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RESEARCH ARTICLE

Complement-mediated 'bystander' damage initiates host NLRP3 inflammasome activation

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ABSTRACT

Complement activation has long been associated with inflammation, primarily due to the elaboration of the complement anaphylotoxins C5a and C3a. In this work, we demonstrate that the phagocytosis of complement-opsonized particles promotes host inflammatory responses by a new mechanism that depends on the terminal complement components (C5b–C9). We demonstrate that during the phagocytosis of complement-opsonized particles, the membrane attack complex (MAC) of complement can be transferred from the activating particle to the macrophage plasma membrane by a 'bystander' mechanism. This MAC-mediated bystander damage initiates NLRP3 inflammasome activation, resulting in caspase-1 activation and IL-1β and IL-18 secretion. Inflammasome activation is not induced when macrophages phagocytize unopsonized particles or particles opsonized with serum deficient in one of the terminal complement components. The secretion of IL-1β and IL-18 by macrophages depends on NLRP3, ASC (also known as PYCARD) and caspase-1, as macrophages deficient in any one of these components fail to secrete these cytokines following phagocytosis. The phagocytosis of complement-opsonized particles increases leukocyte recruitment and promotes T helper 17 cell (T_H17) biasing. These findings reveal a new mechanism by which complement promotes inflammation and regulates innate and adaptive immunity.

KEY WORDS: Complement, Membrane attack complex, NLRP3, Inflammasome, Phagocytosis, IL-1β, IL-18, ASC, Caspase, Bystander activation, T_H17

INTRODUCTION

Phagocytosis is a process whereby cells engulf particulate materials into phagosomes for subsequent degradation. Macrophages, neutrophils and dendritic cells are considered to be 'professional phagocytes' because not only do they express receptors that bind directly to particles but they also have receptors that bind to opsonizing factors, such as IgG and complement. The host response to these particles can differ depending on the nature of the particle or the receptors that these particles engage (Mosser, 1994). In this work, we show that complement-mediated phagocytosis initiates a unique and unexpected inflammatory response from phagocytic cells, through a mechanism that depends, not on ability of the complement pathway to opsonize particles for enhanced uptake, but rather on the terminal complement membrane attack complex (MAC).

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The complement system refers to a group of inactive proteins in the plasma that can be sequentially activated by a number of diverse triggers. Complement activation contributes to host defense through the opsonization of particles and the lysis of target cells through the assembly of the MAC, which is composed of complement proteins C5b–C9 (Kondos et al., 2010; Morgan, 2015). Complement activation also leads to inflammation, and people with genetic deficiencies in complement regulatory proteins frequently suffer from inflammatory immunopathology (Zipfel and Skerka, 2009). Although complement-mediated inflammation has been largely attributed to the generation of the complement anaphylotoxins C5a and C3a, it is currently unclear whether other components of the complement system might also play a role in inflammation. In this study, we report that the complement MAC can induce the release of interleukin (IL)-1β and IL-18 from macrophages to promote inflammatory immune responses.

IL-1β is a cytokine that promotes fever, stimulates the production of acute phase proteins and induces cell adhesion molecules at the site of inflammation to promote cellular infiltration (Dinarello, 2009). Because IL-1β controls many aspects of innate immunity, its production and biological activity is tightly controlled. The IL-1β protein lacks a consensus secretory signal peptide and therefore is produced in a precursor form (pro-IL-1β) that must be proteolytically cleaved in order to be secreted. In most cells, the secretion of IL-1β depends on the activation of a multi protein complex termed the inflammasome (Sutterwala et al., 2014). The inflammasome is composed of an NLR protein (nucleotide-binding domain and leucine-rich repeat containing protein), the adaptor molecule ASC (Apoptosis-associated Speck-like protein containing a CARD; also known as PYCARD) and the zymogen pro-caspase-1. Assembly of the inflammasome induces activation of caspase-1. Caspase-1 then induces the maturation and secretion of biologically active IL-1β. NLRP3, the most well-characterized NLR, is known to be activated by a variety of 'danger' signals that induce cellular injury, including lysosomal destabilizing agents like alum or monosodium urate crystals (Martinon et al., 2006), cholesterol crystals (Samstad et al., 2014) or membrane-permeabilizing agents like bacterial pore-forming toxins (Muñoz-Planillo et al., 2013). Pattern recognition receptors, such as Toll-like receptors (TLRs), can provide the priming signals necessary for inflammasome activation (Fang et al., 2009; Zhang et al., 2007).

Complement components C3a, C5a and their cognate receptors can trigger IL-1β secretion in distinct ways. C3a binding to C3aR, triggers ERK1/2 kinase activation leading to ATP efflux. The high extracellular ATP levels can lead to the activation of P2X7, which in turn triggers NLRP3 activation and IL-1β secretion (Asgari et al., 2013). Soluble C5a can partner with TNF-α and act as a priming signal for inflammasome activation (Samstad et al., 2014), or the crystalline form can deliver the primary signal directly to pro-IL-1β through a caspase-1-dependent mechanism (An et al., 2014). Similar to bacterial pore-forming toxins, the complement MAC can

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damage membranes. Therefore, autoimmune reactions in which auto-antibodies direct complement to host cells can result in inflammasome activation (Lien et al., 2015; Mulla et al., 2013; Savage, 2011).

In the present work, we reveal an unexpected intersection between the complement system and IL-1β production. We demonstrate that during the process of complement-mediated phagocytosis, the complement MAC can 'jump' from the surface of complement-opsonized particles to the plasma membrane of macrophages. This bystander deposition results in the activation of the NLRP3 inflammasome, the activation of caspase-1, and the release of IL-1β and IL-18. This process can influence innate immunity by recruiting leukocytes to the site of phagocytosis and it can influence adaptive immune responses by promoting T_H17 development.

RESULTS

Complement-mediated phagocytosis by macrophages induces the release of IL-1β and IL-18

To study the effect of phagocytosis on inflammasome activation by macrophages, a variety of complement-activating particles were added to lipopolysaccharide (LPS)-primed murine macrophages, and the release of IL-1β was measured in supernatants following phagocytosis. Zymosan is a potent activator of the alternative complement pathway (Fig. S1A). Increasing doses of zymosan were added to macrophages in the presence or absence of normal human serum as a source of complement proteins. Zymosan bound avidly to macrophages and was phagocytosed in the presence or absence of serum (Fig. 1A), but only serum-opsonized zymosan induced robust release of IL-1β from macrophages (Fig. 1B). As a control for these studies, latex beads, which do not activate complement (Fig. S1A), were added to macrophages. These beads bound avidly to macrophages and were phagocytosed in the presence or absence of serum (Fig. 1C), and neither condition resulted in substantial IL-1β release by macrophages (Fig. 1D). An additional control was performed with IgG-opsonized sheep erythrocytes, which bound avidly to macrophages but failed to induce the release of IL-1β (Fig. S1B). The release of IL-18 from macrophages was also examined, and there was a similar dose-dependent release of IL-18 from macrophages following their interaction with zymosan (Fig. 1E) or other complement activators (data not shown) in the presence but not the absence of complement.

Two other complement activators were similarly analyzed. We have previously reported that the protozoan parasite *Leishmania major* is an efficient activator of the alternative complement pathway (Brittingham and Mosser, 1996; Mosser and Edelson, 1987). The addition of *L. major* to macrophages caused a dose-dependent increase in IL-1β release from macrophage in the presence but not the absence of complement (Fig. 1F). Particulate inulin also induced a dose-dependent release of IL-1β in the presence of serum, but not in its absence (Fig. 1G). Thus complement-mediated phagocytosis by macrophages results in the release of IL-1β. The IL-1β that was released from macrophages was in the mature (cleaved) form, because little pro-IL-1β was detected in the supernatants of these cells following the phagocytosis of complement-opsonized inulin (Fig. 1H).

Complement-mediated phagocytosis leads to the activation of the NLRP3 inflammasome

To investigate the role of inflammasome activation following phagocytosis, bone-marrow-derived macrophages (BMMϕ) from mice deficient in NLRP3, NLRC4, ASC or caspase-1 were examined and compared to macrophages from wild-type (WT) mice. LPS-primed macrophages from WT or NLRC4$^{-/-}$ mice secreted IL-1β in response to complement activation by zymosan (Fig. 2A), inulin (Fig. 2B) or *L. major* (Fig. 2C). However, macrophages from mice deficient in NLRP3, ASC or caspase-1 failed to secrete detectable IL-1β. Caspase-1 activation was directly measured by FLICA staining of macrophages. Macrophages responding to complement activation by zymosan (Fig. 2D; Fig. S2A) inulin (Fig. 2D; Fig. S2B) or *L. major* (Fig. 2D) exhibited caspase-1 cleavage. Incubation of these particles in serum deficient in the sixth component of complement failed to induce caspase-1 activation (Fig. 2D). Thus, the phagocytosis of complement activators results in NLRP3-dependent inflammasome activation.

Visualization of inflammasome activation following complement activation

To visualize inflammasome formation, a mouse macrophage-like cell line RAW264.7, which does not express endogenous ASC, was transduced with CFP–ASC and exposed to complement activators. These macrophages were primed with LPS for 4 h and then exposed to fluorescent Zymosan particles in the presence of normal serum. ASC specks (Fig. 3, arrowheads) were observed in macrophages that actively took up zymosan (Fig. 3A,B). The ASC specks were not observed in the cells that received zymosan without normal serum (Fig. 3C). The specks observed were not as plentiful as those in macrophages exposed to the well-known NLRP3 agonist ATP (Fig. 3D). The difference in the appearances of the ASC specks can be interpreted as being the early stages of interaction between NLRP3 and the PYD domain of ASC, when filamentous, and a higher organizational level when compact (Fig. 3). Such differences in ASC speck assembly has been addressed by previous structural studies of ASC mutants (Sahillioglu et al., 2014).

Bystander deposition of the MAC complex causes IL-1β release

The induction of IL-1β by inulin occurred when inulin particles were added to macrophages in the presence of normal serum, but not in serum treated with Compstatin, a cyclic tridecapeptide that is a potent C3 inhibitor (Fig. S1C). To identify the complement components necessary for macrophage inflammasome activation, serum deficient in specific complement components was examined. Macrophages failed to secrete IL-1β in response to complement activation by inulin when any individual complement protein (C3, C5, C8 and C9) was depleted from serum (Fig. 4A). This included serum deficient in the terminal components C8 and C9, indicating a role for the MAC in IL-1β release. To directly show that the MAC complex was required for inflammasome activation, the terminal complement components C5b, C6, C7 C8 and C9 were directly added sequentially to LPS-primed macrophages to assemble the MAC on the macrophage plasma membrane. IL-1β was only detected in cells exposed to the complete C5b–C9 MAC (Fig. 4B).

The MAC can be formed on the surface of host cells adjacent to the target particle through a process termed 'bystander activation' (Lint et al., 1976). The phenomenon of 'bystander activation' was described more than 30 years ago, but the biological significance of bystander activation has remained unclear. To demonstrate bystander activation in this work, the original description of 'reactive lysis' (Thompson and Lachmann, 1970), was adapted to detect macrophage inflammasome activation. In the original reactive lysis experiments, the complement C5 convertase was formed on the surface of a complement-activating particle, such as inulin, by incubating inulin with complete serum in 5 mM Mg^{2+} and

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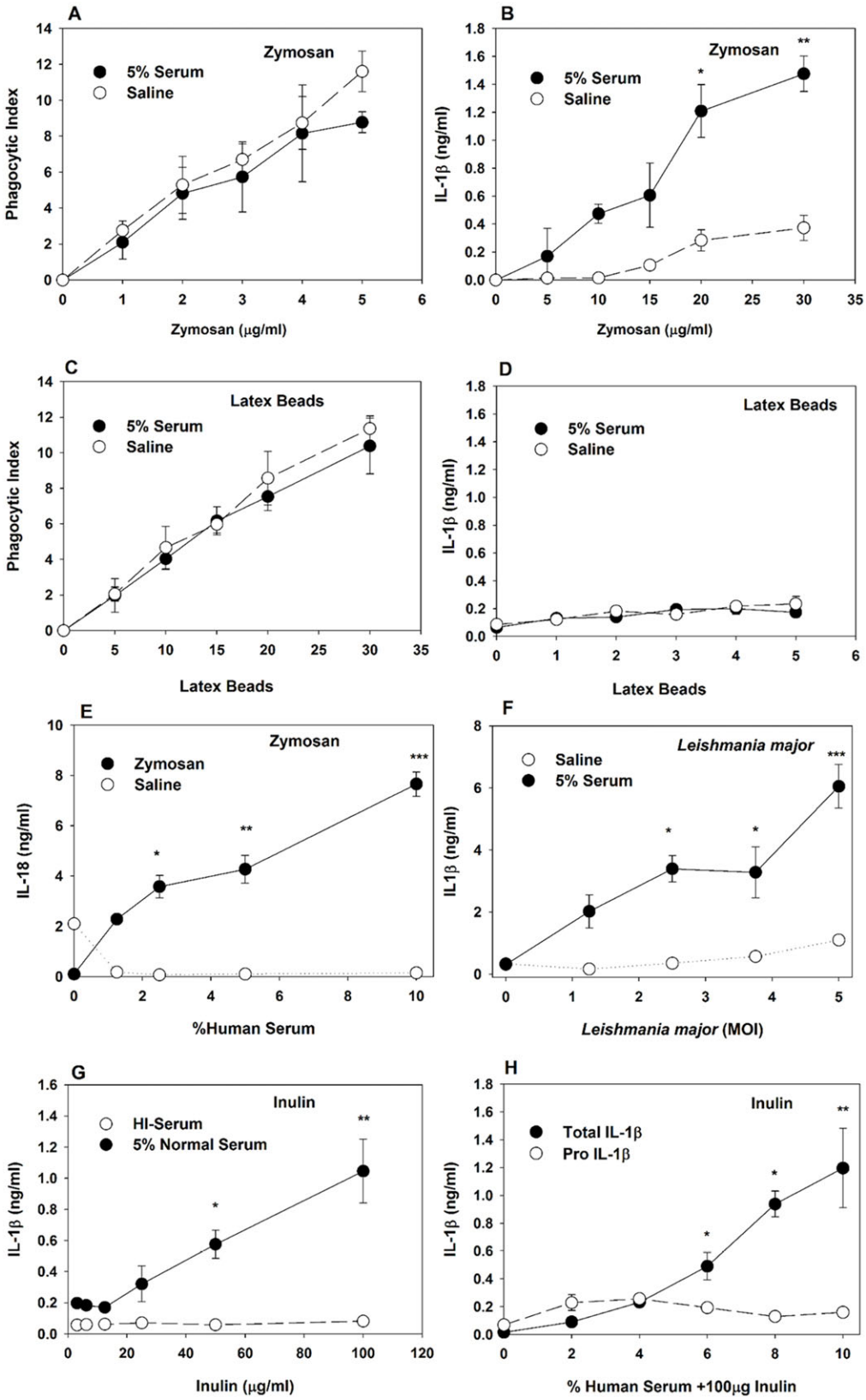

Fig. 1. IL-1β production following complement-mediated phagocytosis. Mouse bone-marrow-derived macrophages (BMMϕ) were pre-treated with LPS for 4–6 h followed by challenges with zymosan (A,B,E), latex beads (C,D), inulin (G,H) or *Leishmania major* (F) for 4 h. (A) Phagocytosis of zymosan by BMMϕ was measured in the presence (black circles) or absence (white circles) of 5% normal human serum. The number of particles per 100 macrophages was counted under a 400× light microscope and the phagocytic index represents the average number of particles/macrophage. (B) IL-1β secretion by LPS primed macrophages was measured 4 h following incubation with increasing amounts of zymosan in the presence (black circles) or absence (white circles) of normal human serum. (C) The phagocytosis of latex beads by mouse bone-marrow-derived macrophages was measured in the presence (black circles) or absence (white circles) of 5% normal human serum. The average number of particles per 100 macrophages was counted and expressed as phagocytic index. (D) IL-1β secretion by LPS primed macrophages was measured 4 h following incubation with increasing amounts of latex beads in the presence (black circles) or absence (white circles) of 5% normal human serum. (E) IL-18 secretion by LPS primed macrophages was measured 4 h following incubation with zymosan (10 μg/ml) (black circles) or vehicle control (white circles) and increasing concentrations of normal human serum. (F) IL-1β secretion by LPS-primed macrophages was measured 4 h following incubation with increasing multiplicities of infection (MOI) of *L. major* in the presence (black circles) or absence (white circles) of 5% normal human serum. (G) IL-1β secretion by LPS-primed macrophages was measured 4 h following incubation with increasing amounts of inulin in the presence (black circles) of 5% normal human serum or 5% heat-inactivated serum (white circles). (H) Total IL-1β (black circles) and pro-IL-1β (white circles) were measured in supernatants by ELISA 4 h after incubating macrophages with 100 μg/ml inulin and increasing concentrations of normal human serum. Data is presented as mean±s.e.m. All experiments were performed a minimum of three times. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ (Student's *t*-test). Where not shown, error bars are smaller than symbols.

EGTA at 17°C. The C5–inulin was then washed and added to unsensitized red blood cells (RBCs) in the presence of serum and 3 mM EDTA, conditions which block complement activation but allow the non-enzymatic formation of the MAC complex. The lysis of 'bystander' RBCs under these conditions (Fig. 4C) indicates that the MAC had been transferred onto RBCs after being activated by the convertase previously formed on inulin. This bystander activation was originally described in 1970 (Thompson and

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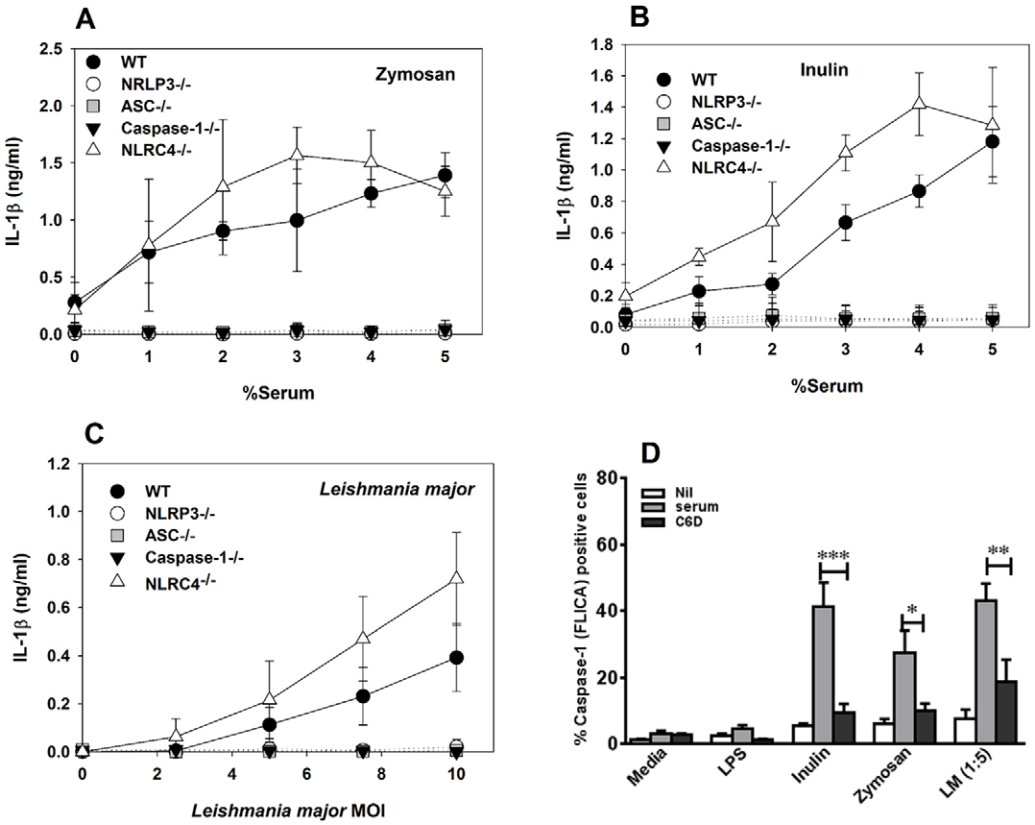

Fig. 2. NLRP3 inflammasome activation during complement-mediated phagocytosis. IL-1β production from LPS-primed bone-marrow-derived macrophages (BMMϕs) from wild-type (WT) mice (black circles) or mice deficient in NLRP3 (white circles), ASC (gray squares), caspase-1 (black inverted triangle) or NLRC4 (white triangle) was measured by ELISA following their incubation with zymosan (A), inulin (B) or *L. major* (C) in the presence of 5% normal human serum (n=4; each panel). (D) The percentage of caspase-1-positive cells was assessed by flow cytometry using a FAM-FLICA dye based assay (n=4). Cells were primed with LPS for 6 h followed by antigen stimulation (LM, *L. major*) in the presence of 5% complete serum or serum deficient in C6 (C6D) human serum. Nil, no serum. Data represent mean±s.e.m. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

Lachmann, 1970). A similar approach was taken, but adding macrophages as the bystander acceptor cell. Rather than measuring RBC lysis, IL-1 secretion from macrophages was measured (Fig. 4D). A serum dose-dependent increase in IL-1β secretion by macrophages was observed under these conditions (Fig. 4D) indicating that complement activation can induce IL-1β release from 'bystander' macrophages.

To demonstrate that this phenomenon occurs across mammalian species, homologous human and mouse systems were compared. Human macrophages release IL-1β in a human serum dose-dependent manner (Fig. 4E), and murine macrophages release IL-1β in a murine serum dose-dependent manner in response to activation by inulin (Fig. 4F). Mouse serum has previously been shown to be particularly labile, exhibiting reduced serum lytic activity *in vitro* (Rosenberg and Tachibana, 1962), therefore it is not surprising that higher concentrations of mouse serum were required to elicit detectable inflammasome activation in this assay.

MAC deposition on the macrophage plasma membrane

To visualize MAC formation on the macrophage plasma membrane during the uptake of complement-activating particles, fluorescence microscopy was used to detect AF-649-tagged C9 deposition. As expected, C9 was easily visualized on the surface of complement-opsonized particles (Fig. 5), but MAC formation was also detected on the surface of bystander macrophages when Ds-Red-expressing *L. major* (Goncalves et al., 2011) (Fig. 5A1,B) or zymosan (Fig. 5A2) was added to macrophages. The bystander MAC deposition occurred primarily on the surfaces of macrophages actively involved in the phagocytosis of complement-opsonized particles (Fig. 5). It also could be visualized on the surface of cells directly adjacent to these cells (Fig. 5A1). Not all of the macrophages undergoing complement-mediated phagocytosis were decorated with C9 (Fig. 5A2). As a control, macrophages phagocytosing in the presence of EDTA, to prevent complement activation, had little to no C9 deposition on their surface (Fig. 5B).

Bystander MAC deposition induces ionic imbalances in cells

MAC pore formation on nucleated cells is known to cause K^+, Ca^{2+} and ATP transport across membranes. Each of these molecules has been shown to influence the activation of the NLRP3 inflammasome. To determine the importance of secreted ATP in complement-mediated secretion of IL-1β, we used macrophages from P2X7R$^{-/-}$ mice that lacked the ability to activate the inflammasome in response to high concentrations of extracellular ATP (Fig. 6A). P2X7R$^{-/-}$ macrophages retained their ability to secrete IL-1β in response to complement activation by particulate inulin (Fig. 6B); indicating that MAC-mediated pore formation does not require the P2X7R for NLRP3 inflammasome activation. The presence of the MAC pore on the cell surface of host cells can also induce reactive oxygen species (ROS) due to mitochondrial stress (Cole and Morgan, 2003). As ROS

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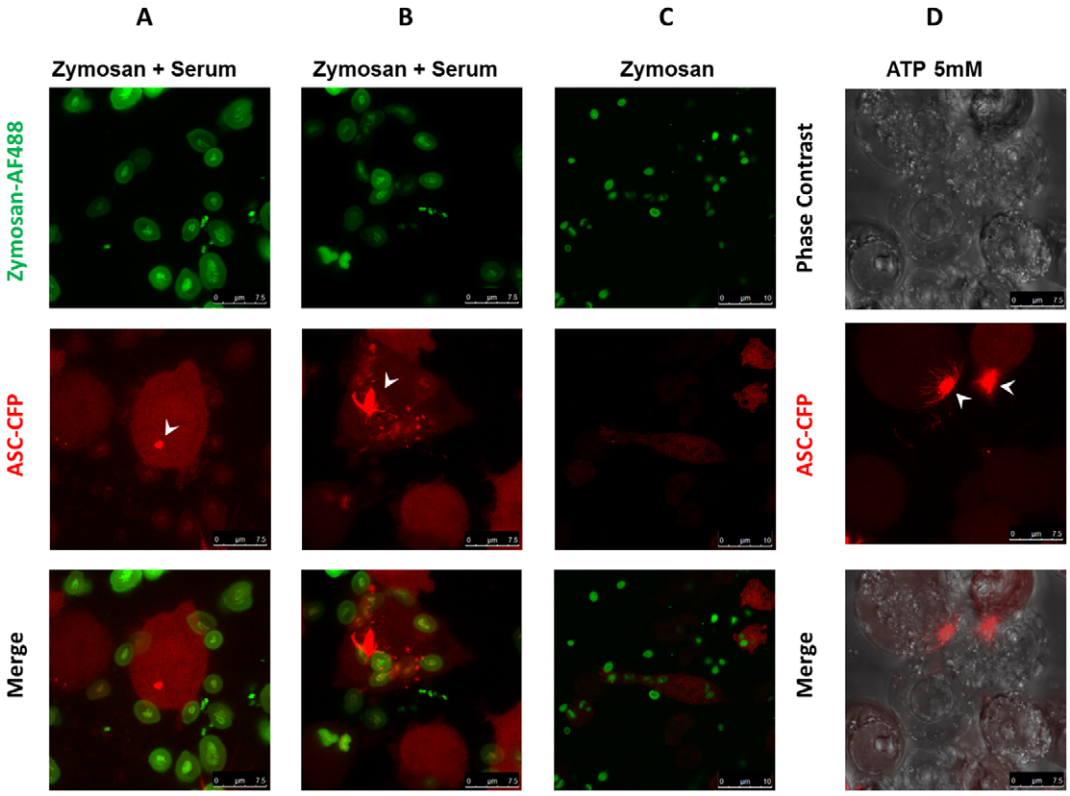

Fig. 3. Visualization of inflammasome activation following complement activators. ASC–CFP transduced macrophages (red) were LPS primed for 4 h. They were then activated by Alexa-Fluor-488-conjugated zymosan (green) and serum (A,B) or zymosan alone for 45 min (C) or by ATP (D) for 20 min. Cells were visualized using Leica SP5X Confocal Microscope. Arrowheads indicate ASC specks. The figures are a representative of images acquired from three independent transductions.

has been implicated in NLRP3 activation (Tschopp and Schroder, 2010), we looked into the effect of ROS inhibition on IL-1β secretion. An increased concentration of the ROS chelator N-acetyl cysteine (NAC) induced a dose-dependent reduction in IL-1β secretion by LPS-primed macrophages in response to inulin and complement (Fig. 6C). Pore formation usually induces K^+ efflux from cells, and this efflux is required for the activation of the inflammasome. To examine the role of K^+ efflux in MAC-pore-induced bystander inflammasome activation, K^+ efflux was blocked by adding the drug glybenclamide (Fig. 6D), or inhibited by increasing the extracellular concentrations of K^+ (Fig. 6E). Both conditions resulted in decreased IL-1β secretion in response to the phagocytosis of complement-opsonized inulin, indicating that K^+ efflux is required for MAC-mediated inflammasome activation.

MAC formation can cause osmotic lysis of cells, and macrophages can also die from pyroptosis following inflammasome activation (Bergsbaken and Cookson, 2007). However, nucleated cells have been shown to be relatively resistant to cell death mediated by the MAC due to membrane repair mechanisms previously described (Kim et al., 1987). To directly monitor macrophage viability following bystander complement activation, macrophages were stained with 7-amino actinomycin D (7-AAD) and annexin V to measure cell viability. The dye 7-AAD is a membrane impermeant DNA-intercalating dye that stains dead cells, and annexin V is a sensitive probe for detecting apoptosis. As shown in Fig. 6F, there was little evidence for cell death by apoptosis in response to complement activation by inulin or zymosan. Thus, macrophages undergoing complement-mediated phagocytosis remain viable even when they cleave caspase-1 to release biologically active IL-1β and IL-18.

Bystander complement activation can influence innate and adaptive immunity

To show that complement activation in mice can lead to increases in serum IL-1β levels, male C57BL/6 mice were injected with LPS. Cobra venom factor was added along with the LPS to systemically activate complement, as previously described (Zhang et al., 2007). The levels of IL-1β in the serum were measured 3 h later. Serum IL-1β levels were significantly increased in wild-type mice relative to mice genetically deficient in the fifth component of complement (C5) (Fig. 7A). Cellular influx into the peritoneum was also measured 6 h following bystander complement activation. In this case, inulin was injected intraperitoneally into mice to locally activate complement in the peritoneal cavity. Inulin administration to complement-sufficient mice resulted in an increase in the number of GR1-positive leukocytes in the peritoneum in response to inulin (Fig. 7B). This induction was blocked by depleting mice of complement (Fig. 7B) prior to the addition of inulin, as previously described (Pagano et al., 2009).

To investigate a role for complement activation in the modulation of T cell responses, we examined the ability of 'bystander-activated' macrophages to serve as antigen-presenting cells. LPS-primed macrophages were pulsed with ovalbumin (Ova) under conditions where the inflammasome was activated, either by the addition of purified MAC components (Fig. 7C) or by bystander inulin activation (Fig. 7D). Ova-specific $CD4^+$ DO.11.10 T cells were added to macrophages and incubated for 7 days. T cells were re-stimulated at day 7 using immobilized antibody to CD3. Cytokine production from T cells was measured 3 days later. Bystander complement activation on macrophages induced T cells to make significantly higher levels of IL-17A (Fig. 7C,D). To verify the

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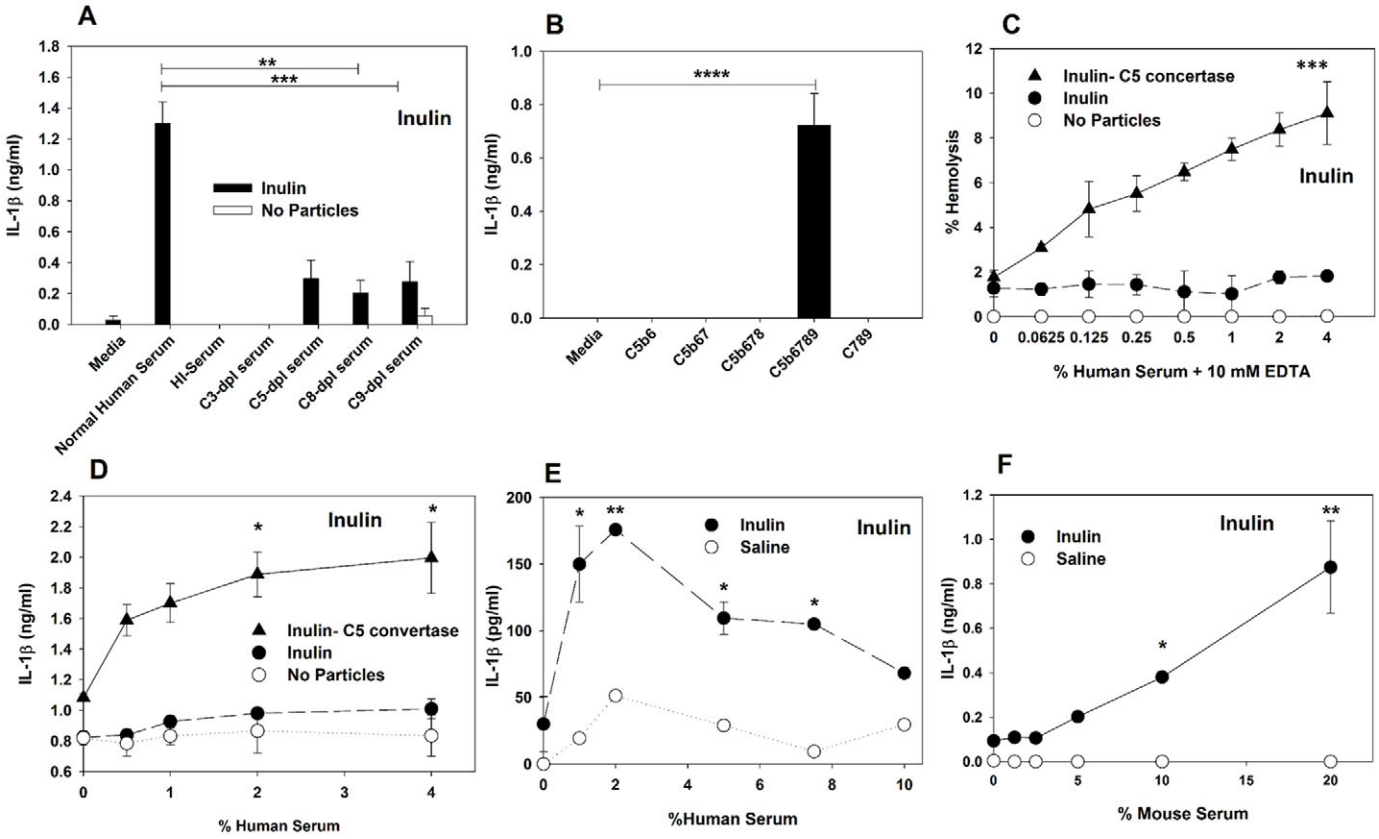

Fig. 4. Bystander activation following complement-mediated phagocytosis. (A) IL-1β secretion was measured in supernatants following the incubation of macrophages with inulin (100 µg/ml) plus normal serum or serum deficient in individual complement components (n=3). (B) IL-1β secretion was measured in supernatants of macrophages after the addition of purified terminal complement components to macrophages (n=3). (C) The 'reactive lysis' of bystander erythrocytes (see Materials and Methods) was measured by quantifying hemoglobin release from lysed RBCs after incubating C5b–inulin in increasing serum concentrations in 3 mM EDTA (n=4). (D) Inflammasome activation by 'bystander' complement activation was assessed by measuring the release of IL-1β following co-incubation of macrophages with C5b–inulin in 3 mM EDTA and increasing amounts of human serum (n=3). (E,F) IL-1β secretion from human (E) or mouse (F) macrophages after incubation of inulin particles with increasing concentrations of human (E) or mouse (F) serum. Bars represent mean±s.e.m. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 (Student's t-test). Where not shown, error bars are smaller than symbols.

importance of inflammasome activation in T cell polarization, LPS-primed wild-type or NLRP3$^{-/-}$ macrophages on a BALB/c background were cultured with inulin and human serum along with 150 µg/ml Ova and added to CD4^{+}OT-II T cells. In primary stimulations, CD4^{+}OT-II T cells co-cultured with wild-type macrophages showed an increase in IL-17A production that was not observed when NLRP3$^{-/-}$ BMMϕ cells were added (Fig. 7E). The re-stimulation of T cells showed a similar NLRP3-dependent increase in IL-17A production (Fig. 7F). These data indicate that IL-1β, produced as a result of bystander complement activation on macrophages can influence T cell polarization.

DISCUSSION

Complement has long been considered to be an integral component of host defense. The third component of complement in particular represents a key opsonin that promotes the uptake of complement activators into professional phagocytes. Complement activation has long been associated with inflammation (Merle et al., 2015; Morgan, 2015) and patients deficient in complement regulatory proteins frequently suffer from inflammatory immunopathology (Grammatikos and Tsokos, 2012; Lewis and Botto, 2006; Sullivan, 1998). The multiple complement activation pathways share a common terminal pathway consisting of C5b–C9, which forms MAC on cells. The MAC creates pores on membranes to cause the osmotic lysis of target particles. 'Self' cells, however, restrict MAC formation either by means of the regulator CD59 or by actively eliminating MAC through ecto- or endocytosis (Morgan, 2015), thus rendering MAC sub-lytic. Such sub-lytic MACs have been shown to trigger pro-inflammatory responses in various cell types (Lueck et al., 2011; Morgan, 1992; Yang et al., 2014). In this study, we demonstrate complement-mediated phagocytosis as the 'kick-start' event leading to the deposition of sub-lethal MAC on macrophages activating host inflammasomes, and thereby affecting innate and adaptive immunity. We show that IL-1β and IL-18 release from macrophages depends on a previously described (Lint et al., 1976) but poorly understood 'bystander' deposition of MAC on the plasma membrane of phagocytic macrophages. The secretion of IL-1β and IL-18 did not occur with macrophages deficient in *Nlrp3*, *Asc* or caspase-1, indicating that the NLRP3 inflammasome is activated during complement-mediated phagocytosis. In this way, complement-mediated phagocytosis of particles generates inflammatory signals that can influence innate and adaptive immunity.

The so-called 'reactive lysis' of unsensitized erythrocytes by the complement MAC was first described by Lachmann and colleagues in 1970 (Thompson and Lachmann, 1970). This early study demonstrated that the terminal complement components were not irreversibly bound to target particles, but rather could

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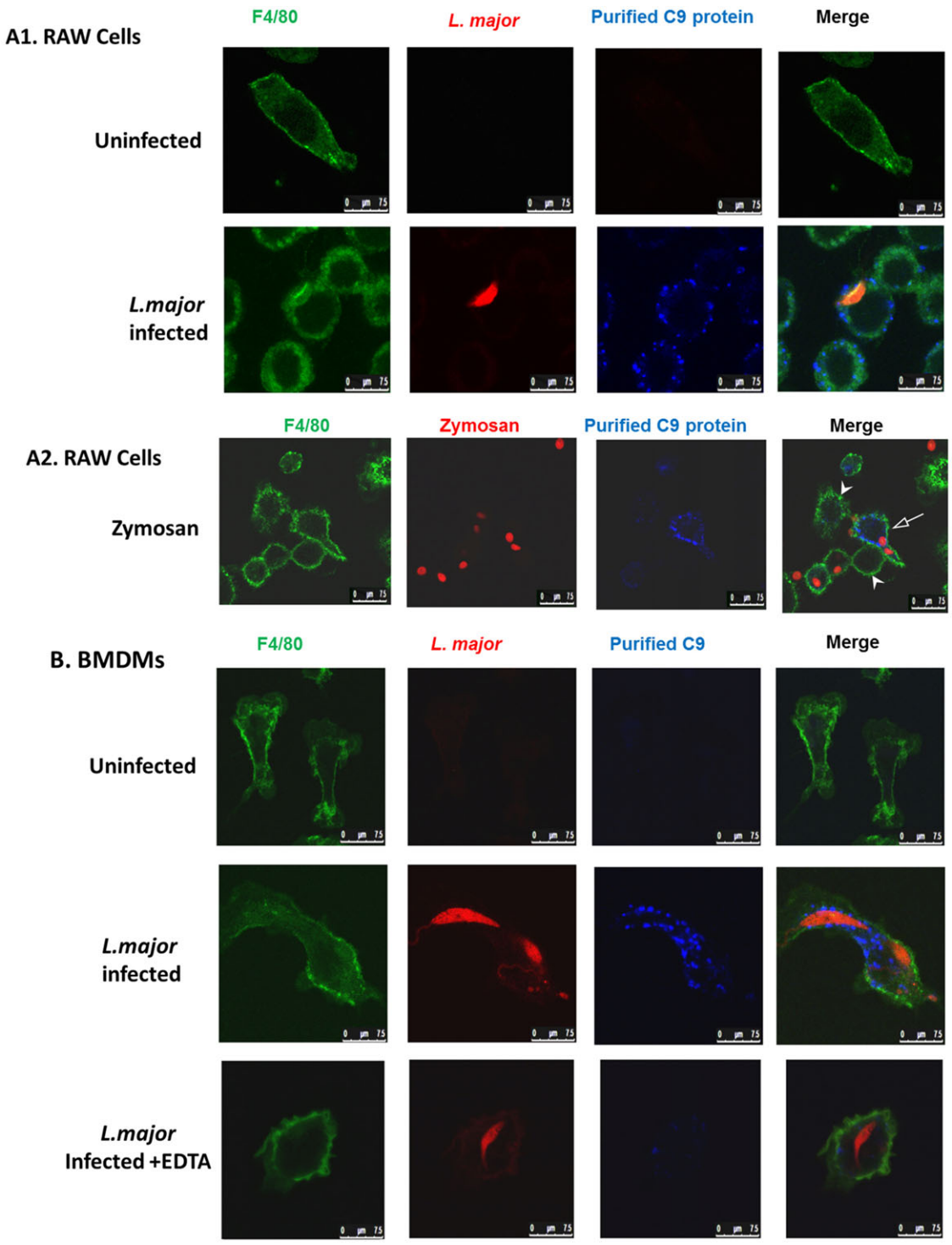

Fig. 5. Bystander MAC deposition on macrophages. LPS-primed RAW cells were infected with Ds Red *L. major* (shown in red; A1) or Alexa-Fluor-488-conjugated zymosan (shown in red; A2) in the presence of Alexa-Fluor-647-labeled C9 protein supplemented with C9-deficient serum (shown in A1 and A2 in blue) and the accumulation of MACs was visualized on macrophages stained for F4/80 (with F4/80–FITC in A1 and F4/80–phycoerythrin in A2; shown in green). The images were taken 3 min after the Alexa-Fluor-647-conjugated C9 was added, using confocal microscopy. The arrow indicates a positive cell with zymosan (red) inside and C9 (blue) deposited on the plasma membrane. Arrowheads indicate negative cells with no zymosan or C9 deposition. (B) Similar imaging experiments to those shown in A were carried out using bone-marrow-derived macrophages (BMMϕ). Ds Red *Leishmania major* parasites either pretreated or not with EDTA were added to macrophages at an MOI of 1:5 for imaging. The MAC deposition was not observed on the cell surface in the macrophages infected with *L. major* pretreated with EDTA, which chelates ions required for MAC deposition. The confocal images are a representative of three independent experiments and images taken at different times of MAC accumulation.

'jump' to neighboring un-sensitized 'bystander' erythrocytes. This could result in the lysis of RBCs that were themselves not activators of complement. Although this bystander mechanism proved to be helpful for the study of the structure and function of the MAC, the physiological relevance of this phenomenon remained unclear. The present study suggests that this bystander

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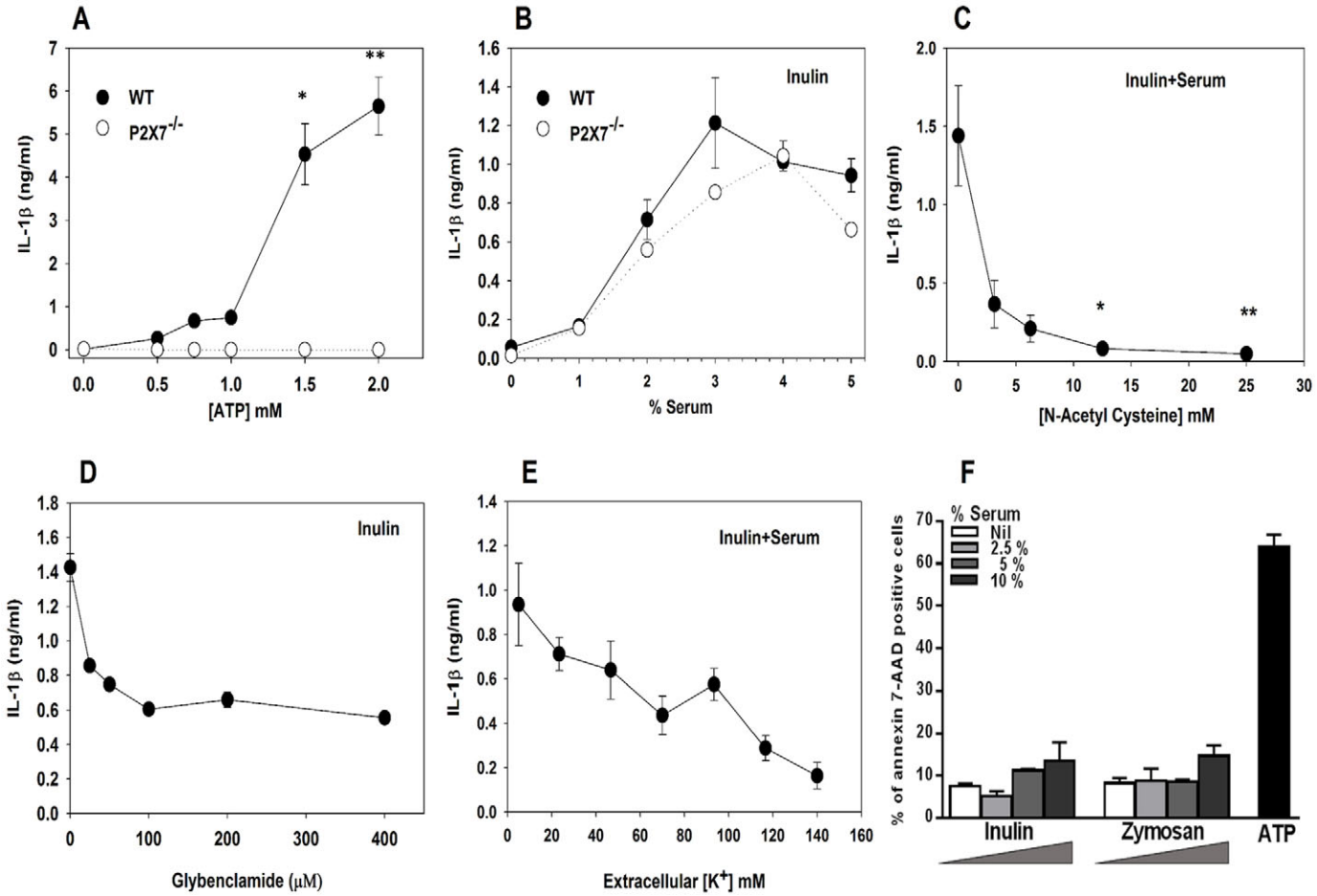

Fig. 6. Bystander MAC deposition is sublytic and induces ionic imbalances in macrophages. (A,B) IL-1β production from macrophages deficient in *P2X7* (white circles) was compared to wild-type (WT) macrophages (black circles) after stimulation with increasing concentrations of ATP (A) or inulin particles (B,C) with increasing concentrations of normal human serum (B). IL-1β production from macrophages was measured by ELISA after inhibiting reactive oxygen species by adding increasing concentrations of N-acetyl cysteine (NAC) (C), or after inhibiting K^+ efflux by incubating macrophages in increasing concentrations of flybenclamide (D), or increasing concentrations of extracellular K^+ (E) during inflammasome activation by inulin particles and 5% serum. (F) Macrophage viability was assessed by measuring the uptake of 7-AAD and annexin V staining after incubating LPS-primed macrophages with various complement activators (n=4). Nil, no serum. Bars represent mean±s.e.m. of a minimum of three independent determinations. *$P\leq0.05$, **$P\leq0.01$ (Student's t-test).

deposition of MAC can occur on macrophages during the process of complement-mediated phagocytosis. Instead of resulting in reactive lysis, it results in inflammasome activation and the secretion of IL-1β and IL-18 by phagocytic macrophages. Thus, this work establishes a new physiological relevance for bystander MAC deposition.

Bacterial pore-forming toxins have been previously associated with the activation of host cell inflammasomes (Muñoz-Planillo et al., 2013). The poly-C9 MAC also forms pores on membranes to cause host cell lysis. However, most eukaryotic cells have efficient membrane repair mechanisms to prevent host cell lysis. In particular, the expression of CD59 on mammalian cells can inhibit increased C9 deposition to prevent cell lysis (Davies and Lachmann, 1993). Despite these regulatory mechanisms, recent studies have shown that directing complement deposition to host cells with antibodies to cell surface proteins, can result in the activation of host inflammasomes (Laudisi et al., 2013; Triantafilou et al., 2013). These studies indicate that in autoimmune diseases, self-reactive antibodies can contribute to inflammation by activating host inflammasomes. In the present work, we demonstrate that complement-mediated activation of inflammasomes can occur in the absence of auto-antibodies during the normal process of complement-mediated phagocytosis. We used a variety of particulate and soluble activators of complement to demonstrate this. The phagocytosis of all of these complement-opsonized particles was sufficient to activate inflammasomes and initiate the secretion of IL-1β and IL-18. These observations might provide another explanation for why complement-mediated phagocytosis is associated with more inflammatory immunopathology than non-opsonic phagocytosis.

The complement MAC has been shown to be involved in exacerbating pathology in a variety of autoimmune and autoinflammatory diseases ranging from gout (Tramontini et al., 2004), to rheumatoid arthritis (Kolb and Müller-Eberhard, 1975) and coronary artery disease (Oksjoki et al., 2007). In many cases, antibodies to terminal complement components have been used to mitigate inflammation. Eculizimab, a humanized antibody targeting complement component C5 is being prescribed for patients with paroxysmal nocturnal hemoglobinuria (PNH) (Hillmen et al., 2007; Mandala et al., 2013), atypical hemolytic uremic syndrome (aHUS) (Mache et al., 2009), glomerulonephritis (Rosenblad et al., 2014), systemic lupus erythematosus (SLE)

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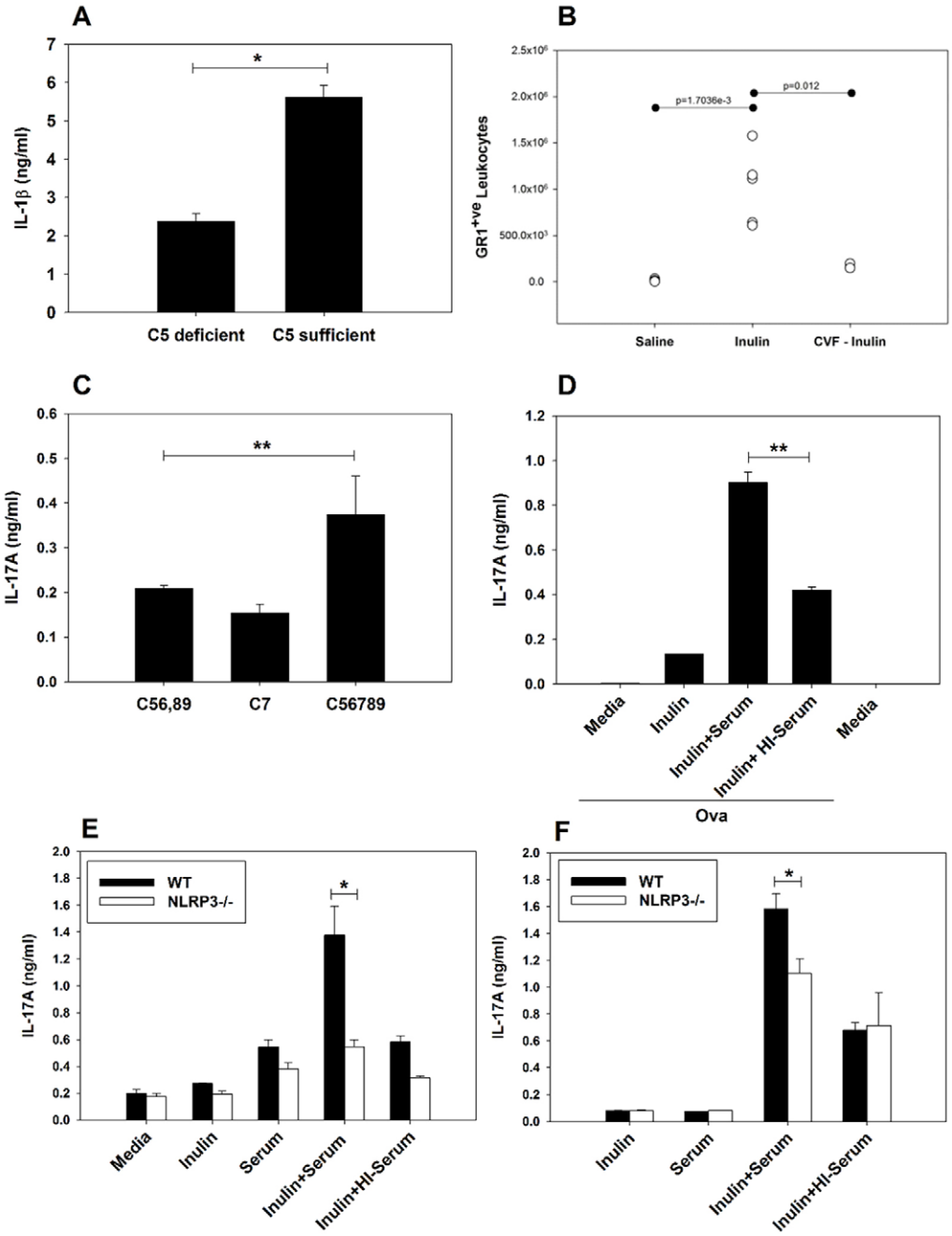

Fig. 7. Bystander complement activation influences innate and adaptive immunity. (A) Male C5-sufficient or C5-deficient mice were treated intraperitoneally with 20 mg/kg LPS and 2 U cobra venom factor (CVF). IL-1β was measured in serum by ELISA 3 h later (*n*=5). (B) Normal or complement-depleted mice were injected with inulin for 6 h. Peritoneal exudate cells were collected and stained for GR1. Complement-depleted mice were treated with 2 U per mouse CVF 24 h prior to the addition of inulin particles (*n*=5). (C,D) IL-17A production from Ova-specific D011.10T cells was measured *in vitro* after stimulation with LPS-primed Ova-pulsed macrophages in which the inflammasome was activated by purified terminal complement components (C) or by inulin particles and serum (D) (*n*=3). (E,F) IL-17A production by OT-II T cells was measured following co-culture with wild-type (WT) (black bars) or NLRP3$^{-/-}$ (white bars) macrophages that were pulsed with Ovaunder conditions that induced inflammasome activation (inulin +serum) or in which the inflammasome was not activated (inulin+HI-serum) following primary (E) or secondary (F) stimulation (*n*=3). Bars represent mean±s.e.m. **P*≤0.05, ***P*≤0.01 (Student's *t*-test).

(Coppo et al., 2015) and even asthma (Smith et al., 2012). Interestingly, the administration of anti-complement antibodies not only prevents host cell lysis, but also reduces inflammatory markers in these patients (Weitz et al., 2012). It is tempting to speculate that part of this might be due to the inhibition of inflammasome induction by bystander deposition of MAC on host cells.

Several groups have proposed that complement can be a natural adjuvant to promote immune responses (Bánki et al., 2010; Dempsey et al., 1996; Haas et al., 2004; Stäger et al., 2003), and that complement activation by adjuvants can enhance vaccine efficacy (Kerekes et al., 2001). Complement activation is thought to play an important role in the adjuvant effects of alum (Güven et al., 2013), and novel adjuvants based on inulin have been shown to be potent inducers of the T_H1 and T_H2 immune response (Silva et al., 2004). Complement-opsonized antigens, like HIV (Bánki et al., 2010; Haas et al., 2004) and anthrax (Kolla et al., 2007), are better at activating T cells than their unopsonized counterparts. In the present studies, we demonstrate that complement activation can be causally linked to IL-1β and IL-18 secretion by macrophages. This can have myriad effects on innate and adaptive immune responses. IL-1β is a potent pro-inflammatory cytokine that is known to induce fever, upregulate acute phase proteins and induce adhesion molecule expression on endothelial cells (Dinarello, 2009). IL-1β is important for the development of T_H17 responses that can confer immunity to fungi, such as *C. albicans* (van de Veerdonk et al., 2011). IL-1β production has been associated with increased resistance to *M. tuberculosis* (Mayer-Barber et al., 2014; van de Veerdonk et al., 2011), and in some scenarios IL-1R1 signaling can also promote T_H2 responses in mouse models of asthma (Johnson et al., 2005). IL-18 is a potent inducer of IFNγ from NK cells and T cells, and in

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the presence of IL-12 or IL-15 can potentiate cell mediated immunity. IL-18 can also combine with IL-23 to promote T_H17 responses (Lalor et al., 2011), and with IL-4 to promote T_H2 responses (Xu et al., 2000). Therefore, the mechanism of phagocytosis might influence the immunogenicity of an antigen and bias immune responses in different directions. For this reason, complement-mediated inflammasome activation might provide a new therapeutic pathway to target to either enhance vaccine responses or to suppress undesirable immunogenicity and inflammation. Our study also suggests that complement opsonization and phagocytosis might fundamentally change the immune response to an antigen.

MATERIALS AND METHODS

Mice

C57BL/6, Balb/c, C-deficient (B10.D2-Hc0 H2d H2-T18c/oSnJ), C-sufficient (B10.D2-Hc1 H2d H2-T18c/nSnJ) and OT-II transgenic mice, which were 6–8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in high efficiency particle-filtered Thoren units (Thoren Caging Systems, Hazleton, PA) at the University of Maryland. All animal studies were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee. The generation of *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice has been described previously (Kuida et al., 1995; Sutterwala et al., 2006).

Reagents

ATP, zymosan, and inulin were purchased from Sigma-Aldrich. Glybenclamide and Compstatin were purchased from Tocris Bioscience (Bristol, UK). Normal human serum, serum deficient for complement proteins C3, C5, C6, C8 or C9, and purified C5b6, C7, C8 and C9 proteins were purchased from Complement Tech (Tyler, TX). Particulate inulin was prepared by dissolving 1 g of inulin in 40 ml water containing 0.1% ammonia and heated at 60°C. Inulin was frozen at −20°C overnight and subsequently precipitated at 37°C for 3 days in the presence of chloroform. The resulting precipitate was centrifuged at 2000 ***g*** for 10 min and re-suspended in water to a concentration of 10 mg/ml and stored as 1 ml aliquots. The particles were sonicated before use.

Murine macrophages

Bone-marrow-derived macrophages (BMMϕ) were prepared as previously described (Goncalves et al., 2011). Bone marrow cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 10 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 20% L929-conditioned medium (LCCM). On day 7, macrophages were removed from Petri dishes and cultured in above mentioned medium without LCCM. RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA).

Human macrophages

Human peripheral blood mononuclear cells were obtained from healthy volunteers with their informed consent in accordance with the Declaration of Helsinki and institutional review board approval. Monocytes were purified by attachment to culture dishes and differentiated into macrophages in the presence of 10% heat inactivated human AB serum for 7 days.

Leishmania parasites

L. major Friedlin strain (MHOM/IL/80/Friedlin), Ds-Red *L. major*, parasites were maintained as previously described (Goncalves et al., 2011; Kimblin et al., 2008). Stationary-phase promastigotes were propagated in 50:50 Schneider's insect medium (Sigma-Aldrich) and M-199 (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine at 25°C. Transgenic Ds-Red *L. major* parasites were grown in the presence of 50 µg/ml of Geneticin (G418; Sigma-Aldrich).

Stimulation of bone-marrow-derived macrophages

BMMϕ cells were primed with 10 ng/ml Ultrapure LPS (InvivoGen, San Diego, CA), for 4–6 h. LPS-primed BMMϕ cells were either challenged with 1:10 multiplicity of infection of *L. major*, or were treated with ATP, zymosan or inulin at the indicated concentrations in the presence or absence of 5% of normal human serum for 4 h. Supernatants were collected and assayed for IL-1β or IL-18. Antibody pairs for IL-1β ELISA were purchased from eBioscience (San Diego, CA) and for IL-18 ELISA from MBL International (Woburn, MA).

Cell viability and FLICA staining

Viability of BMMϕ cells was assessed by 7-AAD and annexin V staining (Molecular Probes). FAM-FLICA™ Caspase-1 assay was performed following the manufacturer's protocol (ImmunoChemistry Technologies, Bloomington, MN). Cells positive for active caspase-1 were quantified by flow cytometric analyses.

Lentiviral transduction

Lentivirus was produced in human embryonic kidney (HEK293T) cells transfected with the FUGW-based expression vector encoding CFP–ASC (Halle et al., 2008) (a kind gift from Katherine Fitzgerald, Division of Infectious Diseases University of Massachusetts Medical School, Worcester, MA), lentiviral packaging plasmid psPAX2 and envelope plasmid pMD2.G using TransIT®-293 Transfection Reagent (Mirus Bio, Madison, WI). Supernatants containing lentivirus were collected after 48 h and were used to infect RAW264.7 cells.

Immunofluorescence

For immunofluorescence imaging BMMϕ cells were cultured on cover slips, LPS-primed and activated with Alexa-Fluor-488-conjugated zymosan A (source *S. cerevisiae*; Life Technologies) or Ds-Red expressing *L. major*. To visualize complement MAC deposition on macrophages, C9-deficient (C9-D) serum was supplemented with Alexa-Fluor-647-labeled C9 protein at the time of image acquisition. Labeling of C9 protein with Alexa-Fluor-647 was performed following the manufacturer's protocol (Alexa Fluor 647 protein labeling kit, Life Technologies, Grand Island, NY). Cells were fixed with 4% paraformaldehyde, 3 min after C9 addition when the MAC deposition was found to occur at maximum on surface. Cover slips were mounted on clean glass slides using Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA).

To visualize inflammasome activation, RAW264.7 cells grown on cover slips were transduced with lentivirus encoding CFP–ASC, LPS-primed and activated with Alexa-Fluor-488-conjugated zymosan A in the presence of 5% normal human serum for 45 min. The cells were then fixed and imaged under a Leica SP5X Confocal Microscope (Leica, Exton, PA), in a 100× oil immersion objective with a numerical aperture of 1.32. The fluorochromes were excited using an argon laser for Alexa Fluor 488 and white light laser for Ds-Red and Alexa Fluor 647 set at appropriate excitation wavelengths. The images acquired using Leica LAS software and processed with LASAF Lite and Adobe Photoshop CS4.

In vivo experiments

For *in vivo* models of inflammasome activation, the complement-activating and complement-depleting functions of cobra venom factor (CVF) were exploited in this study. Age-matched male C5 deficient or sufficient mice were injected with 2 U/mouse CVF (Quidel, San Diego, CA) and 20 mg/kg LPS intraperitoneally. After 3 h, blood was collected by penetrating the retro-orbital sinus of mice to measure serum levels of IL-1β by ELISA. To examine whether complement activation plays a role in leukocyte infiltration, C57Bl/6 mice were injected with saline or 1 mg of inulin particles intraperitoneally and assessed for intraperitoneal infiltration of GR1-positive cells ($1A8^+$) after 6 h in peritoneal lavage fluid, in mice previously complement-depleted with CVF. CVF was administered 24 h prior to inulin administration at a concentration of 2 U per mouse. The percentage of GR1-positive cells was assessed by flow cytometry.

T cell activation

For antigen presentation, macrophages were plated at a concentration of 2×10^5/well and primed with 10 ng/ml LPS for 4 h. Cells were then washed, and activated with 150 µg/ml Ova and inflammasome activators like inulin (100 µg/ml) in the presence of human serum (5%). Alternatively, purified complement proteins C5b6 (2 µg/ml), C7, C8 and C9 (10 µg/ml each) alone were added to macrophages to activate the inflammasome. Macrophages were washed after 2 h and co-cultured with 5×10^5 $CD4^+$ T cells (purified from spleen by positive selection using MACS (Miltenyi Biotec, Germany) for a week. Following primary stimulation, $CD4^+$ T cells were harvested, washed, and re-stimulated with immobilized CD3 antibodies (BD Biosciences, CA) for 48 h. Levels of IL-17 were measured in the supernatants following secondary stimulation (BD Biosciences, CA).

Statistical analysis

Results are reported as a mean±s.e.m. unless otherwise stated. For s.e.m., at least three independent experiments were performed. Data analysis was performed using SigmaPlot (Systat Software, San Jose, CA). Student's *t*-tests were utilized for the comparison of means between two groups. One-way analysis of variance (ANOVA) tests were used when comparing means of more than three comparisons. A P value of less than 0.05 was adopted as the statistical significance level.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors contributed to the writing of manuscript. R.S. developed the research plan, performed experiments and analysis of the data. P.C. performed the membrane attack complex microscopy, flow cytometry assays and prepared knockout BMDMs. F.S.S. provided the knockout mice. D.M.M. conceived the study and directed the research.

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Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.179291/-/DC1

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