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Dora Hancz  
*Lund University*

*Et al.*

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# Streptolysin O Induces the Ubiquitination and Degradation of Pro-IL-1 $\beta$

Dóra Hancz<sup>a</sup> Elsa Westerlund<sup>a</sup> Christine Valfridsson<sup>a</sup>  
Getachew Melkamu Aemero<sup>a</sup> Benedicte Bastiat-Sempe<sup>b</sup> Pontus Orning<sup>c, d</sup>  
Egil Lien<sup>c, d</sup> Michael R. Wessels<sup>b</sup> Jenny J. Persson<sup>a</sup>

<sup>a</sup>Immunology Section, Department of Experimental Medical Sciences, Lund University, Lund, Sweden; <sup>b</sup>Division of Infectious Diseases, Boston Children's Hospital and Department of Pediatrics, Harvard Medical School, Boston, MA, USA; <sup>c</sup>Program in Innate Immunity, Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA; <sup>d</sup>Centre of Molecular Inflammation Research, Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway

## Keywords

Group A *Streptococcus* · Streptolysin O · Ubiquitin, IL-1 $\beta$

## Abstract

Group A *Streptococcus* (GAS) is a common and versatile human pathogen causing a variety of diseases. One of the many virulence factors of GAS is the secreted pore-forming cytotoxin streptolysin O (SLO), which has been ascribed multiple properties, including inflammasome activation leading to release of the potent inflammatory cytokine IL-1 $\beta$  from infected macrophages. IL-1 $\beta$  is synthesized as an inactive pro-form, which is activated intracellularly through proteolytic cleavage. Here, we use a macrophage infection model to show that SLO specifically induces ubiquitination and degradation of pro-IL-1 $\beta$ . Ubiquitination was dependent on SLO being released from the infecting bacterium, and pore formation by SLO was required but not sufficient for the induction of ubiquitination. Our data provide evidence for a novel SLO-mediated mechanism of immune regulation, emphasizing the importance of this pore-forming toxin in bacterial virulence and pathogenesis.

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## Introduction

A common trait among pathogenic microbes is their ability to circumvent or counteract the protective measures induced by an invaded host, often taking advantage of host factors or processes for their own benefit. One network within host cells frequently exploited or manipulated by pathogens is the ubiquitination system [1]. In this study, we demonstrate that the common human pathogen Group A *Streptococcus* (GAS or *Streptococcus pyogenes*) induces ubiquitination and degradation of the inactive pro-form of the inflammatory cytokine IL-1 $\beta$ , an event that is dependent on the bacterial virulence factor streptolysin O (SLO).

Ubiquitin is a small and a highly conserved protein that can be covalently attached to a substrate protein and thereby modify its activity, function or localization, or mark it for secretion or destruction [2]. Ubiquitination occurs in a 3-step enzymatic process and results in either the attachment of a single ubiquitin, or ubiquitin chains that may be linear or branched. Within such chains, ubiquitin proteins can be linked together via any of its 7 lysines (K6, K11, K27, K29, K33, K48, or K63) or via the N-ter-

minimal methionine (M1). The nature of the ubiquitin linkage dictates the fate of the substrate protein; K48-linked poly-ubiquitin chains generally target proteins for proteasomal degradation, whereas M1- (linear) or K63-linked chains are mostly associated with, for example, protein-protein interactions, protein trafficking, or degradation of proteins by autophagy [2, 3].

The Nlrp3 inflammasome is an intracellular protein complex that is assembled and activated in response to a wide range of stimuli, mostly pertaining to disruptions in cellular homeostasis [4]. Within an inflammasome, the cysteine protease caspase-1 may cleave the inactive proforms of the inflammatory cytokines IL-1 $\beta$  and IL-18 into their active forms, and also initiate a pro-inflammatory type of cell death known as pyroptosis through processing of the pore-forming protein gasdermin D [5]. The inflammasome plays an important role in innate immunity and is a key mediator of powerful inflammatory signals upon infection as well as sterile tissue damage. The activity and function of these complexes are tightly regulated, both at the level of generation and through post-translational modification of the pro-IL-1 $\beta$  protein *per se*. Several of these control measures are mediated by ubiquitination and deubiquitination processes [6–11].

GAS is an extracellular bacterial pathogen responsible for superficial as well as severe and sometimes life-threatening diseases, causing around 500,000 deaths annually [12]. This bacterium produces an impressive set of virulence factors, among them a variety of secreted toxins, proteases and superantigens with prominent roles in pathogenesis. The 2 pore-forming proteins SLO and streptolysin S act through different mechanisms but are both membrane-damaging toxins with the ability to lyse red blood cells [13]. Of particular interest for this study is SLO, which belongs to a family of cholesterol-dependent cytolysins that can insert into the lipid bilayer of host cell membranes and may result in pores up to 50 nm in diameter [14]. SLO has been ascribed multiple functions including the induction of oncosis or apoptosis in macrophages [15, 16], and prevention of phagolysosomal acidification and xenophagic killing in keratinocytes [17, 18]. In addition, SLO is a known activator of the inflammasome and generates release of mature IL-1 $\beta$  from macrophages [19, 20]. The role of IL-1 $\beta$  in GAS infection seems complex both in human disease and in murine experimental settings, and presumably appropriate management of the levels of this very potent proinflammatory cytokine is important to the pathogen as well as the infected host [21–25].

**Table 1.** Description and references of *streptococcal* strains used in this study

Strain name	Genotype and properties	Ref.
wt	Wild-type, GAS strain 854	[26]
$\Delta slo$	<i>slo</i> deletion mutant	[17]
<i>slo</i> (Y255A)	SLO Y255A point mutation, lack pore formation ability	[17]

Here, we describe a previously unknown capacity of GAS to induce SLO-dependent ubiquitination of pro-IL-1 $\beta$ . This ubiquitination event results in increased pro-IL-1 $\beta$  degradation, thus limiting the amount of pro-IL-1 $\beta$  available for inflammasome-mediated maturation and reducing the release of pro-inflammatory IL-1 $\beta$ .

## Materials and Methods

### Bacterial Strains and Growth Conditions

The wild type (wt) GAS strain 854 is an M type 1 strain isolated from a patient with a retroperitoneal abscess [26]. The isogenic mutant strains used in this study are listed in Table 1. Bacteria were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) in 5% CO<sub>2</sub> at 37°C. Overnight cultures were reinoculated in THY and grown to late exponential phase (optical density of 1.1–1.3 at 600 nm), washed with phosphate-buffered saline, and diluted before use.

### Mice

Genetically modified mouse strains IL-1 $\beta$ <sup>-/-</sup> [27], caspase-1/11<sup>-/-</sup> [28], *Asc*<sup>-/-</sup> [29], Nlrp3<sup>-/-</sup> [30] and receptor-interacting kinase 3 (Rip3)<sup>-/-</sup> [31] were all on a C57BL/6 (B6) background. B6 mice were bred in-house. Genetically modified mice or cells from such mice were kindly provided by Catharina Svanborg (IL-1 $\beta$ <sup>-/-</sup>), Bengt Johansson-Lindbom (caspase-1/11<sup>-/-</sup>) and Russell E. Vance (*Asc*<sup>-/-</sup>, Nlrp3<sup>-/-</sup>). Rip3<sup>-/-</sup> mice were originally generated by K. Newton and V. Dixit (Genentech), and provided to E. Lien by Drs. Kaiser, Mocarski, Dillon and Green. All animal experiments were conducted in accordance with protocols approved by the Lund/Malmö Animal Ethics Committee.

### Generation and Infection of Bone Marrow-Derived Macrophages

Bone marrow was isolated from murine femurs and tibiae, and progenitor cells were differentiated into bone marrow-derived macrophages (BMDMs) for 7 days (37°C, 5% CO<sub>2</sub>) in RPMI 1,640 (Gibco) supplemented with 10% fetal bovine serum (Sigma), 2.5 mM L-glutamine, and macrophage colony-stimulating factor. Prior to infection, 10 × 10<sup>6</sup> BMDMs were seeded on 90 mm Petri dishes (unless otherwise stated), primed with 1 μg/mL lipopolysaccharide (LPS; Sigma) for 4 h, and infected at a multiplicity of infection of 20 and incubated for 90 min (unless otherwise stated). For longer incubation times, the bacterial suspension was replaced with fresh medium containing 300 μg/mL gentamicin (Sig-

ma) to kill extracellular bacteria at 90 min post infection. When indicated, BMDMs were stimulated with recombinant SLO (rSLO; Bio-Rad) at the concentration of 10  $\mu$ g/mL in the presence or absence of  $\Delta$ slo bacteria. Cell culture medium was supplemented with 50 mM KCl (Sigma) in order to block K<sup>+</sup> efflux. Nigericin (Sigma) was used at 10  $\mu$ M concentration for 60 min. MG-132 (Sigma), 3-Methyladenine (3-MA; Sigma) or Bafilomycin A1 (Sigma) were added to the cell cultures 30 min before infection and were present throughout the whole infection at indicated concentrations.

#### *Soluble IL-1 $\beta$ Measurement*

Supernatants from infected BMDMs were cleared from debris by centrifugation (300 g, 5 min) and analyzed using IL-1 $\beta$  ELISA kits (BD Biosciences or R&D Systems) according to the manufacturer's instructions.

#### *Immunoprecipitation and Deubiquitinase Treatment*

Cells used for infection were lysed with NP-40 cell lysis buffer (150 mM sodium chloride, 1% NP-40 [Sigma], 50 mM Tris, complete protease inhibitor cocktail [Roche]). Lysates were incubated with 0.3  $\mu$ g antibody (IL-1 $\beta$ : AF-401 [R&D Systems], IL-18: 5180R [BioVision])/10<sup>6</sup> cells at 4 °C overnight and precipitated using protein G Dynabeads (Thermo Scientific) incubated at 4 °C for 1 h. When indicated, precipitated samples were digested with deubiquitinase enzymes (DUB) using the UbiCREST DUB Set (Boston Biochem) according to the manufacturer's instructions. Precipitated proteins were eluted by incubation at 70 °C for 10 min using elution buffer containing lithium dodecyl sulfate (Thermo Scientific) under reducing conditions.

#### *Protein Separation and Immunoblot Assays*

Following infection of 5  $\times$  10<sup>5</sup> BMDMs as described above, cell lysates were prepared by using NP-40 lysis buffer followed by the addition of lithium dodecyl sulfate sample buffer and sample reducing agent (Life Technologies). Whole cell lysates or precipitated samples were separated by SDS-PAGE using NuPAGE 12% bis-Tris gels (Life Technologies) under reducing conditions and transferred to hybond polyvinylidene difluoride. For the analysis presented in Figure 5, whole cell lysates were run on a 4–15% TGX Stain Free Gel (Bio-Rad), and transferred to polyvinylidene difluoride membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). This system allows UV-based detection of total protein content in each lane after transfer to the membrane. For detection, the following primary and horseradish peroxidase-conjugated secondary antibodies were used: anti-IL-1 $\beta$  (AF-401; R&D Systems), anti-IL-18 (5180R; BioVision), anti-caspase-1 p20 (AG-20B-0042; AdipoGen), anti-caspase-8 (ALX 804-448; Enzo), anti-Rip1 (clone D94C12; Cell Signaling), anti-Rip3 (2283; ProSci), anti-A20 (56309; Cell Signaling), anti-pan-ubiquitin (clone P4D4; Santa Cruz Biotechnology), anti-GAPDH (G9545; Sigma), rabbit anti-goat IgG (61-1620; Thermo Scientific or 705-035-003; Jackson Immuno Research), donkey anti-mouse IgG (715-036-151; Jackson Immuno Research), donkey anti-rat IgG (712-036-153; Jackson), and goat anti-rabbit IgG (111-035-144; Jackson). Stained membranes were incubated in Clarity Western ECL blotting substrate for chemiluminescence (Bio-Rad) and developed. Blots were documented and quantified using ChemiDoc imaging system (Bio-Rad), Quantity One and Image Lab software.

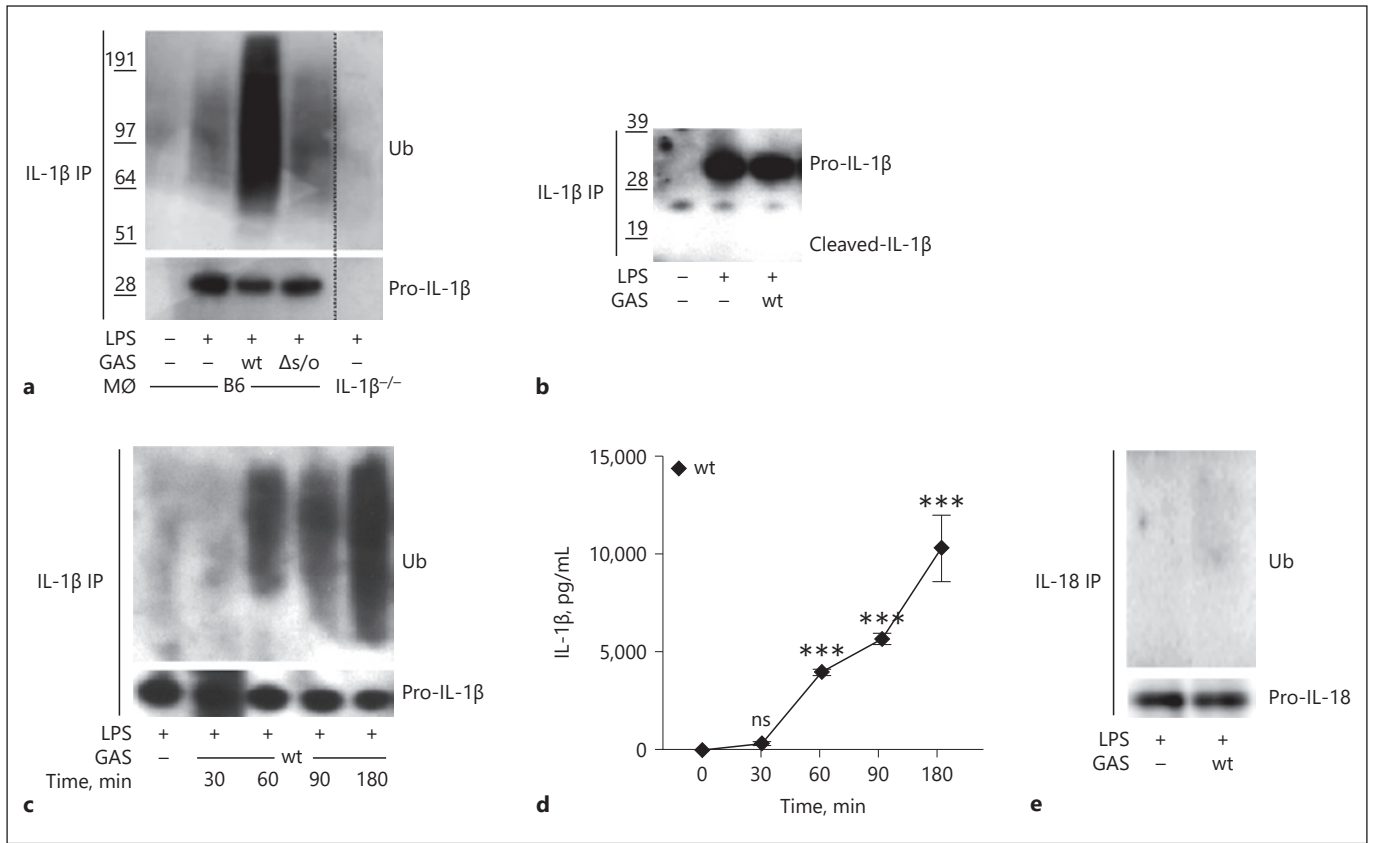
#### *Statistical Analysis*

Statistical calculations were performed using Prism version 7.0 (GraphPad software). Data were analyzed using 1-way ANOVA (3 or more groups with 1 categorical variable) or 2-way ANOVA (3 or more groups with 2 categorical variables): ns, not significant, \*\*\*  $p < 0.001$ .

## **Results**

### *GAS Expressing SLO Induces Ubiquitination of Pro-IL-1 $\beta$ in Macrophages*

Nlrp3 inflammasome activation and maturation of IL-1 $\beta$  requires 2 sequential signals [32], the first one (referred to as “priming”) mediates the upregulation of the Nlrp3 and pro-IL-1 $\beta$  proteins in addition to multiple post-translational modifications of proteins involved in the inflammasome complex. Priming is commonly a response to a microbial ligand or endogenous danger signal, and can be achieved through receptors activating the transcription factors NF- $\kappa$ B and AP-1 [32]. In vivo, priming is likely to occur through multiple receptors, in vitro however priming is often mediated by LPS through TLR4. The second signal (“activation”) leads to the assembly of the inflammasome complex, caspase-1 activation and cleavage and secretion of mature IL-1 $\beta$ , and can be mediated by a wide array of stimuli, including SLO [19, 20]. As ubiquitination has previously been reported to influence levels of secreted IL-1 $\beta$  [7, 9, 10], we set out to investigate the potential role for ubiquitination in the regulation of mature IL-1 $\beta$  release during GAS infection. We used immunoprecipitation to isolate pro-IL-1 $\beta$  from LPS-primed, GAS-infected murine BMDMs and revealed possible ubiquitination of the precipitate by western blotting. In agreement with existing data [9, 10], LPS priming induced a low level of pro-IL-1 $\beta$  ubiquitination (Fig. 1a). This post-translational modification has previously been shown to support more efficient maturation and secretion of IL-1 $\beta$  [9]. Upon infection with wt GAS, pro-IL-1 $\beta$  ubiquitination was markedly increased, while this was not observed in cells infected with an isogenic bacterial mutant lacking SLO expression ( $\Delta$ slo; Fig. 1a). As expected, no ubiquitinated protein could be detected after IL-1 $\beta$  immunoprecipitation of primed IL-1 $\beta$ <sup>-/-</sup> BMDMs (Fig. 1a), confirming the specificity of the IL-1 $\beta$  antibody. Of note, this antibody recognizes pro- and mature IL-1 $\beta$  alike, implying that both forms may be precipitated in our assays. However, cleaved IL-1 $\beta$  is rapidly released from the cell and consequently mature IL-1 $\beta$  is not readily detectable in lysates of cells where the inflammasome has been activated. Indeed, we did not



**Fig. 1.** Group A *Streptococcus* (GAS) expressing streptolysin O (SLO) induces ubiquitination of pro-IL-1 $\beta$  in macrophages. **a** B6 macrophages were primed with lipopolysaccharide (LPS) or primed and infected with GAS as indicated and cell lysates were subjected to IL-1 $\beta$  immunoprecipitation (IP) and immunoblot analysis for ubiquitin (Ub) and (pro-)IL-1 $\beta$ . Primed IL-1 $\beta$ <sup>-/-</sup> macrophages were used as a control for specificity. **b** IL-1 $\beta$  IP and immunoblot analysis of lysates from LPS-primed or primed and infected macrophages, as indicated. **c** Macrophages were primed with LPS or primed and infected with wild type (wt) GAS for the

indicated times. IL-1 $\beta$  was precipitated from cell lysates and analyzed for Ub and (pro-)IL-1 $\beta$  by immunoblot. **d** LPS-primed macrophages were infected by wt GAS and levels of released IL-1 $\beta$  were assessed by ELISA. **e** IL-18 was precipitated from cell lysates of LPS-primed or primed and infected macrophages, as indicated, followed by immunoblot analysis for Ub and (pro-)IL-18. Blots show 1 representative of 2 or 3 independent experiments. Graph shows means plus SD for triplicate samples and is representative of 2 independent experiments.

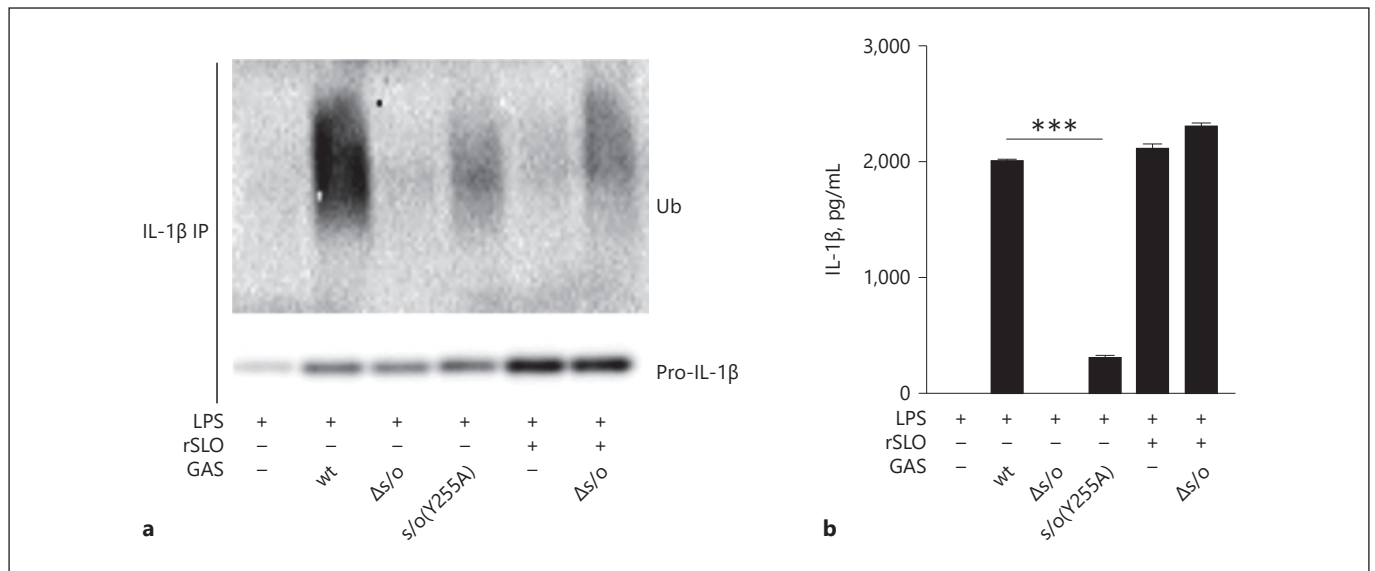
observe any cleaved IL-1 $\beta$  in the precipitated samples from cells infected with wt GAS (Fig. 1b), suggesting that the detected ubiquitination pattern mostly reveals post-transcriptional modifications of the pro-form (Fig. 1a). This does not exclude the possibility that released mature IL-1 $\beta$  may also be ubiquitinated, although ubiquitin is commonly detached from target proteins before secretion as part of the maintenance of ubiquitin homeostasis in the cell [33]. The ubiquitination of pro-IL-1 $\beta$  was visible after 60 min and increased up to 180 min of infection (Fig. 1c), demonstrating kinetics similar to that of inflammasome-dependent IL-1 $\beta$  release after GAS infection of macrophages (Fig. 1d). Nlrp3 inflammasome activation may also lead to the maturation and secretion of

IL-18 [34], the pro-form of this cytokine, however, does not seem to be ubiquitinated upon GAS infection of BM-DMs (Fig. 1e).

Taken together, these data suggest that GAS infection of macrophages rapidly induces increased ubiquitination of pro-IL-1 $\beta$ , but not pro-IL-18, and that this process is dependent on the cytolysin SLO.

#### *Pro-IL-1 $\beta$ Ubiquitination Induced by SLO Requires Pore Formation*

SLO is secreted from the bacteria as monomers, which oligomerize into a prepore on the host cell membrane. The prepore complex then inserts fully into the membrane to form a pore that can reach as much as 50 nm in



**Fig. 2.** Pro-IL-1 $\beta$  ubiquitination is dependent on bacterial secretion of and pore formation by streptolysin O (SLO). LPS-primed B6 macrophages were infected with wild type (wt),  $\Delta$ slo or slo(Y255A) Group A *Streptococcus* (GAS) or stimulated with 10  $\mu$ g/mL recombinant SLO (rSLO) for 90 min in the presence or absence of  $\Delta$ slo bacteria, as indicated. **a** IL-1 $\beta$  precipitates from cell

lysates were assayed for ubiquitin (Ub) and (pro-)IL-1 $\beta$  by immunoblot. **b** Supernatants were analyzed for IL-1 $\beta$  by ELISA. Blot shows 1 representative of at least 3 independent experiments. Graph shows means plus SD for triplicate samples and is representative of 3 independent experiments.

diameter [14, 35]. Complete pore formation is required for the induction of cell lysis and inflammasome activation [35], which seems to be the case also for similar toxins found in other bacterial pathogens, such as the *Listeria monocytogenes* listeriolysin O [36] and pneumolysin secreted by *S. pneumoniae* [37].

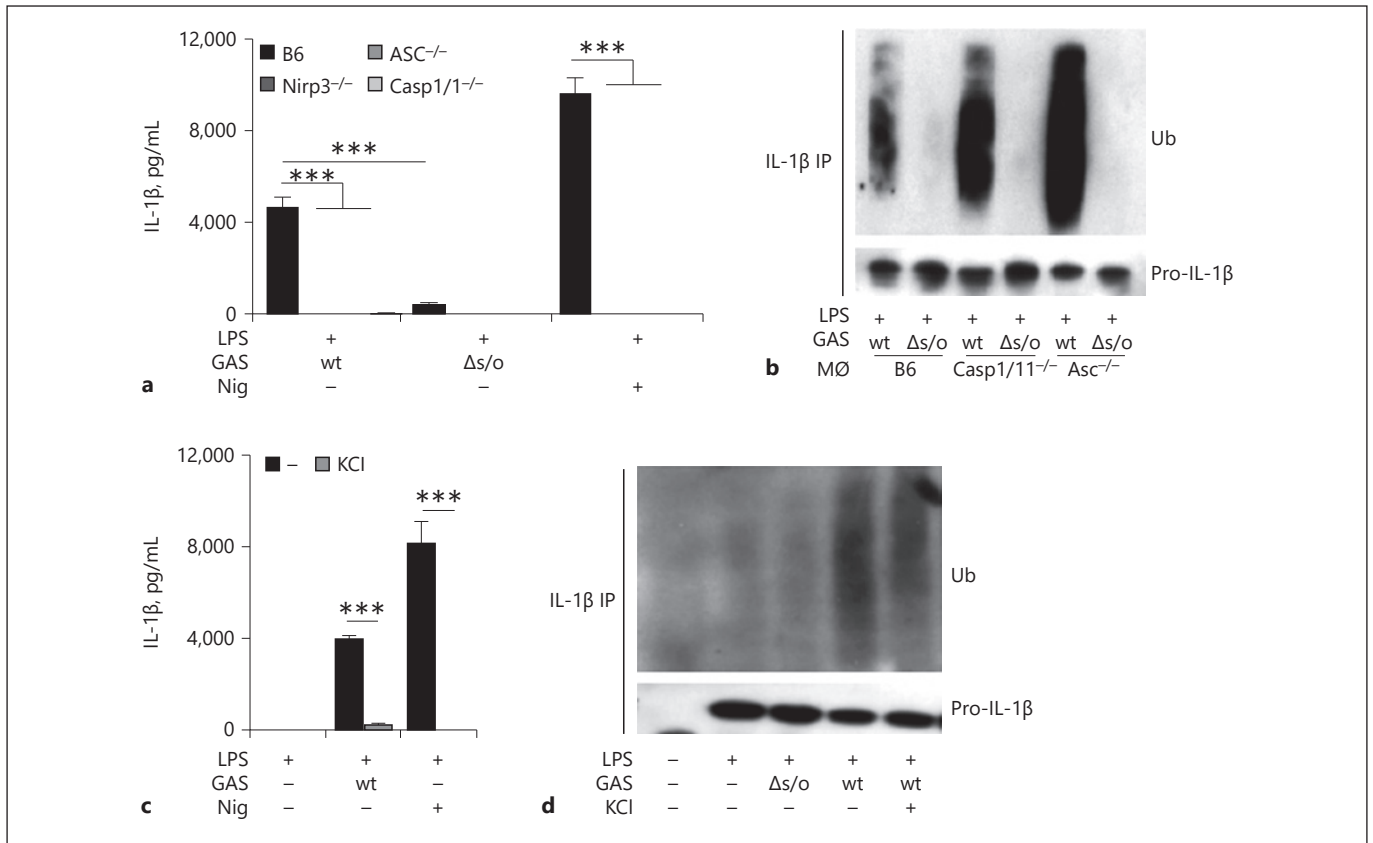
Through the introduction of point mutations into SLO, pore formation can be blocked at different steps of the oligomerization-insertion process [38]. To study the effect of the pore forming capacity of SLO on pro-IL-1 $\beta$  ubiquitination, we took advantage of an isogenic bacterial strain expressing a mutated form of SLO that is locked in the prepore stage (slo[Y255A]), thus secreting SLO monomers that oligomerize on the host cell membrane but are not able to fully insert and form pores [17]. We observed decreased pro-IL-1 $\beta$  ubiquitination (Fig. 2a), and as expected also reduced IL-1 $\beta$  release (Fig. 2b), in BMDMs infected with the mutant strain compared to wt GAS, suggesting that pore-formation is important not only for inflammasome activation by SLO but also for SLO-mediated ubiquitination of pro-IL-1 $\beta$ .

Pore-formation by SLO induces multiple processes in the affected cell [13]. To investigate whether ubiquitination of pro-IL-1 $\beta$  was a response induced primarily by the membrane damage caused by SLO, or by the strepto-

coccal infection, we treated LPS-primed BMDMs with rSLO in the absence of bacteria. While rSLO efficiently induced the release of mature IL-1 $\beta$  (Fig. 2b), it was not able to promote ubiquitination of the IL-1 $\beta$  proform (Fig. 2a). Similarly, the addition of rSLO during an infection with the  $\Delta$ slo bacterial strain did not generate pro-IL-1 $\beta$  ubiquitination (Fig. 2a) but as expected resulted in efficient IL-1 $\beta$  release (Fig. 2b). These observations allow us to conclude that SLO needs to be expressed and secreted from the bacterium during infection in order to support pro-IL-1 $\beta$  ubiquitination and that pore-formation by SLO is required, but not sufficient, for the ubiquitination process. Further, in contrast to the baseline ubiquitination of pro-IL-1 $\beta$  that is induced by LPS priming [9], the additional ubiquitination of pro-IL-1 $\beta$  induced by SLO-proficient streptococcal infection does not seem to be required to facilitate subsequent release of mature IL-1 $\beta$ .

#### *Streptococcus-Induced Ubiquitination of Pro-IL-1 $\beta$ Occurs Independently of SLO-Mediated Inflammasome Signaling*

Inflammasome activation and IL-1 $\beta$  release induced by GAS is known to be mostly due to pore-formation by SLO and to be dependent on the inflammasome sensor



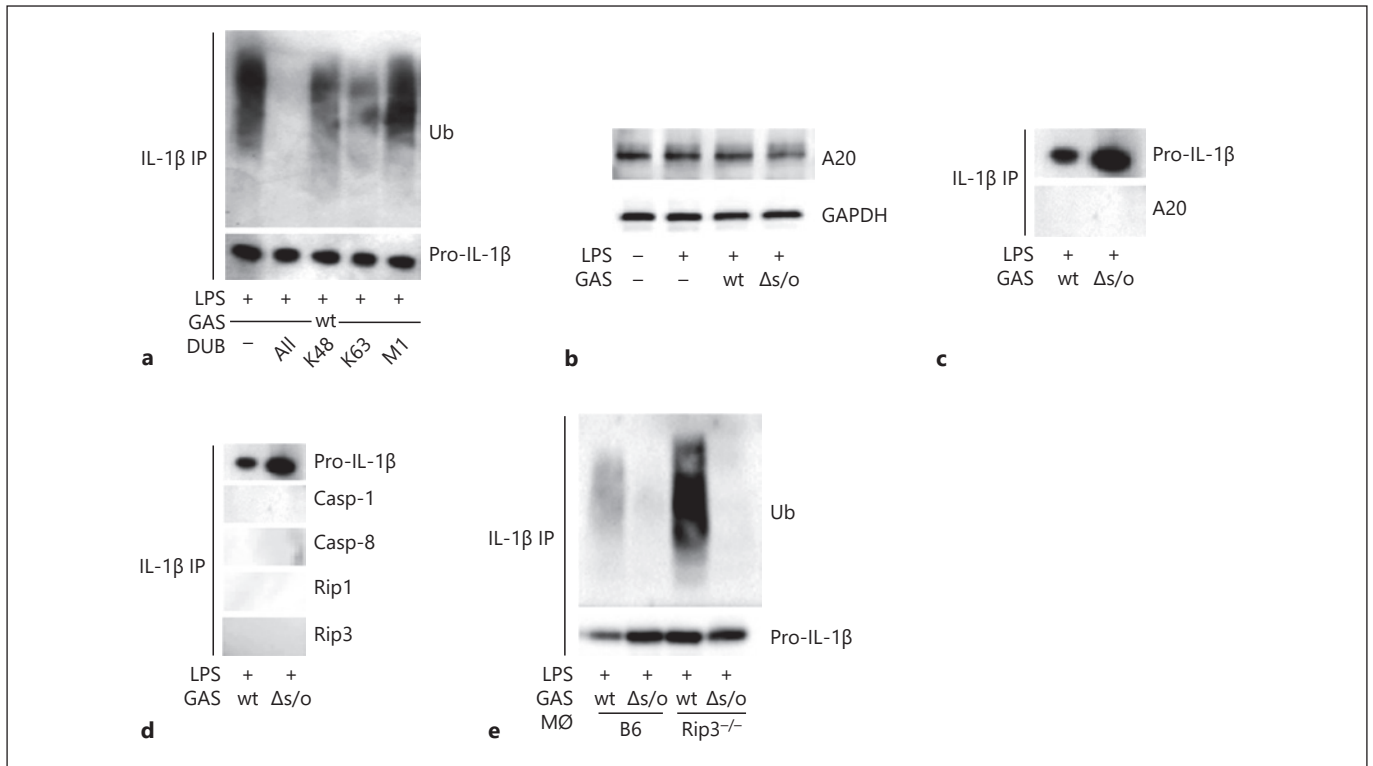
**Fig. 3.** *Streptococcus*-induced ubiquitination of pro-IL-1 $\beta$  occurs independently of SLO-mediated inflammasome signaling. **a** Primed macrophages of different genotypes were infected with wild type (wt) or  $\Delta$ slo Group A *Streptococcus* (GAS), as indicated. The Nlrp3 inflammasome-activating toxin Nigericin (Nig) was used as a control. Supernatants were assessed for IL-1 $\beta$  by ELISA. **b** Different genotype macrophages were primed and infected with wt or  $\Delta$ slo GAS. IL-1 $\beta$  was precipitated from cell lysates and ubiquitin (Ub) and (pro-)IL-1 $\beta$  were assessed by immunoblot. **c** B6

macrophages were primed or primed and infected or treated with Nig, as indicated, in the presence or absence of KCl and IL-1 $\beta$  in the supernatant was assessed by ELISA. **d** B6 macrophages were primed or primed and infected, as indicated, in the presence or absence of KCl and IL-1 $\beta$  was precipitated from cell lysates and ubiquitin (Ub) and (pro-)IL-1 $\beta$  were assessed by immunoblot. Graphs show means plus SD for triplicate samples and are representatives of at least 3 independent experiments. Blots show 1 representative of 3 independent experiments.

protein Nlrp3, the linker protein ASC and the cysteine protease caspase-1 [19, 20]. Because we found a requirement for SLO in pro-IL-1 $\beta$  ubiquitination, we hypothesized that ubiquitination may occur as a response to SLO-mediated inflammasome activation. Thus, we infected B6 (wt) BMDMs or BMDMs deficient for the components required for inflammasome activation by GAS (Nlrp3, ASC and caspase-1, knock-outs on B6 background) and analyzed them for IL-1 $\beta$  release and pro-IL-1 $\beta$  ubiquitination. As expected, Nlrp3, ASC, and caspase-1 were all required for release of mature IL-1 $\beta$  upon GAS infection (Fig. 3a). However, pro-IL-1 $\beta$  was ubiquitinated also in their absence (Fig. 3b), suggesting that this ubiquitination event is not a process mediated by inflammasome activation per se. Although the amounts of pro-IL-1 $\beta$  were sim-

ilar in cell lysates of different genotype BMDMs, the ubiquitination pattern appeared stronger in caspase-1-, and particularly ASC-deficient as compared to B6 macrophages (Fig. 3b), suggesting that these well-known inflammasome components may affect the regulation of the ubiquitination process.

The Nlrp3 inflammasome can be activated by a number of bacterial toxins [39], and although the actual molecular mechanisms governing its activation are still largely unknown, in most cases, it can be blocked by the inhibition of K<sup>+</sup> efflux from the cell, suggesting the disruption of intracellular ion homeostasis may be an activating trigger [4]. To explore whether the ion flux conduits affecting inflammasome activation may be involved in pro-IL-1 $\beta$  ubiquitination, BMDMs supplemented with



**Fig. 4.** Pro-IL-1 $\beta$  ubiquitination after Group A *Streptococcus* (GAS) infection is of heterogeneous linkage specificity. **a** lipopolysaccharide (LPS)-primed macrophages were infected with wild type (wt) GAS followed by precipitation of IL-1 $\beta$ . Precipitates were subjected to deubiquitinase (DUB) proteolysis removing ubiquitin (Ub) of all linkages (“All”) or specifically K48, K63 or M1 linkages, as indicated, and ubiquitin and (pro-)IL-1 $\beta$  were assessed by immunoblot. **b** Immunoblot for A20 in lysates of untreated, primed or primed, and infected (wt or  $\Delta$ s/o GAS) macrophages, as indi-

cated. **c** Immunoblot for A20 in IL-1 $\beta$  precipitates from macrophages primed and infected with wt or  $\Delta$ s/o GAS. **d** IL-1 $\beta$  was precipitated from whole cell lysates from macrophages after priming and infection with wt or  $\Delta$ s/o GAS and analyzed for co-precipitation of the indicated proteins. **e** B6 and receptor-interacting kinase 3 (Rip3)<sup>-/-</sup> macrophages were primed and infected with wt or  $\Delta$ s/o GAS. Precipitates of IL-1 $\beta$  from cell lysates were assayed for ubiquitin (Ub) and (pro-)IL-1 $\beta$ . Blots show 1 representative of 2 or 3 independent experiments.

extracellular K<sup>+</sup> to block efflux were infected and analyzed as described above. While K<sup>+</sup> efflux was essential for SLO-mediated inflammasome activation and release of mature IL-1 $\beta$  (Fig. 3c), it was dispensable for ubiquitination of the pro-form (Fig. 3d), which is in line with our data suggesting that Nlrp3 inflammasome activation is not required for pro-IL-1 $\beta$  ubiquitination.

#### *Pro-IL-1 $\beta$ Ubiquitination after Streptococcal Infection Is of Heterogeneous Linkage Specificity*

Ubiquitin monomers can be linked together through any of their 7 internal lysines or their N-terminal methionine residues, and the nature of this linkage decides the fate of the ubiquitinated target [2]. To investigate which mode of ubiquitination is present on pro-IL-1 $\beta$  after GAS infection, we took advantage of the so-called DUBs. The DUBs are a family of enzymes that can spe-

cifically cleave 1 or more type of intra-ubiquitin bonds, for example, all linkages or K48 or K63 linkages only [2]. Thus, we pulled down pro-IL-1 $\beta$  from BMDMs infected with wt GAS and treated the precipitate with different DUB enzymes. While the non-specific linkage enzyme USP2 as expected detached all ubiquitin moieties on pro-IL-1 $\beta$ , the K48- and K63-specific DUBs, OTUB1, and AMSH, both only partially reduced associated ubiquitin signals, suggesting the presence of both types of linkages. In contrast, the M1 linkage-specific enzyme Otulin did not appear to change the level of ubiquitination, implying ubiquitin is not attached to pro-IL-1 $\beta$  by M1 linkage (Fig. 4a).

The enzyme A20 was first described as an inhibitor of the NF- $\kappa$ B activation and has as such a prominent role in immune homeostasis [40]. A20 has both E3 ligase and deubiquitinase activity and has subsequently been impli-



cated in multiple immune signaling pathways, for example, regulating ubiquitination of pro-IL-1 $\beta$ . More specifically, A20 seems to restrict the ubiquitination of pro-IL-1 $\beta$  through a mechanism dependent on Rip3, thereby limiting secretion of mature IL-1 $\beta$  [9]. Therefore, we investigated the possibility that GAS infection might reduce A20 protein levels, which in turn could allow for increased pro-IL-1 $\beta$  ubiquitination. However, LPS-primed, wt- or  $\Delta slo$ -infected BMDMs all expressed comparable levels of A20, excluding this possibility (Fig. 4b). We further found, contrary to previous observations [9], that pro-IL-1 $\beta$  did not co-precipitate with A20, caspases or Rip kinases (Fig. 4c, d). In addition, our data indicate that pro-IL-1 $\beta$  ubiquitination was not abolished, but rather increased, by the absence of Rip3 (Fig. 4e). These findings agree with the observation that GAS-induced ubiquitination, contrary to the LPS-mediated, A20-restricted ubiquitination of pro-IL-1 $\beta$ , does not seem to be required for the maturation and secretion of this cytokine, and suggests that the infection-driven mechanism governing the ubiquitination of pro-IL-1 $\beta$  described here differs from mechanisms previously reported.

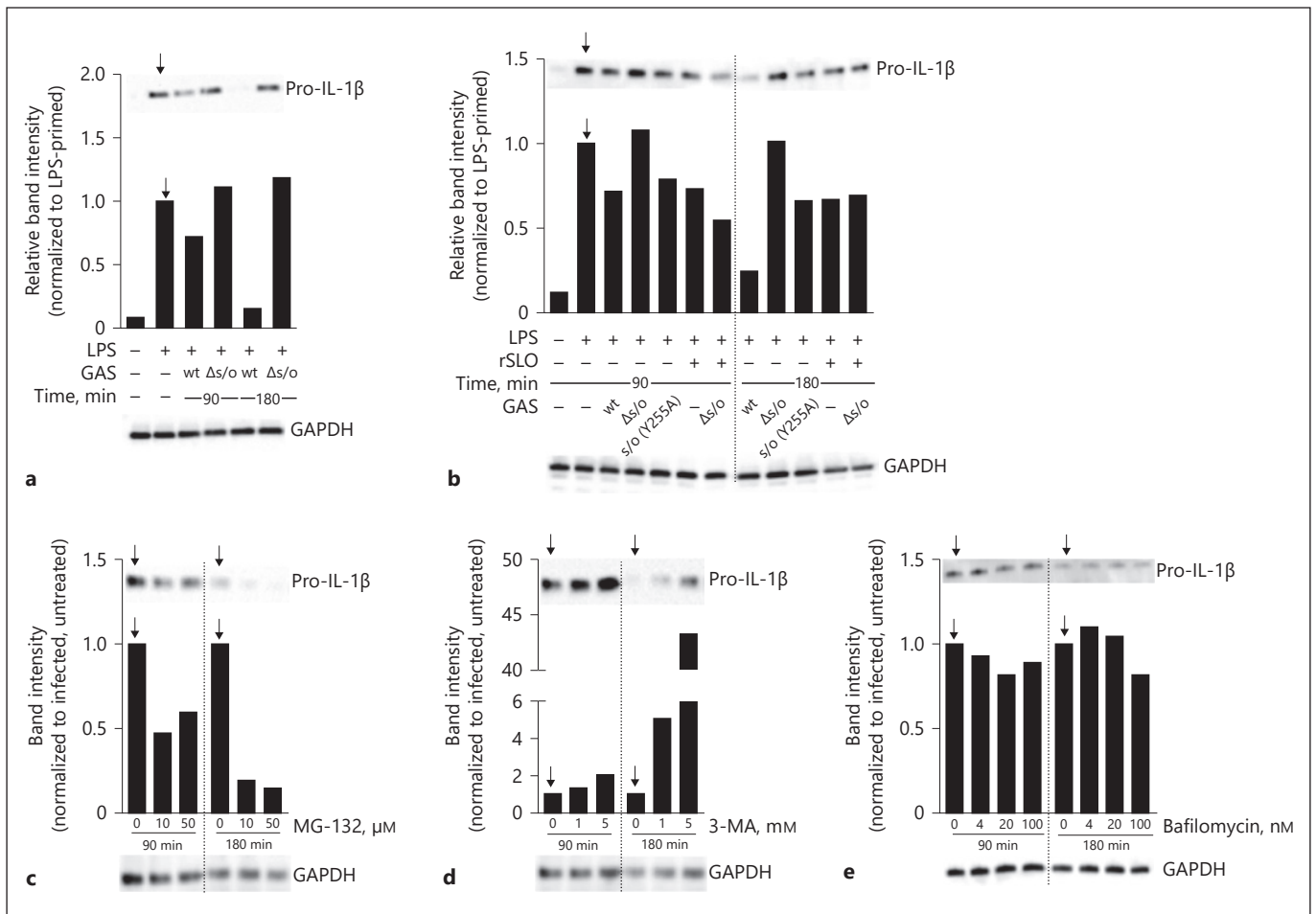
#### *Pro-IL-1 $\beta$ is Degraded upon Infection with SLO-Proficient GAS*

Previous studies have proposed that ubiquitinated pro-IL-1 $\beta$  protein may be degraded in the proteasome [7, 10] or by autophagy [41]. Thus, we set out to investigate whether GAS infection induces pro-IL-1 $\beta$  degradation, a possible outcome downstream of ubiquitination. In order to avoid the inflammasome-dependent processing of pro-IL-1 $\beta$ , we infected BMDMs lacking the integral inflammasome component ASC and observed a decrease in pro-IL-1 $\beta$  levels upon infection with wt bacteria as compared to the strain lacking SLO, or uninfected cells, and this decrease became more pronounced with time (Fig. 5a). In contrast, in macrophages that were infected with the SLO prepore-locked mutant *slo* (Y225A), treated with rSLO or infected with  $\Delta slo$  bacteria in the presence of rSLO (all stimuli that induce reduced or no ubiquitination of pro-IL-1 $\beta$  [Fig. 2a]) degradation was significantly reduced (Fig. 5b). As this result supports the hypothesis that SLO-dependent pro-IL-1 $\beta$  ubiquitination increases protein degradation, we carried out infections in the presence of well-established inhibitors of the proteasome or autophagy, expecting that inhibition of the responsible degradative pathway would hamper the reduction of pro-IL-1 $\beta$ . The proteasome inhibitor MG-132 was not able to inhibit, but rather increased, the degradation of pro-IL-1 $\beta$  (Fig. 5c). However, 3-MA, which blocks autophagy by

inhibiting phosphatidylinositol 3 kinases, did reduce pro-IL-1 $\beta$  degradation in wt-infected cells (Fig. 5d), suggesting that the ubiquitination induced by SLO-proficient GAS infection may promote increased autophagy-mediated degradation of pro-IL-1 $\beta$ . Because 3-MA is a broad inhibitor potentially disturbing multiple cellular processes, we performed an additional set of experiments using the V-ATPase pump inhibitor Bafilomycin, which prevents the acidification of lysosomes leading to a blockage in autophagosome-lysosome fusion and inhibition of autophagic flux. Surprisingly, under the conditions tested, Bafilomycin had minimal or no effect on the process (Fig. 5e), suggesting either that autophagy may not be responsible for the breakdown of pro-IL-1 $\beta$  and that 3-MA is blocking another degradative process, or that the inhibitors are differentially able to inhibit autophagic processes under the conditions tested. Neither of the inhibitors induced cell death as determined by LDH release (data not shown).

## Discussion

During the mid-80s, invasive and life-threatening GAS disease increased significantly and it was found that these infections were mainly caused by a single clone of serotype M1 that was rapidly disseminated worldwide [42]. More recently, a similar propagation of an M89 clone has been observed [43]. Interestingly, both these clonal expansions were preceded by the acquisition of a genetic region that in these strains conferred increased expression of the pore-forming protein SLO and its co-toxin NADase [43–45], indicating that SLO and NADase make important contributions to virulence. In the current report, we describe a property of SLO that is likely to play an important role in virulence; the ability of SLO to drive ubiquitination and degradation of pro-IL-1 $\beta$  in infected macrophages, thus limiting the pool of pro-IL-1 $\beta$  available for inflammasome-mediated maturation and release. This previously unknown function for SLO may limit the innate immune response evoked by the infection and may at least partly explain why increased SLO expression levels correlate with enhanced fitness. It is interesting to note that while our data indicate that the ubiquitination triggered by SLO limits the release of processed IL-1 $\beta$ , SLO also induces secretion of mature IL-1 $\beta$  from macrophages [19, 20, 35]. This may reflect the activation and simultaneous control of IL-1 $\beta$  release, resulting in optimal circumstances for successful infection [22].



**Fig. 5.** Pro-IL-1 $\beta$  is degraded upon streptolysin O (SLO) proficient Group A *Streptococcus* (GAS) infection. **a** Macrophages deficient for the inflammasome linker protein ASC were left untreated, primed or primed, and infected with wild type (wt) or  $\Delta$ slo Group A *Streptococcus* (GAS) for the designated time points, as indicated, and (pro-)IL-1 $\beta$  levels in whole cell lysates were evaluated by immunoblot. **b** Macrophages deficient for ASC were left untreated, primed or primed, and infected with wt,  $\Delta$ slo, or slo(Y255A) GAS in the presence or absence of 10  $\mu$ g/mL recombinant SLO (rSLO) for the designated time points, as indicated, and pro-IL-1 $\beta$  levels in whole cell lysates were evaluated by im-

munoblot. Lipopolysaccharide (LPS)-primed ASC deficient macrophages were infected with wt GAS for the indicated times in the presence of specific inhibitors as follows; **c** MG-132 (proteasome), **d** 3-MA (autophagy, PI3K inhibitor) or **e** Bafilomycin (autophagy, V-ATPase inhibitor). Band intensities were quantified based on whole protein content of cell lysates loaded onto gels followed by normalization to infected cells (without inhibitor) at each time point (indicated by arrows). The housekeeping protein GAPDH was used as a visual loading control but not as a basis for quantification. Figure shows representative blots of at least 3 independent experiments.

We found that SLO, pure or added to an infection with SLO-deficient bacteria, did not increase the level of pro-IL-1 $\beta$  ubiquitination, suggesting that SLO has to be secreted from the infecting bacterium and that pore-formation as such is not sufficient, although it is required, for the ubiquitination of pro-IL-1 $\beta$ . It is interesting to note the resemblance between this observation and the ones that have previously been made for SLO-mediated translocation of NADase, that is, both toxins have to be expressed and secreted by the same bacteri-

um for translocation to occur, and translocation cannot be rescued in trans by co-infection with 2 bacterial mutants deficient in either toxin [46]. In contrast to the pro-IL-1 $\beta$  ubiquitination observed here however, translocation is independent of SLO pore formation [38]. Clearly, the potential involvement of NADase in pro-IL-1 $\beta$  ubiquitination is compelling and requires investigation. However, this problem is complicated by the fact that SLO functionality is reduced in the absence of NADase and by the finding that the SLO:NADase in-

teraction is partly dependent on a NADase protein domain that is removed in translocation mutants [47, 48]. In addition, although NADase is currently the only known substrate for SLO-mediated translocation, it remains possible that there are other, yet unknown substrates translocated that are involved in the ubiquitination process.

Our analysis of GAS-infected caspase-1- or ASC-deficient macrophages show that pro-IL-1 $\beta$  ubiquitination proceeds independently of these inflammasome components, and thus does not require inflammasome formation and activation. Instead, the knockout BMDMs, in particular the ASC-deficient cells, exhibited significantly increased levels of ubiquitinated pro-IL-1 $\beta$ . This observation could be explained by an increase in the amount of ubiquitin conjugated to each molecule of pro-IL-1 $\beta$  and/or an increased ratio of ubiquitinated to non-ubiquitinated pro-IL-1 $\beta$  in these cells. These options require further investigation, and presently we can only speculate that our data may propose a role for caspase-1 and especially ASC in the regulation of the ubiquitination-degradation process. Interestingly, there are several reports describing novel roles for the components of the Nlrp3 inflammasome, for example, in transcription or regulation of phagocytosis and metabolic pathways [49–51], providing precedence for non-inflammasome-related functions of these proteins.

Ubiquitination of target proteins was initially believed to result exclusively in cellular signaling or proteasomal degradation as a result of K63 or K48 linkages respectively. However, as the field expanded it became obvious that the ubiquitin system is complex and dynamic. For example, it has been shown that non-degradative ubiquitin signals can be modified by the addition of further ubiquitin chains leading to the degradation of the target protein [52], and it is possible that our observation described here relates to this finding. Indeed, we found that LPS induced a basal level of ubiquitination of pro-IL-1 $\beta$ , which has previously been described to facilitate cytokine maturation and secretion, and that subsequent infection with SLO-proficient streptococci significantly increases pro-IL-1 $\beta$  ubiquitination. The ubiquitin chain(s) linked to pro-IL-1 $\beta$  as a result of GAS infection contains a heterogeneous mixture of K48 and K63 linkages, and although our current analyses do not allow us to draw conclusions about relative levels of these 2, or presence of other lysine linkage types, our data indicate that the final ubiquitin composition is a signature for cytokine degradation rather than maturation and secretion. Indeed, rSLO efficiently activates the inflammasome and generates the release

of mature IL-1 $\beta$ , but does not induce increased ubiquitination of the pro-form, suggesting that the GAS-mediated ubiquitination of the cytokine is not essential for its secretion.

We find that infection with GAS expressing SLO leads to the degradation of pro-IL-1 $\beta$ , which can be blocked using the autophagy inhibitor 3-MA. Of note, 3-MA is a broad phosphatidylinositol 3 kinases inhibitor known to affect multiple cellular processes. As Bafilomycin, which is also commonly used to block autophagy, does not prevent the decay of pro-IL-1 $\beta$ , we believe it is still an open question whether the degradation truly ensues through autophagy or by another yet to be determined pathway. Interestingly, the inhibition of the proteasome during infection increased the degradation of pro-IL-1 $\beta$ , which may imply that the proteasome normally degrades a yet unknown regulating factor of pro-IL-1 $\beta$  stability or turnover.

It has previously been reported that several pathogens exploit the ubiquitination to interfere with host immune responses. For example, human papilloma virus induces ubiquitination and proteasome-dependent degradation of pro-IL-1 $\beta$  [53], enteropathogenic *E. coli* downregulates the inflammasome activity by preventing Nlrp3 deubiquitination [54], and *Shigella* translocates a bacterial E3 ubiquitin ligase into the host cell and promotes the dissemination of the pathogen by inducing cell death [55]. Our results identify another mechanism by which pathogenic bacteria may exploit ubiquitin pathways of the host in order to dampen inflammation. The nature of the E3 ligase mediating this ubiquitination event and whether it is derived from the host or the bacterium remain to be explored. Based on our observations we propose that GAS-induced pro-IL-1 $\beta$  ubiquitination and degradation may represent a previously unrecognized immune evasion strategy, designed to directly dampen the innate immune response evoked by the invading bacterium.

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## Statement of Ethics

Animals were housed at the BMC facility at Lund University, and all experiments were performed in accordance with national regulations and the approval of the Lund/Malmö local Ethics Committee.

## Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

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## Authors Contribution

D.H., E.W., and J.J.P. conceived and designed the experiments and analyzed the data; D.H., E.W., C.V., and G.M.A. performed the experiments; B.B.-S., P.O., E.L., and M.R.W. generated and contributed essential materials; D.H. and J.J.P. wrote the paper; all authors critically reviewed the study.

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