, for the first time documented that the complement system is crucial for CC-induced inflammasome/caspase-1 activation.

Our data demonstrate that CC induced a robust complement activation that resulted in high amounts of C3bc and the soluble TCC. Previous studies have suggested that CC activate the alternative pathway (22–24). Our data confirm these findings, as the amount of C3b-Bb-properdin was strongly increased by the CC. Notably, we also found a clear activation of the classical pathway by C1q binding to the CC surface. Further studies are needed to clarify whether C1q binds directly to the CC, or indirectly via
other molecules such as Igs or C-reactive protein. It cannot be excluded that also the lectin pathway may contribute to CC-induced complement activation. However, because the CC lack carbohydrates, the contribution of the lectin pathway is likely to be minimal. Moreover, methods to detect specific complement activation products for the lectin pathway are, to our knowledge, not available. The alternative pathway may play an important role in the downstream effect of initial classical pathway activation by the CC, as the alternative pathway has been reported to amplify the outcome of total complement activation initially triggered by the classical or lectin pathway by 80–90% (25, 26).

The human whole blood model is a unique tool to explore the role of the complement system in CC-induced inflammation. It allows inflammatory cross-talk by using an anticoagulant that does not affect complement activation (16). In this model we demonstrate a marked release of proinflammatory cytokines, in particular TNF, when CC is added. Minimal cytokine response and strong myeloperoxidase release were observed from granulocytes upon addition of CC, suggesting that monocytes are the primary cell type that responds to CC by releasing cytokines. By adding infliximab, some of the CC-induced cytokines were reduced, suggesting that TNF may act as an amplifier of CC-induced cytokine release. The inflammatory cytokine response to CC was strongly dependent on complement activation, as both C3 and C5 inhibitors attenuated the response. Furthermore, the CC-induced cytokines were lower in whole blood from a C5-deficient person, which were restored by reconstitution with purified C5.

Among the complement activation products, C5a is described as the most potent inflammatory peptide, with a broad spectrum of functions in immune activation and inflammation (11, 27, 28). Early studies have shown that C5a can potentiate secretion of TNF by human PBMCs (29) whereas others have shown that C5a stimulates transcription of TNF (30). Among other effector functions of complement is the ability of the terminal pathway to form sublytic TCC that can trigger cell activation, including the release of inflammatory cytokines such as TNF, without inducing cell death (31–34). The sublytic TCC may also trigger intracellular Ca²⁺ fluxes leading to NLRP3 activation (35). However, addition of an anti-C7 Ab that inhibited the TCC formation by 50–60% did not reduce either CC-induced IL-1β or TNF release. Notably, the CC-induced TNF and IL-1β responses were reduced with the C5a receptor antagonist, suggesting that C5a is a key player in the induction of proinflammatory cytokines induced by CC.

Adding TNF and C5a together to monocytes in vitro enhanced the pro–IL-1β mRNA; however, the NLRP3 mRNA was not increased by this combined treatment. Fully differentiated macrophages have modest levels of basal NLRP3 expression whereas monocytes show high endogenous NLRP3 levels (36). Thus, induction of pro–IL-1β may be sufficient as a priming signal in monocytes for CC-induced NLRP3 activation. Furthermore, C5a has the ability to increase the expression of its own signaling receptor CD88 (37). The priming effect was strongest for the C5a/TNF combination, as only minimal priming was observed for the C5a/TNF combination. These findings suggest that TNF, which is released early during an inflammatory response, may act together with C5a as an endogenous primer for CC-induced NLRP3 activation in monocytes by enhancing the transcripts of pro–IL-1β.

Activation of the NLRP3 inflammasome is also dependent on ROS generation. Many of the NLRP3 activators, including ATP and crystalline structures such as asbestos and monosodium urate, are triggers of short-lived ROS (38). Our data from the whole blood assay show that monocytes and granulocytes produce ROS in response to CC in a complement-dependent manner, which may contribute to inflammasome activation. Indeed, we show in the same model that CC also activated caspase-1 in a complement-dependent manner; however, little caspase-1 activation occurred in granulocytes compared with monocytes, which corresponds well with the low CC-induced IL-1β release from granulocytes. Moreover, our data suggest that monocytes and granulocytes employ CR3 for the phagocytosis of CC. CR3 is expressed on monocytes, macrophages, and granulocytes and is activated during the innate immune response to microbes. C3b, a cleavage product of C3, coats invading microbes and is further cleaved to form C3bi, which is the main ligand for CR3, resulting in a phagocytic response. CR3 is a central receptor that mediates phagocytosis of E. coli by monocytes and granulocytes (18). Several lines of evidence suggest that CR3 is an essential phagocytosis receptor also for CC. Addition of CC to whole blood resulted in C3 opsonization and a substantial upregulation of CR3 (detected by CD11b) in monocytes and granulocytes. Complement inhibitors virtually abolished both the CR3 expression and phagocytosis of CC, and a C5-deficient donor had blunted CR3 expression and phagocytosis in response to CC. Moreover, C5a was found to be a mediator of both the CC phagocytosis and CR3 expression, which is in line with previous data showing that C5a directly induces CR3 expression in monocytes and granulocytes (18).

The results in the present study demonstrate that the complement system participates in CC-induced inflammasome activation in monocytes by regulating several cellular responses. The combination of C5a and TNF acts as a primer for inflammasome/caspase-1 activation, and complement activation leads to C5a generation with subsequent upregulation of CR3, CC phagocytosis, ROS production, and formation of functional caspase-1. We suggest that the connection observed between CC-induced complement activation and inflammasome activation is important for development of atherosclerosis. Complement components are detected in atherosclerotic plaques, and circulating levels of C5a are increased in persons with advanced atherosclerosis (39). Moreover, a C5a inhibitor has been reported to reduce atherosclerosis in apolipoprotein E–deficient mice (40). Interestingly, treatment with this inhibitor did not reduce the number of immune cells in lesion sites, but instead significantly inhibited the lipid content within the plaques. The lowering effect of C5a inhibition on cholesterol content in local atherosclerotic lesions may be attributed to the effect of C5a on phagocytosis of CC, suggesting a role for complement not only in inflammation, but also in lipid accumulation. The bidirectional interaction between lipids and inflammation is a hallmark of atherosclerosis, and our findings suggest that CC and complement are important mediators in these processes.

IL-1β is proposed to be involved in the development of atherosclerosis. A clinical trial is currently testing the long-term effect of an IL-1β Ab on recurrent cardiovascular events (41). In summary, our data demonstrate that CC induce cytokine responses that are dependent on complement activation, and that C5a and TNF together play a role in controlling inflammasome activation by the crystals. Furthermore, we describe data implying that CR3 is a candidate receptor for mediating phagocytosis of CC. Altogether, results in the present study point to the complement system as a key trigger involved in CC-induced inflammation and suggest that complement inhibition, in particular at the level of C5, is an interesting target for treatment of cardiovascular disease.

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Disclosures
J.D.L. is the holder of several patent applications on complement inhibitors and the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors for clinical applications. The other authors have no financial conflicts of interest.

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