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**The murine neutrophil NLRP3 inflammasome is activated by soluble but not particulate or crystalline agonists**

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**Abbreviations:** AO: acridine orange, NLR: NOD-like receptor.

## **Abstract**

Neutrophils express pattern recognition receptors (PRRs) and regulate immune responses via PRR-dependent cytokine production. An emerging theme is that neutrophil PRRs often exhibit cell type-specific adaptations in their signalling pathways. This prompted us to examine inflammasome signalling by the PRR NLRP3 in murine neutrophils, in comparison to well-established NLRP3 signalling pathways in macrophages. Here, we demonstrate that while murine neutrophils can indeed signal via the NLRP3 inflammasome, neutrophil NLRP3 selectively responds to soluble agonists but not to the particulate/crystalline agonists that trigger NLRP3 activation in macrophages via phagolysosomal rupture. In keeping with this, alum did not trigger IL-1 $\beta$  production from human PMN, and the lysosomotropic peptide Leu-Leu-OMe stimulated only weak NLRP3-dependent IL-1 $\beta$  production from murine neutrophils, suggesting that lysosomal rupture is not a strong stimulus for NLRP3 activation in neutrophils. We validated our in vitro findings for poor neutrophil NLRP3 responses to particles in vivo, where we demonstrated that neutrophils do not significantly contribute to alum-induced IL-1 $\beta$  production in mice. In all, our studies highlight that myeloid cell identity and the nature of the danger signal can strongly influence signalling by a single PRR, thus shaping the nature of the resultant immune response.

## Introduction

Inflammasomes are cytoplasmic multi-protein complexes that drive the maturation of specific interleukin-1 (IL-1) family cytokines and induce an inflammatory form of cell death called pyroptosis. Inflammasomes consist of a sensor protein (e.g. a Nod-like receptor (NLR) protein), the caspase-1 protease, and often contain the common inflammasome adaptor, ASC [1]. The NLRP3 inflammasome is of particular importance because it has the capacity to sense a wide variety of structurally unrelated molecules, including whole pathogens, bacterial toxins, metabolic products, insoluble molecules (particles, crystals and protein aggregates), and alarmins released from damaged tissues. Consequently, the NLRP3 inflammasome is central to host defence but also mediates pathological immune responses in numerous inflammatory and metabolic disorders such as Alzheimer's Disease, silicosis, asbestosis and gout [1]. How a single protein responds to such a diverse range of agonists remains an enigma. Many studies posit that these distinct agonists elicit convergent cell stress signals that trigger NLRP3 activation and inflammasome assembly. Proposed mechanisms include  $K^+$  efflux [2], lysosomal rupture by crystals, particles and protein aggregates [3], reactive oxygen species production [4] and calcium mobilisation [5]. However, the precise nature of the NLRP3-activating stress signal, and the mechanism by which NLRP3 senses such a signal, remains unknown. It is also unclear how a single receptor signalling module can tailor an in vivo immune response that is appropriate to such diverse cues such as tissue injury, infection, metabolic stress or environmental irritants; one possibility is that distinct NLRP3 agonists trigger NLRP3 signalling in different immune cell suites.

While the biology of NLRP3 has been widely studied in macrophages and dendritic cells, increasing evidence suggests that these cells are not the sole cell types driving NLRP3-dependent responses in vivo. Two recent studies demonstrated that neutrophils are major contributors to NLRP3-dependent IL-1 $\beta$  production during murine infection with *Staphylococcus aureus* [6] and *Streptococcus pneumoniae* [7]. NLRP3 signalling pathways are poorly characterised in neutrophils, and it is increasingly appreciated that immune signalling pathways often display cell type-specific effects. For example, we recently reported that neutrophils selectively trigger caspase-1-dependent cytokine processing but not pyroptosis following NLRC4 activation by *Salmonella* [8], while in macrophages, the NLRC4 inflammasome drives both cytokine processing and pyroptosis. Here, we investigated NLRP3 signalling in neutrophils, with the hypothesis that like NLRC4, the NLRP3 pathway may exhibit neutrophil-specific adaptations. In doing so, we demonstrate that soluble but not particulate or crystalline NLRP3 agonists trigger NLRP3-dependent responses in neutrophils. Our findings highlight cell type specificity in the ability to respond to individual NLRP3 agonists, and provide a possible mechanism by which NLRP3-dependent immune responses are tailored to the specific danger encountered.

## **Results and discussion**

### *The neutrophil NLRP3 inflammasome selectively responds to soluble agonists*

The neutrophil NLRP3 inflammasome is reported to trigger caspase-1-dependent IL-1 $\beta$  cleavage and release following infection with whole

pathogen (*S. aureus* and *S. pneumoniae*) or bacterial pore-forming toxins [6, 7, 9]. However, it was unclear whether other compounds established to activate NLRP3 in macrophages, such as insoluble compounds that cause phagolysosomal rupture, also activate NLRP3 in neutrophils. We investigated this possibility by stimulating purified bone marrow neutrophils and bone marrow-derived macrophages (BMDM) with a range of NLRP3 agonists. Prior to challenge with NLRP3 agonists, cells were exposed to lipopolysaccharide (LPS) to upregulate NLRP3 and pro-IL-1 $\beta$  [10, 11]. The bacterial pore-forming toxin and potassium ionophore, nigericin, triggered IL-1 $\beta$  secretion from both wild type (WT) neutrophils and macrophages (**Fig. 1A–C**). IL-1 $\beta$  production by neutrophils required an inflammasome, as IL-1 $\beta$  secretion was markedly reduced in caspase-1/11-deficient *Ice*<sup>-/-</sup> neutrophils (**Fig. 1A**), similar to *Ice*<sup>-/-</sup> macrophages (**Fig. 1B**). Moreover, caspase-1/11 deficiency ablated IL-1 $\beta$  processing by neutrophils (**Fig. 1C**). Other soluble activators of the NLRP3 inflammasome (e.g. extracellular ATP, R837) also triggered NLRP3/caspase-1-dependent IL-1 $\beta$  release in neutrophils (**Fig. S1A–B**), as for macrophages [12, 13]. We previously found that IL-1 $\beta$  was the dominant inflammasome target cytokine produced upon neutrophil NLRC4 activation [8], and in keeping with this, NLRP3 activation was not a strong stimulus for neutrophil IL-18 release (**Fig. S1C**). In macrophages and dendritic cells, a range of insoluble compounds can also trigger NLRP3 activation by rupturing phagolysosomes [3, 14]. We next investigated if this pathway is also active in neutrophils. Surprisingly, neither alum nor silica particles, nor MSU crystals, triggered caspase-1-dependent IL-1 $\beta$  secretion from murine neutrophils (**Fig. 1D–F**), while all elicited strong caspase-1-dependent IL-1 $\beta$  secretion from

macrophages as anticipated (**Fig. 1G–J**). We next investigated whether human blood polymorphonuclear cells (PMNs) phenocopied murine neutrophils in their response to soluble versus insoluble agonists. Human neutrophils express inflammasome components such as NLRP3, ASC and caspase-1 [8, 15], and produce IL-1 $\beta$  upon exposure to the *S. pneumoniae* toxin, pneumolysin [7]. In keeping with this, nigericin induced IL-1 $\beta$  production from LPS-primed human PMNs, and this response was suppressed by VX-765, a caspase-1/4 inhibitor [16]. Surprisingly, LPS alone triggered IL-1 $\beta$  secretion from human PMN (**Fig. 1J**), similar to human monocytes [17]. Alum did not elicit IL-1 $\beta$  production from LPS-primed human PMN (**Fig. 1J**), similar to murine neutrophils (**Fig. 1D**). Thus, neutrophils can mount robust NLRP3 inflammasome responses to soluble but not insoluble NLRP3 agonists.

*The phagolysosomal rupture pathway is a weak stimulus for neutrophil NLRP3*

Insoluble NLRP3 agonists trigger NLRP3 activation in macrophages by destabilising phagolysosomes [3]. The observed lack of inflammasome activation in neutrophils stimulated with insoluble NLRP3 agonists could either reflect ineffective phagocytosis of these stimuli, or a deficiency in the NLRP3-activating phagolysosomal rupture pathway. We first investigated the cellular uptake of silica particles by live cell imaging. Neutrophils were incubated with silica in the presence of the fluid phase marker, Dextran, over 2 h. Silica particles were detected in Dextran-positive compartments (**Fig. 2A, S2**), indicating that neutrophils phagocytose these particles, as for MSU crystals [18]. We next examined the lysosomal rupture pathway in neutrophils versus

macrophages by treating the cells with the small lysosomotropic peptide L-leucyl-L-leucine methyl ester (Leu-Leu-OMe) to induce phagolysosomal damage [3]. Phagolysosomal integrity can be measured using acridine orange (AO), which fluoresces red in the low pH of lysosomes [3]. AO fluorescence was 3-fold higher in bone marrow macrophages compared to neutrophils (**Fig. 2–C**), suggesting that macrophages harbour a larger, or more acidic, lysosomal compartment than neutrophils. Leu-Leu-OMe treatment triggered a loss of AO fluorescence in both cell types (**Fig. 2B–C**), although the effect was much more prominent in macrophages. The loss of AO fluorescence correlated inversely with IL-1 $\beta$  secretion for both neutrophils and macrophages (**Fig. 2D–E**). However, Leu-Leu-OMe elicited a poor IL-1 $\beta$  response in neutrophils, while producing a robust IL-1 $\beta$  response in macrophages. This indicates that while Leu-Leu-OMe can elicit phagolysosome rupture and resultant IL-1 $\beta$  production in neutrophils, this pathway is a very weak stimulus for neutrophil NLRP3 activation as compared to macrophages.

*Neutrophil depletion does not alter IL-1 $\beta$  production in alum-induced peritonitis in mice*

We, and others, demonstrated that neutrophils are a major cellular compartment for caspase-1-dependent IL-1 $\beta$  production during in vivo bacterial infection [6-8]. Given our surprising observation that alum did not trigger IL-1 $\beta$  production by the neutrophil NLRP3 inflammasome *in vitro* (**Fig. 1D**), we anticipated that, unlike the case for in vivo bacterial challenge [6-8], neutrophils would not be a significant cellular source of IL-1 $\beta$  during alum-

induced peritonitis. To examine this hypothesis, we administered C57BL/6 mice with an isotype control antibody or an  $\alpha$ -Ly6G (1A8) antibody to specifically deplete neutrophils [8]. At 16 - 24 h post-depletion, mice were challenged with 350  $\mu$ g alum for 6 h and peritoneal IL-1 $\beta$  levels were quantified. Alum triggered neutrophil recruitment and IL-1 $\beta$  production in mock-depleted mice (**Fig. 3A**), but neutrophil depletion did not affect peritoneal IL-1 $\beta$  levels (**Fig. 3B**). This indicates that, as anticipated, neutrophils are not a significant cellular source of IL-1 $\beta$  in this setting.

### **Concluding remarks**

Neutrophils have been traditionally viewed as anti-microbial effector cells, but an emerging literature documents the wide diversity of neutrophil functions, including their role as important modulators of inflammation [19]. The NLRP3 inflammasome is a key pathway for eliciting inflammatory responses, but the circumstances in which neutrophils contribute to NLRP3-driven inflammation were poorly characterised. Here we challenged neutrophils with a panel of 7 different NLRP3 agonists that trigger NLRP3 activation by phagolysosomal rupture (MSU, alum, silica, Leu-Leu-OMe) or by mechanisms independent of phagolysosomal rupture (nigericin, ATP, R837). Human and mouse neutrophils express NLRP3 [8], and in keeping with this, we and others [9] observe robust NLRP3-dependent responses to soluble agonists (e.g. nigericin, ATP, R837) that signal the presence of infection or tissue damage. Surprisingly, we discovered that the neutrophil NLRP3 inflammasome was poorly responsive to insoluble NLRP3 agonists such as alum, silica and MSU that trigger macrophage NLRP3 activation via phagolysosomal rupture.

Murine neutrophils were likewise poorly responsive to the lysosomotropic peptide Leu-Leu-OMe, suggesting that lysosomal rupture is not a prominent pathway for NLRP3 activation in these cells. Accordingly, we found that neutrophils did not significantly contribute to IL-1 $\beta$  production during alum-induced peritonitis in mice. This is in sharp contrast with in vivo bacterial challenge models, where neutrophils were major producers of IL-1 $\beta$  [6-8] and thereby drove an amplification loop to ensure large numbers of activated neutrophils were recruited to resolve the infection [8].

Given the diversity of NLRP3 activating signals, a key outstanding question is how the immune system tailors an in vivo response that is appropriate to each stimulus. For example, in scenarios where neutrophil-mediated clean up is important (e.g. infection, injury), one might expect that the immune response will be dominated by neutrophils. Neutrophil recruitment may be less important in other scenarios of NLRP3 activation, such as metabolic stress (e.g. MSU) or exposure to environmental irritants (e.g. alum, silica). Our data broadly support such a model, as intraperitoneal *Salmonella* infection was a more potent stimulus for neutrophil recruitment than alum injection (**Fig. 3A** and [8]). The finding that neutrophils produce IL-1 $\beta$  in response to NLRP3 agonists indicative of infection or injury, but not those signalling metabolic stress or irritant exposure, suggests a possible mechanism by which immune responses are tailored according to stimulus; neutrophil-derived IL-1 $\beta$  and the ensuing positive feedback loop of neutrophil recruitment and activation likely allows for a strong neutrophil response when it is appropriate, but this positive feedback loop is not engaged to prevent collateral damage in circumstances

where neutrophil function is less important. Our proposed model is noteworthy from a clinical perspective, as the NLRP3 pathway reportedly contributes to the adjuvant activity of alum [20], and is central to a number of inflammatory disorders triggered by insoluble aggregates, such as gout and silicosis. Our data suggest that other cell types (e.g. macrophages, dendritic cells) are responsible for driving inflammasome-mediated inflammatory responses in these settings.

Our finding that the signalling pathways eliciting NLRP3 activation in macrophages are not always conserved in neutrophils emphasises the strong influence of cell identity on innate immune signalling pathways. Instances of such cell type-specificity in signalling pathways are already appreciated within the myeloid compartment; for example, neutrophils exhibit various cell type-specific adaptations to the Toll-like receptor signalling pathways described for macrophages [21]. We speculate that cell type-specific tailoring of immune signalling networks may be common, and warrants further investigation to gain a more nuanced mechanistic understanding of immune response sculpting in health and disease.

## **Materials and methods**

### *Mice*

C57BL/6 and *Ice*<sup>-/-</sup> [22] mice were housed in specific-pathogen-free facilities at the University of Queensland. *Ice*<sup>-/-</sup> mice were backcrossed at least ten times to C57BL/6, and all experiments were conducted with age- and sex-

matched mouse cohorts. The University of Queensland's animal ethics committee approved all experimental protocols.

#### *Murine primary cell culture and inflammasome assays*

Bone marrow neutrophils were surface labelled using  $\alpha$ -Ly6G-FITC (1A8, Biolegend), and purified by MACS with  $\alpha$ -FITC beads. MACS-purified fractions were assayed for cell purity (>98%) by flow cytometry. Macrophages were differentiated from bone marrow as previously described [23]. Bone marrow neutrophils were always used for experiments on the day of purification and were plated in OPTI-MEM (Life Technologies) supplemented with 0.4  $\mu$ g/mL aprotinin (Sigma) at a density of  $3.3 \times 10^6$  cells/ml, except in Figure S2B where  $5 \times 10^6$  cells/ml were used. Bone marrow-derived macrophages were cultured at a density of  $1 \times 10^6$  cells/ml in OPTI-MEM (Life Technologies) supplemented with 100 ng/ml units/ml recombinant human M-CSF (ImmunoTools). To activate the NLRP3 inflammasome, neutrophils and macrophages were primed with 100 ng/ml ultrapure *E. coli* K12 LPS (Invivogen) for 4 h to induce the expression of pro-IL-1 $\beta$  before stimulation with NLRP3 agonists. IL-1 $\beta$  and IL-18 levels in cell-free supernatants were analysed by ELISA (eBioscience). Cell culture supernatants were precipitated with chloroform and methanol as previously described [24], and western blots on cell extracts and precipitated supernatants were performed using standard procedures [24]. Antibodies included IL-1 $\beta$  (polyclonal goat, R&D Systems), caspase-1 (Casper-1, Adipogen), ASC (polyclonal rabbit, Santa Cruz), and tubulin (B-5-1-2, Sigma).

### *Human PMN preparation and inflammasome assays*

Fresh peripheral blood was collected with informed consent from healthy human donors, under approval from the University of Queensland Institutional Human Research Ethics Committee. Bulk polymorphonuclear cells (PMN) were prepared using PolymorphPrep (Progen Biotechnik) according to published procedures [25]. PMNs were cultured in RPMI-1640 (Life Technologies) supplemented with 2% heat-inactivated autologous serum, GlutaMAX (Life Technologies) and 100 U/ml penicillin-streptomycin (Life Technologies) at a density of  $1 \times 10^6$  cells/ml. PMNs were primed with 100 ng/ml ultrapure *E. coli* K12 LPS for 3-4h prior to stimulation with nigericin (5  $\mu$ M) or alum (600  $\mu$ g/ml) for 5 h. VX-765 (25  $\mu$ M) was applied to PMN 30 mins prior to the addition of nigericin or alum. IL-1 $\beta$  production was quantified in cell-free supernatants by ELISA (eBioscience).

### *Live cell imaging of neutrophil uptake of silica*

Purified neutrophils were plated on Poly-L-Lysine (Sigma) treated Mat-tek dishes for live cell imaging experiments. Cells were incubated with silica (15  $\mu$ g/ml) particles and Dextran (200  $\mu$ g/ml; 10,000 MW, Alexa Fluor® 555). After 2 hours incubation, cells were washed three times with warm OPTI-MEM (Life Technologies) and imaged live using a Zeiss 710 confocal microscope.

### *Acridine orange labelling and flow cytometry*

Lysosomes were loaded with acridine orange as previously described [3]. In brief, cells were incubated with acridine orange (Santa Cruz, 1  $\mu$ g/ml) for 15 min and washed thrice prior to stimulation with Leu-Leu-OMe. Lysosomal

rupture was quantified using flow cytometry at a loss of emission at 600 – 650 nm. Data were acquired on BD Canto II and were analysed with FlowJo software (Tree Star).

#### *Neutrophil depletion and in vivo alum challenge*

Neutrophils were depleted or mock-depleted by i.p. administration of 100 µg α-Ly6G antibody (endotoxin-free 1A8 clone, BioXcell) or isotype control antibody (endotoxin-free 2A3 clone, BioXcell). 16 - 24 h post depletion, mice were challenged with i.p with 350 µg alum (Pierce) and sacrificed after 6 h. The peritoneal cavity was flushed with 10 ml of ice-cold DPBS. Collected cells were analysed for neutrophil abundance by flow cytometry, and cell-free supernatants were subject to IL-1β ELISA.

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### **Conflict of interest**

The authors declare no commercial or financial conflict of interest.

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### Figure legends.

**Figure 1. Neutrophils produce mature IL-1 $\beta$  in response to soluble but not particulate/crystalline agonists of the NLRP3 inflammasome.** Bone marrow neutrophils (A, C, D–F) or bone marrow-derived macrophages (B, G–I) were primed with 100 ng/ml LPS for 4 h before challenge with (A–C) nigericin (Nig) (A–B: 0, 1.25, 2.5, 5  $\mu$ M; C: 5  $\mu$ M), (D, G) alum (0, 150, 300, 600  $\mu$ g/ml), (E, H) silica (0, 150, 300, 600  $\mu$ g/ml) or (F, I) MSU (0, 150, 300, 600  $\mu$ g/ml). IL-1 $\beta$  secretion was measured by ELISA at (A, B) 1, 3, 5 h or (D–I) 7 h post challenge. (C) Neutrophil supernatants (SN) and cell extracts (XT) were harvested at 5 h post-nigericin treatment, and examined by western blot. GAPDH was used as a loading control. All murine ELISA data are shown as mean + SD of technical triplicate cell stimulations, and are from single experiments representative of 3 independent experiments. (J) Human PMN were primed with 100 ng/ml LPS for 3–4 h before challenge with nigericin (5  $\mu$ M) or alum (600  $\mu$ g/ml) for 5 h. VX-765 (25  $\mu$ M) was applied to cells 30 min prior to nigericin. Data are derived from PMN from n=5 individual donors (each represented by different symbols), collected in two independent experiments. A non-parametric, paired t-test was used to assess significance (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.005$ ).

**Figure 2. The phagolysosomal rupture pathway is a weak stimulus for neutrophil IL-1 $\beta$  production.** (A) Bone marrow neutrophils were exposed to silica (15  $\mu$ g/ml) for 2 h in the presence of Dextran555 (200  $\mu$ g/ml) and silica internalisation was analysed by confocal microscopy. Brightfield image shows

refractive silica particles with one particle (arrow) co-localising with the fluid phase marker, dextran. Scale bar = 5  $\mu$ M. (B, D) Bone marrow neutrophils or (C, E) bone marrow-derived macrophages were primed with 100 ng/ml LPS for 4 h before exposure to increasing doses of Leu-Leu-OMe (0, 0.5, 1, 2 mM). Acridine orange (AO) staining and IL-1 $\beta$  secretion was measured at 7 h by flow cytometry and ELISA, respectively. (B–E) Data are shown as mean + SD of technical triplicate cell stimulations, and are from single experiments representative of (A) 2 or (B–E) 3 independent experiments.

**Figure 3. Neutrophils do not significantly contribute to IL-1 $\beta$  production during alum challenge in vivo.** Mice were i.p injected with 100  $\mu$ g of  $\alpha$ -Ly6G (1A8) or isotype antibody. After 16-24 h, mice were challenged i.p with 350  $\mu$ g alum for 6 h. (A) Absolute abundance of CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup> neutrophils in the peritoneal exudate was determined by flow cytometry, and (B) peritoneal exudate IL-1 $\beta$  levels were determined by ELISA. Each symbol represents an individual mouse (n=4-5 mice per condition), combined from 2 independent experiments. Statistical analyses were performed using the non-parametric Mann-Whitney t-test. Data were considered significant when  $p \leq 0.05$  (\*).

Figure 1

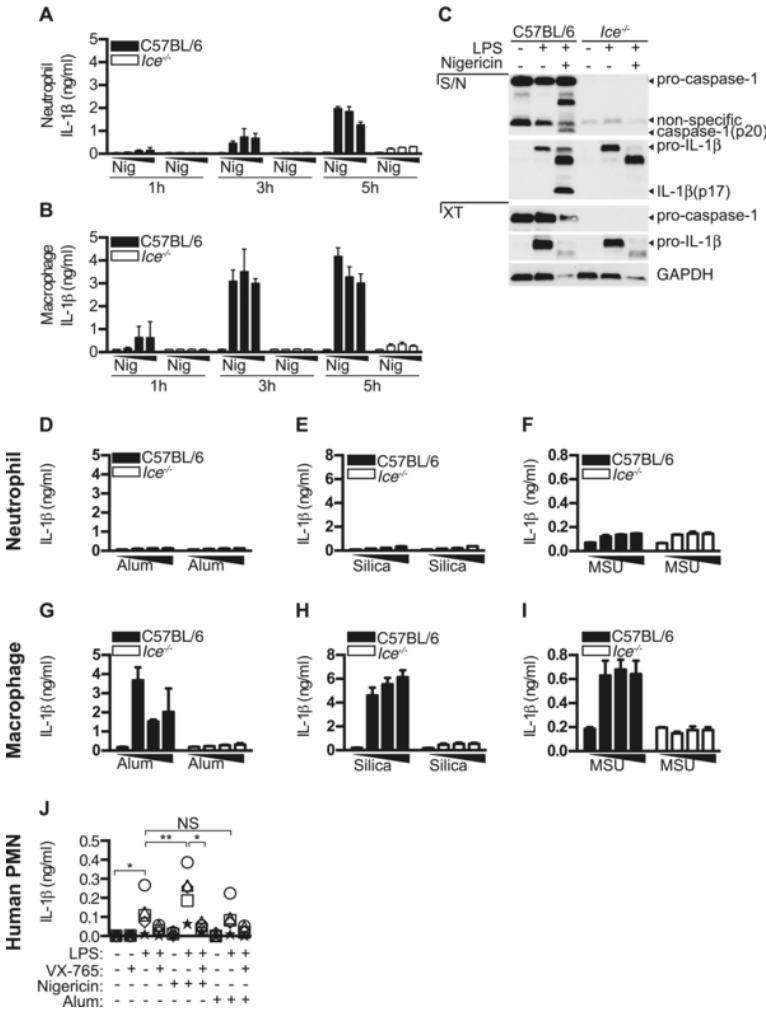


Figure 2

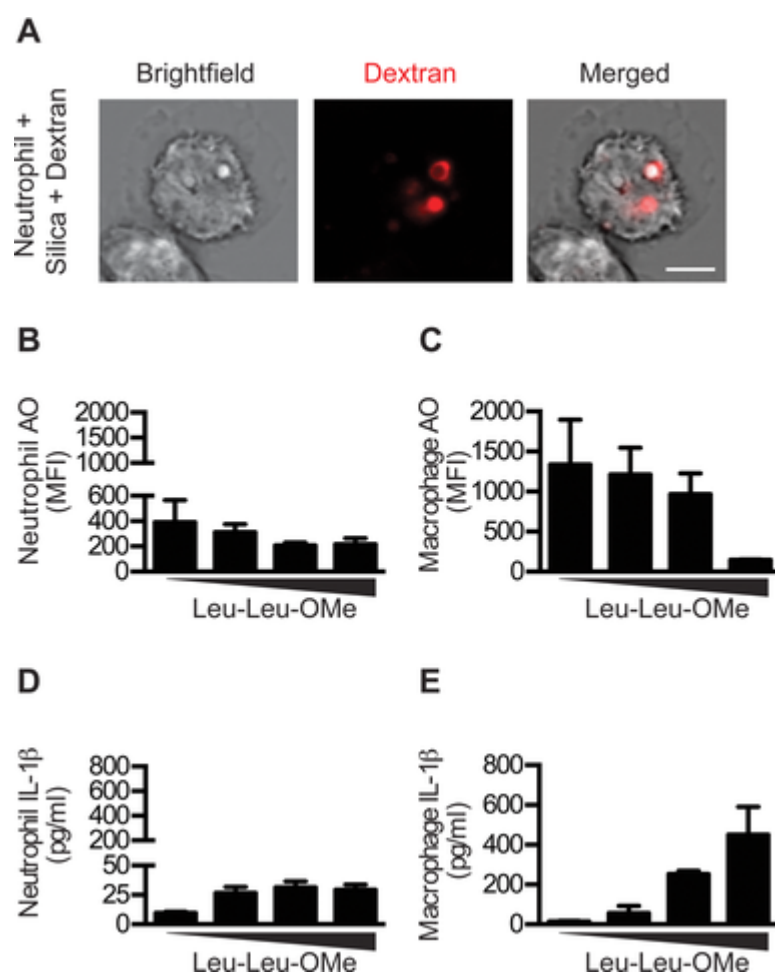
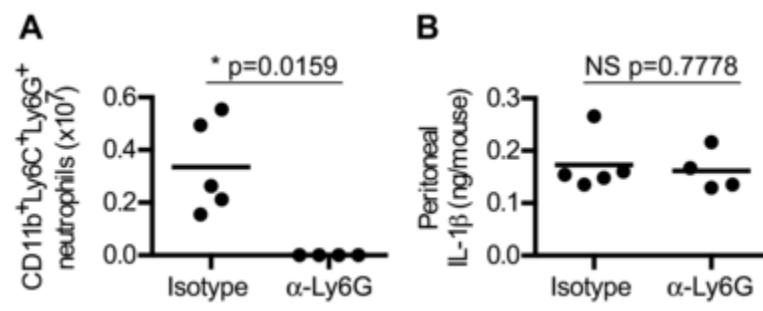
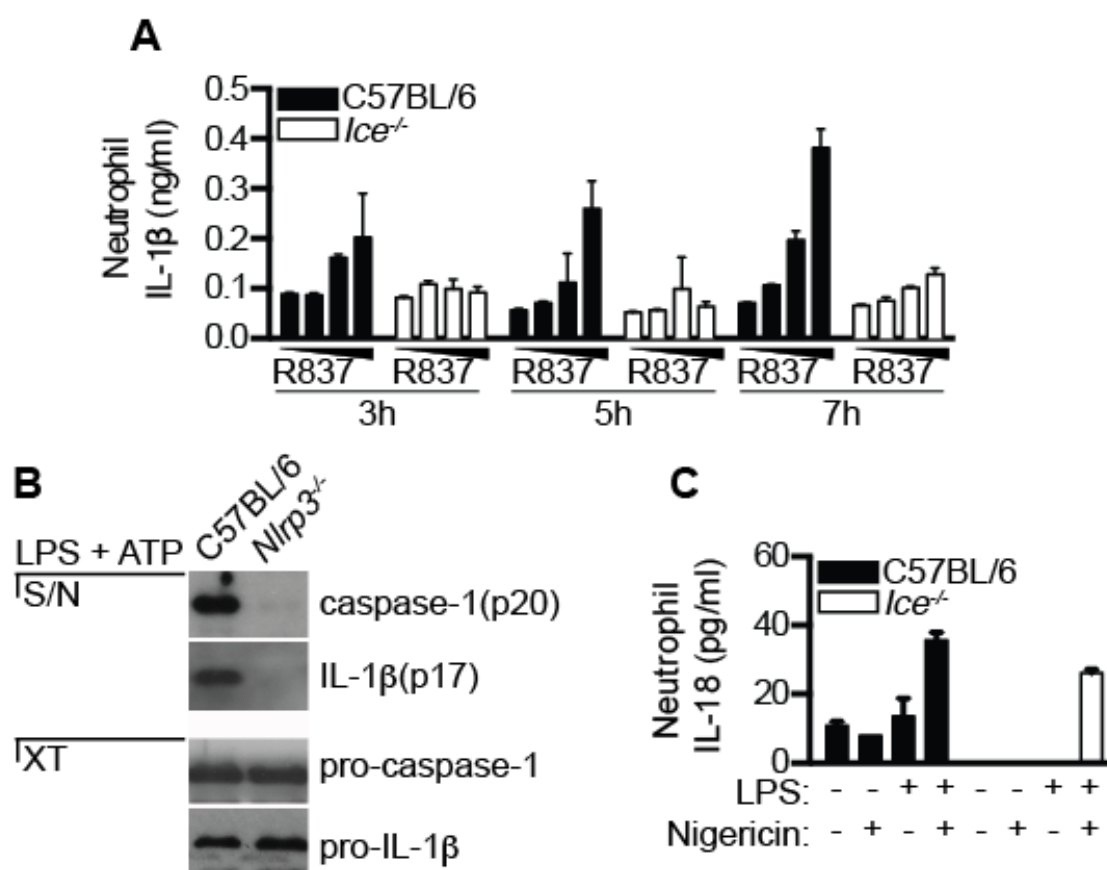


Figure 3



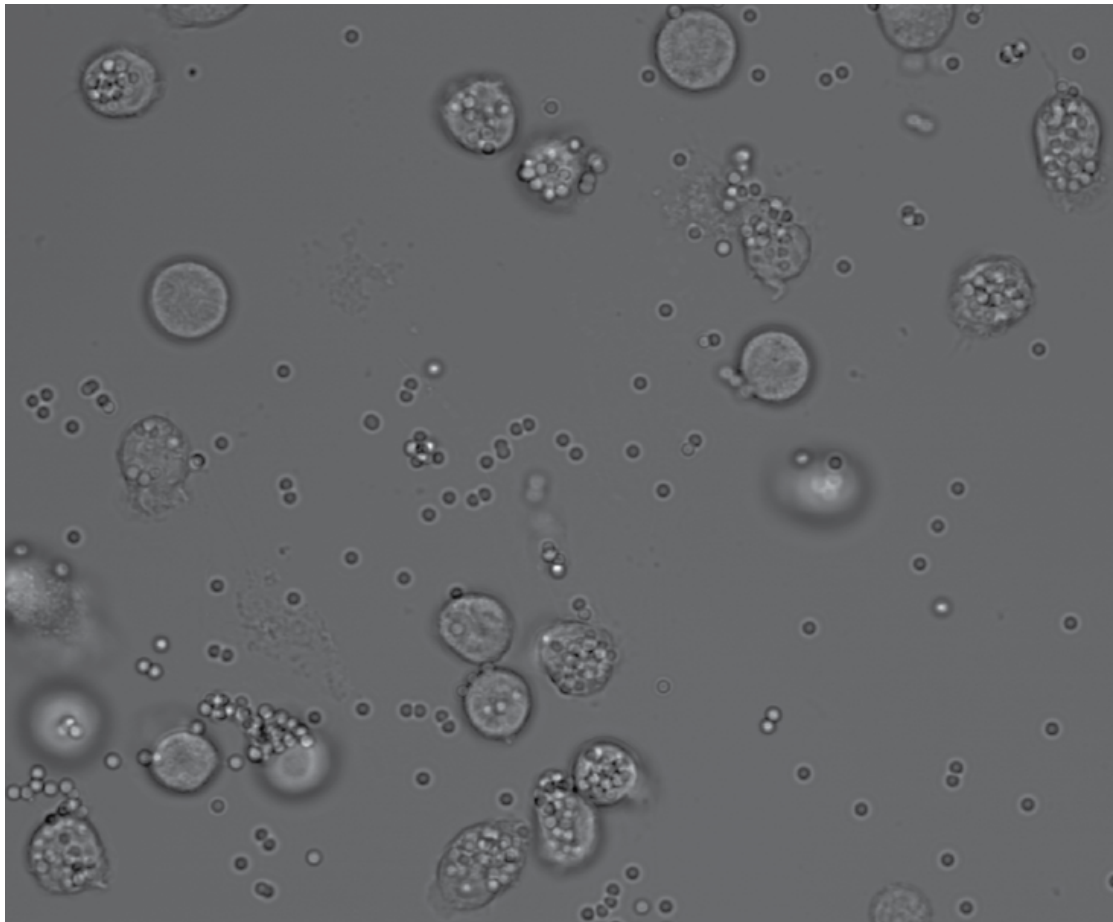
Supporting Information Figure 1



**Neutrophils produce mature IL-1 $\beta$  in response to soluble agonists of the NLRP3 inflammasome.**

(A) Bone marrow neutrophils were primed with 100 ng/ml LPS for 4 h before challenge with R837 (0, 5, 10, 20  $\mu$ g/ml) and IL-1 $\beta$  secretion was measured by ELISA at 3, 5, and 7 h post-challenge. Data are mean + SD of technical triplicate cell stimulations from a single experiment, and are representative of 3 independent experiments. (B) Wild type versus *Nlrp3*<sup>-/-</sup> neutrophils were primed with 100 ng/ml LPS for 3 h and challenged with 5 mM ATP for 1 h. Supernatants (SN) and cell extracts (XT) were harvested and examined for pro-IL-1 $\beta$  and pro-caspase-1 cleavage by western blot. (C) Bone marrow neutrophils were primed with 100 ng/ml LPS for 4 h before stimulation with nigericin (5  $\mu$ M) for 5 h. IL-18 secretion was measured by ELISA. Data are mean + SD of cell stimulations in technical triplicate from a single experiment

## Supporting Information Figure 2



### **Neutrophils internalise silica particles.**

Neutrophils were incubated with silica (15  $\mu\text{g/ml}$ ) for 2 h. Uncropped brightfield image shows refractive silica particles within neutrophils.