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Cutting Edge: The NLRP3 Inflammasome Links Complement-Mediated Inflammation and IL-1 β Release

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The complement system is a potent component of the innate immune response, promoting inflammation and orchestrating defense against pathogens. However, dysregulation of complement is critical to several autoimmune and inflammatory syndromes. Elevated expression of the proinflammatory cytokine IL-1 β is often linked to such diseases. In this study, we reveal the mechanistic link between complement and IL-1 β secretion using murine dendritic cells. IL-1 β secretion occurs following intracellular caspase-1 activation by inflammasomes. We show that complement elicits secretion of both IL-1 β and IL-18 in vitro and in vivo via the NLRP3 inflammasome. This effect depends on the inflammasome components NLRP3 and ASC, as well as caspase-1 activity. Interestingly, sublethal complement membrane attack complex formation, but not the anaphylatoxins C3a and C5a, activated the NLRP3 inflammasome in vivo. These findings provide insight into the molecular processes underlying complement-mediated inflammation and highlight the possibility of targeting IL-1 β to control complement-induced disease and pathological inflammation. *The Journal of Immunology*, 2013, 191: 1006–1010.

Inflammation is an integrated immune process that is rapidly initiated either when tissues are damaged or by pathogenic infections. Two main players of the inflammatory process are the complement system and innate phagocytes (macrophages and dendritic cells [DCs]). Complement is a large collection of plasma proteins whose activation culminates in the assembly and deposition of membrane attack complexes (MACs) that kill infected or damaged cells through disrupting membrane integrity. Nonlethal amounts of MAC induce profound phagocyte activation, with the production of inflammatory mediators, such as PGs, leukotrienes,

and reactive oxygen species (1). Alongside MAC formation, the production of bioactive complement fragments C3a and C5a, known as anaphylatoxins, promotes local inflammatory events, including recruitment of phagocytic cells to the site of inflammation (2). These phagocytes cooperate with complement, binding and internalizing the infectious agents via cell surface receptors (TLRs, lectins, complement and Fc receptors) and releasing proinflammatory cytokines (IL-1, TNF- α , and IL-6). Although inflammation is crucial for effective resolution of infection, it also has the capacity to damage cells and tissue if inappropriately regulated. Unrestrained activation generates a harmful chronic inflammatory environment that can result in conditions such as rheumatoid arthritis, lupus, multiple sclerosis, and Alzheimer's disease (3). As such, there is a pressing need to understand the complex process of inflammation and to identify new targets for therapeutic intervention in the treatment of autoimmune and autoinflammatory disorders.

The complement system itself has recently begun to attract interest as a therapeutic target. In particular, it seems that complement components can enhance proinflammatory TLR-mediated signaling in phagocytes, leading to increased production of IL-1 β (4, 5). IL-1 β is critically involved in several inflammatory diseases and is elevated in many conditions characterized by complement overactivation (4, 5). However, it is unknown whether, or how, complement and IL-1 β are linked.

One possibility is that the interaction of complement system with TLRs on phagocytes also enhances TLR cross-talk with members of the intracellular nucleotide-binding oligomerization domain-like receptor family. Some nucleotide-binding oligomerization domain-like receptors assemble with the adaptor protein ASC to form the caspase-1-activating complexes, which are responsible for the production of active IL-1 β . Activation of the NLRP3 inflammasome promotes maturation of the proinflammatory cytokines IL-1 β and

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Abbreviations used in this article: B6, C57BL/6; Cf, complement factor; Cvf, cobra venom factor; DC, dendritic cell; HI-NRC, heat-inactivated normal rabbit complement; MAC, membrane attack complex; MSU, monosodium urate; NRC, normal rabbit complement; WT, wild-type.

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IL-18 (6). Thus, IL-1 β production occurs in two steps: pro-IL-1 β synthesis is triggered by pattern recognition receptor ligation, leading to NF- κ B activation, before being cleaved by inflammasome-activated caspase-1 to produce the active cytokine (7). The NLRP3 inflammasome is activated by diverse stimuli, including microbial molecules, host-derived molecules associated with stress or danger, and crystalline or particulate substances (8, 9); many of these are also able to induce complement activation.

Thus, we hypothesized that complement and IL-1 β were linked through activation of the inflammasome. In this study, we show that complement induces IL-1 β and IL-18 maturation and release from DCs, dependent on the NLRP3 inflammasome. MAC deposition at sublethal levels, but not C3a or C5a, activated the NLRP3 inflammasome in vivo. Thus, our results reveal a new molecular mechanism linking complement with proinflammatory cytokine production from immune phagocytes. This brings a new perspective to the search for therapeutic targets in diseases characterized by abnormal complement and IL-1 β activity.

Materials and Methods

Mice

C57BL/6 (B6) and BALB/c mice were purchased from the Biological Resource Center (Agency for Science, Technology, and Research), and *C3ar*^{-/-} and *C5ar*^{-/-} mice on the BALB/c background were from The Jackson Laboratory. *Nlrp3*^{-/-}, *Asc*^{-/-}, and *C66*^{-/-} (B6 background) mice were described previously (10–12). All mice were backcrossed onto the B6 or BALB/c background for at least eight generations. Experiments were conducted with 8–10-wk old female mice bred under specific pathogen-free conditions, under the approval of the local ethics committee.

Cell culture and stimulation

Bone marrow DCs, prepared as described (13), were primed with ultrapure LPS (0.1 μ g/ml, *Escherichia coli* 055:B5; Alexis) in Opti-MEM medium (Life Technologies) for 3 h. Medium was replaced by adding normal baby rabbit serum as a source of complement (Cedarlane Laboratories) or monosodium urate (MSU; 250 μ g/ml; Alexis) for 4 h. In some experiments, cells were preincubated with the caspase-1 inhibitor Z-YVAD-FMK after LPS priming. To inhibit MAC formation, normal rabbit complement (NRC) was preincubated with anti-C6 mAb (1.25 μ g/ml, clone WU 6-4; Hycult), alone or with anti-C9 mAb (7.5 ng/ml), for 30 min at 37°C before adding to DCs.

ELISA

Murine IL-1 β , IL-1 α , and IL-6 were measured in cell-free supernatants and sera using a DuoSet ELISA kit (R&D Systems), according to the manufacturer's instructions. IL-18 was measured by ELISA, as described (13).

MAC deposition

DCs were stimulated with LPS in combination with NRC or heat-inactivated (HI)-NRC for 4 h. Cells were collected and washed, and Fc γ II/Fc γ IIIr were blocked by anti-mouse CD16/32 (clone 93; eBioscience). Anti-human C9 Ab and anti-mouse IgG1 were then used to detect MAC formation on cell membrane. Single events were acquired using a BD LSR II flow cytometer (BD Biosciences) and analyzed by FlowJo software (TreeStar).

Immunoblot analysis

Supernatants were immunoprecipitated with anti-mouse IL-1 β Ab (clone B122; BioLegend), followed by Protein G Sepharose beads (GE Healthcare), according to the manufacturer's instructions. Protein pellets were resuspended in sample loading buffer and resolved by 15% SDS-PAGE. Immunoblotting of cell lysates was performed following standard procedures. Membranes were immunoblotted with goat anti-mouse IL-1 β or anti-mouse caspase-1 p10 Ab (clone M-20; Santa Cruz Biotechnology).

In vivo complement activation

Mice were injected i.p. with 20 mg/kg LPS (*E. coli* 0111:B4; Sigma). Where indicated, mice also received 60 μ g cobra venom factor (CVF; Quidel) i.p.

After 3 h, mice were sacrificed, and blood was collected. IL-1 β , IL-6, and IL-18 levels in sera were measured by ELISA.

Statistical analysis

The data shown in each figure represent the mean of three or more independent experiments. Error bars indicate SD or SEM, as indicated. Data were analyzed using Prism 5 software (GraphPad), and statistical significance was determined by an unpaired two-tailed Student *t* test, or multiple test corrections when testing more than two conditions.

Results and Discussion

Complement induces IL-1 β maturation and caspase-1 processing

We first asked whether complement could induce the release of mature IL-1 β from phagocytes. Murine bone marrow-derived DCs in serum-free media were primed with LPS to induce pro-IL-1 β production. Cells were then washed and treated with NRC at different concentrations (Fig. 1A). Complement or LPS alone induced barely detectable amounts of IL-1 β secretion; however, when combined, IL-1 β was produced in a dose- and time-dependent manner (Fig. 1A, Supplemental Fig. 1). IL-1 α was also released by LPS and complement-treated DCs. The levels of IL-1 β and IL-1 α released upon NRC treatment were comparable to those from DCs treated with MSU crystals, a classical NLRP3 inflammasome activator (Fig. 1A, Supplemental Fig. 1). Heat-inactivated NRC (HI-NRC) was included as a control; it induced barely detectable IL-1 β and IL-1 α production, indicating that complement activation was the genuine trigger for IL-1 release. IL-6 production by LPS-primed DCs in response to NRC or HI-NRC was similar, confirming that

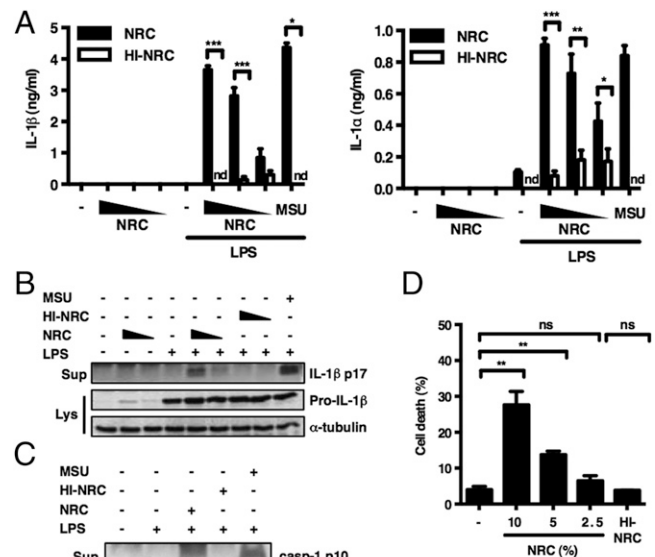


FIGURE 1. LPS-primed DCs treated with NRC rapidly release IL-1 β and process caspase-1. (A) ELISA of IL-1 β and IL-1 α in cell-free supernatants from LPS-primed DCs treated with different concentrations (1.25, 2.5, 5%) of NRC or HI-NRC. Cytokines released by LPS-primed DCs treated with MSU (250 μ g/ml) were included. Data represent mean $\pm$ SD for at least three independent experiments. (B) Immunoblot detection of mature IL-1 β p17 in cell-free supernatant from LPS-primed DCs stimulated with NRC or HI-NRC (5 and 2.5%) or MSU. Levels of pro-IL-1 β were also assessed in total cell lysates; α -tubulin was used as loading control. (C) Caspase-1 activation was detected by immunoblot in DCs activated with NRC (2.5%), HI-NRC, or MSU. (D) Lactate dehydrogenase analysis was performed to determine cell viability after DCs were stimulated with different concentrations of NRC (2.5, 5, 10%) or HI-NRC (10%). Data shown are representative of three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. ns, Not significant.

NRC does not influence DCs' ability to respond to LPS stimulation (data not shown).

IL-1 β secretion was further confirmed by immunoblot of cell supernatants, which detected active IL-1 β p17 only in samples from LPS-primed DCs stimulated with NRC or MSU but not with HI-NRC (Fig. 1B). Complement-induced IL-1 β maturation was associated with activation of caspase-1 cleavage, measured by immunoblot of caspase-1 p10 in cell-free supernatants (Fig. 1C).

When we measured cell death, using lactate dehydrogenase release by complement-treated DCs, we noticed that it was proportional to complement dose, as expected (Fig. 1D). However, at a complement concentration of 2.5%, we did not observe a significant increase in cell death over background (Fig. 1D), yet IL-1 β release was consistent at this concentration (Fig. 1A), indicating that IL-1 β secretion was not merely triggered by cell necrosis (14). Therefore, we selected the sublethal complement concentration of 2.5% to investigate the molecular mechanism underlying complement-mediated caspase-1 activation in subsequent experiments.

The NLRP3 inflammasome is required for complement-induced caspase-1 activation and IL-1 β release

To determine whether the NLRP3 inflammasome is crucial for IL-1 β production in response to complement, we stimulated

LPS-primed DCs from wild-type (WT), *Nlrp3*^{-/-}, and *Asc*^{-/-} mice with a sublethal complement dose, as above. WT DCs secreted readily detectable amounts of mature IL-1 β and IL-18 (Fig. 2A), whereas DCs deficient for *Nlrp3* or *Asc* did not, confirming the role of the NLRP3 inflammasome. *Nlrp3*^{-/-} and *Asc*^{-/-} DCs produced IL-6 amounts similar to WT DCs, indicating that their functionality was not affected by treatment (Fig. 2A). In *Nlrp3*^{-/-} and *Asc*^{-/-} cells, cleaved IL-1 β p17 was completely absent after complement exposure (Fig. 2B). Similarly, the caspase-1 inhibitor Z-YVAD abrogated maturation and release of IL-1 β in DCs treated with complement (Fig. 2C, 2D). Complement-mediated activation of NLRP3 inflammasome was dependent on K⁺ and Ca²⁺ fluxes (Supplemental Fig. 2). Indeed, blockade of K⁺ efflux or Ca²⁺ influx suppressed the release of IL-1 β by DCs after complement stimulation (Supplemental Fig. 2).

We further evaluated the role of the NLRP3 inflammasome in complement-mediated IL-1 β and IL-18 production in vivo. We evoked complement activation in mice by i.p. administration of LPS (15). Serum IL-1 β and IL-18 levels 3 h after LPS treatment were significantly higher than were those in untreated mice. Notably, serum IL-1 β and IL-18 were markedly decreased in *Nlrp3*^{-/-} and *Asc*^{-/-} mice (Fig. 2E). In addition, we caused enhanced complement activation in vivo by administration of LPS with C566, which causes

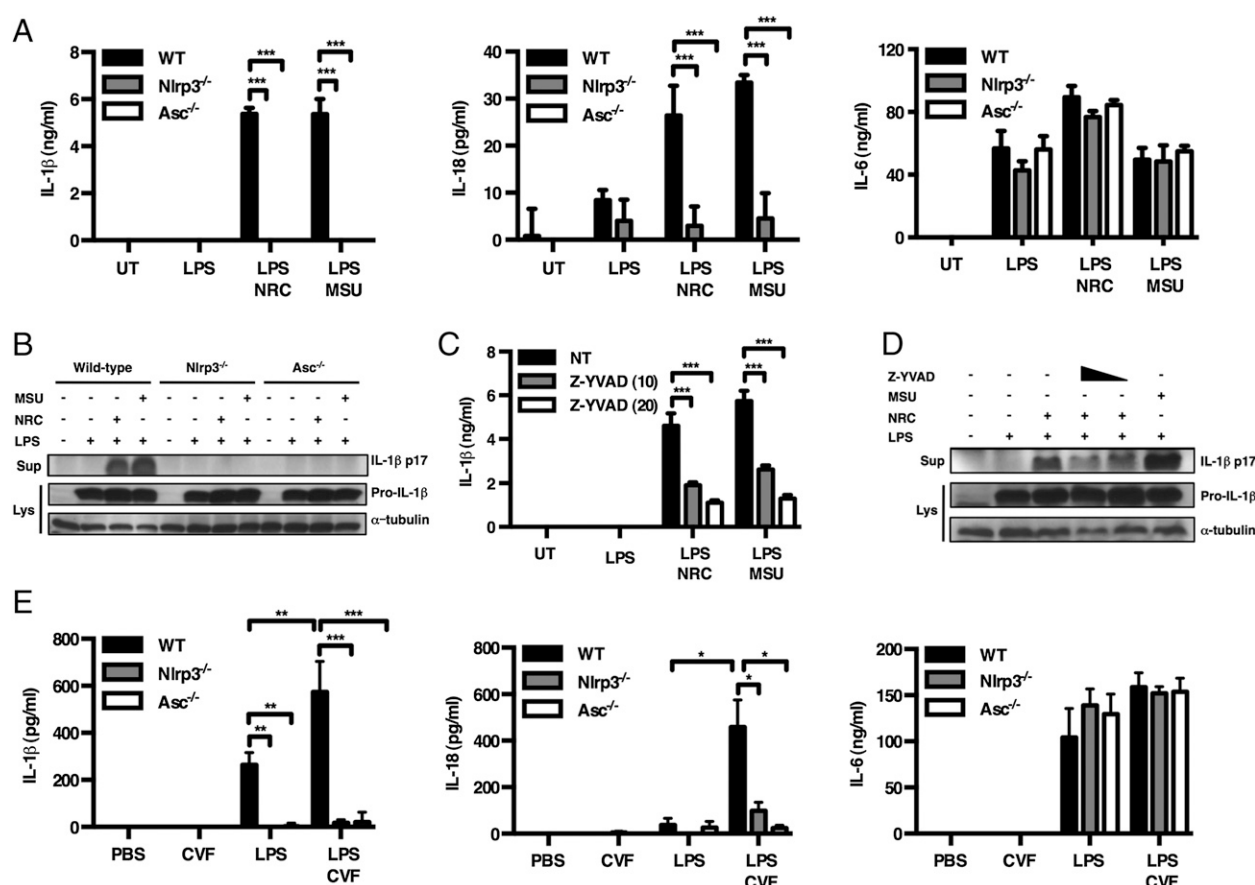


FIGURE 2. IL-1 β release induced by complement is NLRP3 dependent. (A) IL-1 β , IL-18, and IL-6 levels in supernatants of LPS-primed WT, *Nlrp3*^{-/-}, and *Asc*^{-/-} DCs treated with NRC (2.5%) or MSU (250 μ g/ml) for 4 h. Data represent mean $\pm$ SD for at least three independent experiments. (B) Mature IL-1 β p17 and pro-IL-1 β were detected in precipitated supernatants and cell lysates, respectively, from WT, *Nlrp3*^{-/-}, and *Asc*^{-/-} DCs by immunoblot. Data shown are representative of three independent experiments. (C and D) IL-1 β released by WT LPS-primed DCs pretreated with the caspase-1 inhibitor Z-YVAD (10 and 20 μ M) for 1 h and then stimulated with NRC or MSU for 4 h. ELISA (C) and immunoblot (D) were performed to detect mature IL-1 β p17, intracellular pro-IL-1 β , and α -tubulin. (E) Serum cytokine levels of WT, *Nlrp3*^{-/-}, and *Asc*^{-/-} mice receiving i.p. injections of LPS alone or with C566 (60 μ g/mouse). * p < 0.05, ** p < 0.01, *** p < 0.001.

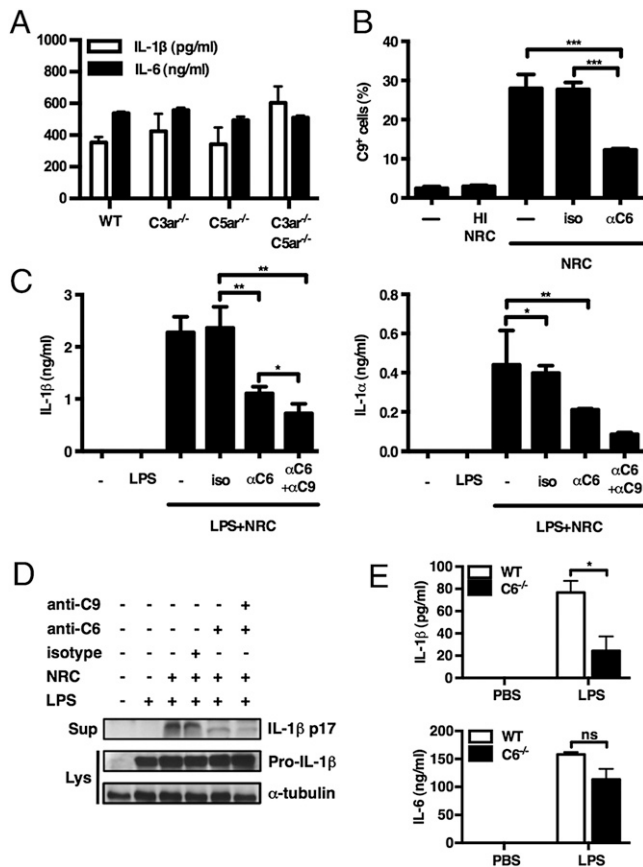


FIGURE 3. Sublethal MAC deposition activates NLRP3 inflammasome and IL-1 β maturation. **(A)** IL-1 β and IL-6 levels in serum from WT mice and mice deficient in *C3ar*, *C5ar*, or *C3ar*, and *C5ar*, receiving LPS injections i.p. Data represent mean $\pm$ SEM of five to six mice/group. **(B)** MAC deposition measured by C9⁺ cells was assessed by flow cytometry of DCs treated with HI-NRC or NRC after preincubation with anti-C6 Ab or isotype control for 4 h. Data represent mean percentage $\pm$ SEM for at least four independent experiments. **(C)** IL-1 β and IL-1 α release was assessed in supernatants from LPS-primed DCs treated with NRC (1.25%) in the presence of anti-C6 Ab (1.25 μ g/ml), alone or with an anti-C9 Ab (7.5 ng/ml). Treatment with NRC together with isotype controls is also shown. **(D)** Mature IL-1 β p17 released by LPS-primed DCs after NRC treatment was detected in precipitated supernatants by immunoblot. Intracellular pro-IL-1 β and α -tubulin were also analyzed in cell lysates. **(E)** Serum IL-1 β and IL-6 levels from WT and C6^{-/-} mice injected i.p. with LPS (20 mg/kg). * p < 0.05, ** p < 0.01, *** p < 0.001. ns, Not significant.

systemic hyperactivation of complement. LPS/CVF coadministration boosted serum IL-1 β and IL-18 levels in WT mice but not in mice deficient for *Nlrp3* or *Asc* (Fig. 2E). These data corroborate the results obtained using LPS alone. Serum IL-6 levels in response to LPS/CVF remained unaltered. Collectively, these data indicate that IL-1 β and IL-18 secretion induced by complement involves the NLRP3/ASC/caspase-1 pathway in vitro and in vivo.

Sublethal MAC formation regulates NLRP3 inflammasome activation

We went on to identify the mechanisms involved in NLRP3 inflammasome activation by complement in vivo, beginning with the possible role of C3a and C5a anaphylatoxins. Serum IL-1 β and IL-6 levels were similar in WT, *C3ar*^{-/-}, *C5ar*^{-/-}, and *C3ar*^{-/-}*C5ar*^{-/-} mice after complement activation induced by LPS, indicating that anaphylatoxins play no major role in NLRP3 inflammasome induction by complement

(Fig. 3A). However, the NLRP3 inflammasome can also be activated through pore-forming toxins, leading us to wonder whether the complement system's pore-forming complex (MAC) might be involved (10). MAC formation was suppressed by incubating NRC with an anti-C6-neutralizing Ab before incubation with DCs. This significantly reduced both the percentage and the mean fluorescence intensity of cells positive for C9 (Fig. 3B, data not shown). IL-1 β and IL-1 α , but not IL-6, release was significantly inhibited by treatment with anti-C6 Ab, alone or in combination with an anti-C9 Ab (Fig. 3C, data not shown). Blocking MAC formation also markedly suppressed IL-1 β p17 maturation, as visualized by immunoblot (Fig. 3D).

We evaluated the role of MAC in vivo using C6^{-/-} mice in which MAC deposition is impaired. The complement cascade was triggered in WT and C6^{-/-} mice by i.p. LPS injections. In mice lacking C6, serum IL-1 β was significantly reduced compared with WT littermates, whereas IL-6 levels remained unchanged (Fig. 3E). Collectively, these data indicate that the MAC is indispensable for NLRP3 inflammasome-mediated IL-1 β maturation.

In this study, we defined a new mechanism triggered by complement to promote inflammation. We provide clear evidence that sublethal MAC formation activates the NLRP3 inflammasome to enable caspase-1 processing and maturation of IL-1 β and IL-18.

MAC has been implicated in the pathology of chronic inflammatory disorders and diseases, including atherosclerosis, reperfusion injury, and glomerular damage (16, 17). Moreover, deficiency in decay accelerating factor, a negative regulator of both the classical and alternative pathways of complement activation, increases complement activation, which results in higher LPS-induced IL-1 β production in vivo (5).

Inhibition of MAC formation may provide a therapeutic approach for the treatment of chronic inflammatory diseases, not only by preventing cell lysis and tissue damage, but also by reducing IL-1 β release and inflammation. Prevention of C5 activation through administration of an anti-mouse C5 Ab abrogated inflammation in complement factor (Cf) H^{-/-} mice that were developing spontaneous membranoproliferative glomerulonephritis (18). Ongoing phase II clinical trials of Soliris (eculizumab), a fully humanized anti-C5 Ab for the treatment of atypical hemolytic uremic syndrome, which is associated with mutations in *CfH*, have achieved remarkable results (19). Moreover, eculizumab can also limit glomerular inflammation in a subset of patients with the most inflammatory forms of C3 glomerulonephritis, which is accompanied by circulating MAC (20). It would be interesting to measure serum IL-1 β levels in atypical hemolytic uremic syndrome/C3 glomerulonephritis patients to understand whether neutralization of C5, which blocks both C5a and C5b, critical components for MAC deposition, could also normalize serum IL-1 β levels. Another productive approach might be to combine eculizumab with IL-1 β blockers, such as anakinra and the anti-human IL-1 β Ab (canakinumab, Ilaris), or to target C6 or C9 directly. IL-1 β neutralization by anakinra successfully decreased C3 deposition, MAC formation, and IL-1 in the brains of two patients with acute hemorrhagic leukoencephalitis, a rare demyelinating disorder associated with partial *CfI* deficiency. Based on the known clinical facts and the findings shown in this study, IL-1 β

modulators should be investigated as an adjunct therapy for the management of diseases caused by complement dysregulation.

Acknowledgments

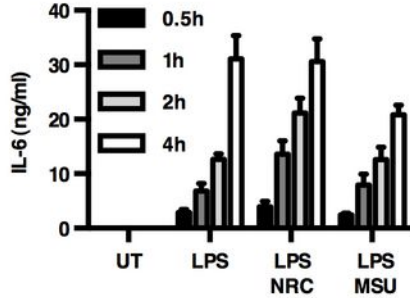
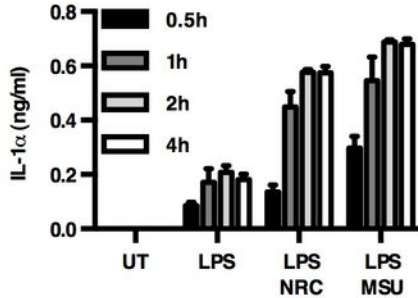
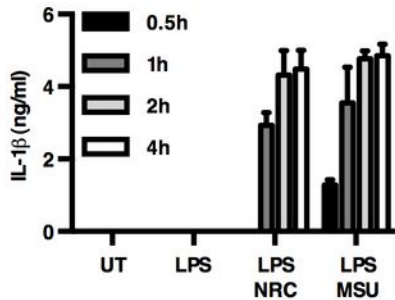
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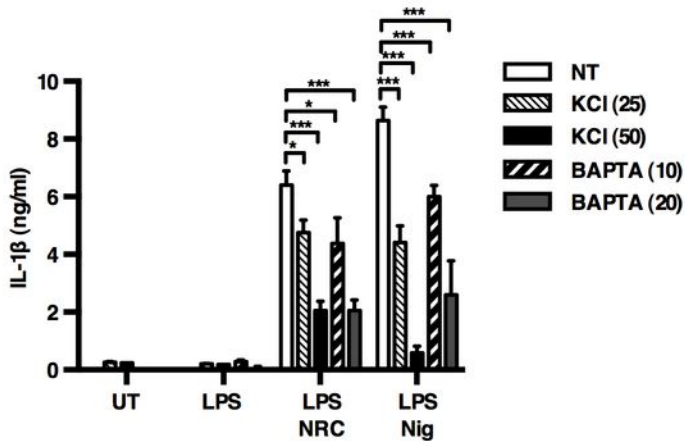
Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figure legends

SUPPL. FIGURE 1. Time-course analysis of IL-1 β , IL-1 α and IL-6 release by primed-DCs in response to NRC (2.5%) or MSU. Data represent mean $\pm$ SD from at least 3 independent experiments.

SUPPL. FIGURE 2. NLRP3 inflammasome activation by complement depends on K⁺ efflux and Ca²⁺ influx. IL-1 β released by wild-type primed DCs pre-treated with the intracellular Ca²⁺ chelator BAPTA (10 and 20 μ M) for 1h or cultured in high [K⁺] medium (KCl, 25 and 50 mM), then stimulated with NRC (2.5%) or MSU (250 μ g/ml) for 4h. Data represent mean $\pm$ SD from 3 independent experiments.