Group A streptococcal M protein activates the NLRP3 inflammasome

J. Andrés Valderrama1,2, Angelica M. Riestra1, Nina J. Gao1, Christopher N. LaRock1, Naveen Gupta3, Syed Raza Ali1, Hal M. Hoffman1,3, Partho Ghosh2* and Victor Nizet2,4*

Group A Streptococcus (GAS) is among the top ten causes of infection-related mortality in humans. M protein is the most abundant GAS surface protein, and M1 serotype GAS strains are associated with invasive infections, including necrotizing fasciitis and toxic shock syndrome. Here, we report that released, soluble M1 protein triggers programmed cell death in macrophages (Mφ). M1 served as a second signal for caspase-1-dependent NLRP3 inflammasome activation, inducing maturation and release of proinflammatory cytokine interleukin-1β (IL-1β) and macrophage pyroptosis. The structurally dynamic B-repeat domain of M1 was critical for inflammasome activation, which involved K+ efflux and M1 protein internalization by clathrin-mediated endocytosis. Mouse intraperitoneal challenge showed that soluble M1 was sufficient and specific for IL-1β activation, which may represent an early warning to activate host immunity against the pathogen. Conversely, in systemic infection, hyperinflammation associated with M1-mediated pyroptosis and IL-1β release could aggravate tissue injury.

**Group A Streptococcus (GAS) is among the top ten causes of infection-related mortality in humans. M protein is the most abundant GAS surface protein, and M1 serotype GAS strains are associated with invasive infections, including necrotizing fasciitis and toxic shock syndrome.** Here, we report that released, soluble M1 protein triggers programmed cell death in macrophages (Mφ). M1 served as a second signal for caspase-1-dependent NLRP3 inflammasome activation, inducing maturation and release of proinflammatory cytokine interleukin-1β (IL-1β) and macrophage pyroptosis. The structurally dynamic B-repeat domain of M1 was critical for inflammasome activation, which involved K+ efflux and M1 protein internalization by clathrin-mediated endocytosis. Mouse intraperitoneal challenge showed that soluble M1 was sufficient and specific for IL-1β activation, which may represent an early warning to activate host immunity against the pathogen. Conversely, in systemic infection, hyperinflammation associated with M1-mediated pyroptosis and IL-1β release could aggravate tissue injury.
Results

GAS M1 protein triggers macrophage cell death. While probing the effects of soluble recombinant M1 protein on host innate immune and inflammatory responses, we surprisingly observed that M1 induced a rapid (within 2 h) and dose-dependent loss of plasma membrane integrity in cultured human THP-1 macrophage-like cells (THP-1 Mφ), as monitored by intracellular lactate dehydrogenase (LDH) release (Fig. 1a). This cytolytic phenotype was independent of the previously described M1–fibrinogen interaction (Supplementary Fig. 1a). Compared to M1, additional recombinant M protein types (M2, M4, M5, M6) induced similar levels of THP-1 Mφ cytolyis, whereas other M types elicited significantly lower (M22) or markedly lower (M28) cytotoxicity (Fig. 1b).

Additionally, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) showed dose-dependent M1-induced DNA fragmentation in THP-1 Mφ (Fig. 1c). Propidium iodide (PI) uptake, assessed by fluorescence microscopy and quantified by flow cytometry, confirmed significant membrane damage in M1-treated THP-1 Mφ versus untreated cells (Fig. 1c–e).

Significantly lower (M22) or markedly lower (M28) cytotoxicity (Fig. 1b). Therefore, M1 does not cause nonspecific cell membrane permeabilization, but somehow provokes macrophages to undergo a form of programmed cell death.

M1 protein promotes IL-1β signalling in macrophages. GAS can trigger cell death in macrophages by pyroptosis, but no role for M1 protein in this process has been reported. Pyroptosis is accompanied by the release of characteristic inflammatory cytokines, most prominently IL-1β. Soluble M1 was sufficient to induce dose-dependent release of IL-1β from THP-1 Mφ, as detected by enzyme-linked immunosorbent assay (ELISA; Fig. 2a). ELISA could not discriminate between pro-IL-1β and mature IL-1β, so we assessed signalling activity using HEK-Blue IL-1β reporter cells (Fig. 2b), confirming dose-dependent release of mature IL-1β upon M1 treatment. M2, M4, M5 and M6 proteins triggered a similar release of functional IL-1β from THP-1 Mφ, while decreased IL-1β activity was seen with M22 or M28 exposure (Fig. 2c), paralleling cytolytic effects.

M1 protein did not stimulate THP-1 Mφ to release IL-6, a cytokine activated in response to toll-like receptor (TLR) signalling but unrelated to the pyroptosis machinery (Fig. 2d). In contrast, classical activators of TLR signalling (for example, lipopolysaccharide, peptidoglycan, lipoteichoic acid) triggered IL-6 release (Fig. 2d) under assay conditions yielding similar macrophage viabilities (Supplementary Fig. 2). Thus, soluble M1 protein was sufficient to trigger macrophage cell death by pyroptosis, a form of programmed cell death.

Fig. 1 | GAS M1 protein promotes cell death in macrophages. a, Percentage of LDH released from THP-1 human macrophages (THP-1 Mφ) after 2 h treatment with increasing concentrations of recombinant purified M1 protein. b, Percentage of LDH released from THP-1 Mφ incubated for 2 h with M1 protein (2 μM) or other M proteins (2 μM). c, Fluorescence microscopy of propidium iodide (PI, red) uptake by THP-1 Mφ that were untreated (−M1, top) or treated for 2 h with 2 μM M1 protein (+M1, bottom). Fluorescence images are shown on the left and merged fluorescence and phase contrast images on the right. Scale bars, 30 μm. d, e, Flow cytometry analysis of PI uptake by THP-1 Mφ that were untreated (−M1) or treated for 2 h with 2 μM M1 protein (+M1). A representative flow cytometry histogram of PI fluorescence is shown in d and quantification of PI uptake as measured by percentage of cells with PI uptake (%) in e. f, g, Fluorescence-activated cell sorting (FACS) analysis of a TUNEL assay of THP-1 Mφ treated with M1 (0.2 or 2 μM) or untreated (O) for 4 h: a representative flow cytometry histogram of DNA fragmentation (f) and the percentage of cells with DNA fragmentation (%) compared with untreated cells (O) (g). h, LDH release from THP-1 macrophages (THP-1 Mφ), THP-1 monocytes (THP-1 Mo), A549 human lung epithelial cells (A549), primary human neutrophils (PMNs) and primary human peripheral blood mononuclear cells (PBMCs) treated for 2 h with M1 (2 μM). Data in a–g are plotted as the mean ± s.e.m. and represent three independent experiments performed in triplicate and analysed by Student’s t-test. NS, not significant (P > 0.05); ***P < 0.001. Panels d, e, f, g, h show representative images and flow cytometry histograms of three independent experiments.
specifically induce IL-1β signalling, with pyroptosis the likely cell death pathway concurrently activated in macrophages.

**M1 activates the NLRP3 inflammasome and pyroptosis in a caspase-1-dependent manner.** The synthesis, processing and release of mature IL-1β by macrophages depend on two independent signals. An inflammatory signal is provided by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) recognized by specific receptors, including TLRs. These first signals, known as priming signals, induce transcription and synthesis of pro-IL-1β and initiate expression of inflammasome components. A second signal is subsequently required for inflammasome assembly and activation, resulting in maximal generation of mature IL-1β. In our experiments with cultured THP-1 Mφ, phorbol 12-myristate 13-acetate (PMA) was used to differentiate cells into the macrophage phenotype. PMA provided signal 1, as IL-1β transcript increased over 1,000-fold compared to undifferentiated THP-1 Mφ (Supplementary Fig. 3), and we hypothesized that M1 was a second signal required for efficient inflammasome activation. Murine bone marrow-derived macrophages (BMDMs) primed with a variety of TLR stimuli, but not unprimed BMDMs, responded to M1 protein by releasing IL-1β (Fig. 2e). In contrast, signal 1 alone was sufficient to trigger IL-1β in human primary PBMCs, and addition of M1 did not further boost release of the cytokine (Fig. 2f). This observation correlates with recent studies that demonstrate human monocytes engage an alternative inflammasome pathway, wherein TLR agonists by themselves trigger IL-1β in a process independent of K+ efflux and pyroptosis.

M1 protein strongly induced IL-1β release from primed wild-type (WT) BMDMs but not primed casp1Δ/11/Δ MDMs, which cannot form functional inflammasomes (Fig. 3a). Nigericin, an ionophore and established canonical NLRP3 inflammasome activator, served as a positive control in this experiment. Pretreatment of THP-1 Mφ with Ac-YVAD-cmk, a specific and irreversible pharmacological inhibitor of caspase-1, nearly abolished M1-mediated release of IL-1β (Fig. 3b) and macrophage cytolysis (Fig. 3c). Thus, M1 protein-triggered IL-1β release and pyroptosis are caspase-1-dependent.

We found that BMDMs from NLRP3-deficient mice failed to secrete significant IL-1β after M1 stimulation (Fig. 3d). As expected, NLRP3−/− cells stimulated with nigericin also produced minimal IL-1β (Fig. 3d). CRID3, a powerful and specific NLRP3 inhibitor, also blocked macrophage release of IL-1β (Fig. 3c) and pyroptosis (Fig. 3f) in response to M1 protein. A drop in cytosolic K+ serves as a common step required to activate the NLRP3 inflammasome. M1-mediated macrophage IL-1β production was markedly reduced when the gradient for K+ efflux was eliminated using K+-rich media (Fig. 3g). This reduction resembled that observed with nigericin (Fig. 3g), whose NLRP3 activation is also mechanistically dependent on K+ efflux. M1 thus activates the NLRP3 inflammasome in a scenario involving K+ efflux, activation of caspase-1, processing of pro-IL-1β and secretion of mature IL-1β, all accompanied by macrophage pyroptosis.

**The B-repeat domain of M1 is critical for NLRP3 inflammasome activation.** To map the domain(s) of M1 protein required for NLRP3 inflammasome activation, we expressed and purified a set of previously described M1 truncations as, depicted schematically in Fig. 3h. Quantifying both IL-1β production and cytotoxicity (LDH release) from THP-1 Mφ, we found the N-terminal AB

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**Fig. 2 | M1 protein provides a second signal that specifically triggers IL-1β signalling.** a, b, Total IL-1β (a) and the processed form of IL-1β (b) produced by THP-1 Mφ incubated for 2 h with increasing concentrations of M1 protein, as measured by ELISA or by HEK-Blue IL-1R reporter cells, respectively. r.u., relative units. c, Comparison of total IL-1β measured by ELISA from THP-1 Mφ incubated for 2 h with M1 (2 μM) or other M proteins (2 μM). d, IL-6 production assessed by ELISA from THP-1 Mφ that were unstimulated (Uns) or stimulated for 15 h with M1 (0.02 μM), lipopolysaccharide (LPS, 100 ng ml−1), peptidoglycan (PGN, 1 μg ml−1), or lipoteichoic acid (LTA, 1 μg ml−1). e, f, IL-1β release measured by ELISA from mouse BMDMs (e) or human PBMCs (f) that were unprimed (Uns) or primed for 16 h with different TLR agonists: LPS (10 ng ml−1), PGN (1 μg ml−1), LTA (1 μg ml−1), R848 (1 μg ml−1) or Pam3CSK4 (10 ng ml−1). BMDMs and PBMCs were then left untreated (−M1) or treated with 2 μM of M1 protein (+M1) for 2 h. Data are plotted as mean ± s.e.m. and represent three independent experiments performed in triplicate and analysed by Student’s t-test. NS, not significant (P > 0.05); *P < 0.05, **P < 0.01, ***P < 0.001.
fragment of M1 protein induced similar levels of IL-1β (Fig. 3i) and LDH release as intact M1 protein (Fig. 3j). In contrast, the C-terminal SCD fragment (truncated M1 protein containing S, C and D regions) did not induce inflammasome activation and pyroptosis. Deletion of the majority of the A region (Δ98) failed to block M1-induced macrophage IL-1β and LDH release, but eliminating either the B1 or B2 repeat region led to significantly less inflammasome activation, with a deletion of both B regions (ΔB1ΔB2) yielding an even greater reduction (Fig. 3i,j). Thus, the B-repeat region of M1 protein plays a key functional role in NLRP3 inflammasome activation, IL-1β release and macrophage pyroptosis.

M1 uptake by macrophages is required for NLRP3 inflammasome activation. To determine if macrophage uptake of M1 was required to stimulate IL-1β, THP-1 Mφ were treated with well-characterized inhibitors of endocytic pathways. Neither wortmannin, a covalent inhibitor of phosphoinositide 3-kinases used to block macrophage endocytosis, nor methyl-β-cyclodextrin, Pitstop-2, a small molecule that selectively binds the C-terminal domain of clathrin to inhibit clathrin pit-mediated dynamics and endocytosis (CME), clearly reduced macrophage IL-1β release and processing upon M1 exposure, as measured by ELISA (Fig. 4a) and HEK-Blue IL-1β reporter cells (Fig. 4b).

To visualize M1 protein uptake into macrophages, we fused M1 protein and fluorescent mCherry (M1-mCherry). Confocal microscopy revealed progressive uptake of M1-mCherry into macrophages over 1 h (Fig. 4c). No significant difference in M1 uptake was observed between primed cells and unprimed BMDMs, indicating that M1 endocytosis proceeded independently of inflammasome signal one (Supplementary Fig. 4). In most treated macrophages, M1-mCherry assembled into intracellular speckles as early as 10 min, after which the number and size of the speckles increased (30 min), eventually reaching a diffuse distribution throughout the cytosolic compartment (1 h), suggesting a rapid and dynamic cellular uptake process (Fig. 4c). Neither macrophage uptake nor intracellular speckle formation was observed with recombinant mCherry alone (Supplementary Fig. 5). Intracellular speckles were also observed with a fusion construct between M1ΔB1ΔB2 protein and mCherry (Supplementary Fig. 6), suggesting that macrophage internalization of M1 occurs independently of its B-repeat region.

M1-mCherry co-localized with early endosome marker EEA1 and as little as 10 min (Fig. 4d). Mander’s coefficients analysis indicated a reciprocal degree of overlapping signal between the M1-mCherry and EEA1 signals, and vice versa (Supplementary Fig. 7), suggesting that M1 is internalized through a specific endocytic pathway. Incubation of macrophages with CME inhibitor Pitstop-2 prevented formation of M1-mCherry intracellular speckles (Fig. 4e). Single-cell analysis confirmed a significant (~65%) decrease in M1-mCherry signal in Pitstop-2 treated cells (Fig. 4f), indicating that macrophage M1 protein uptake is strongly dependent on CME. Pitstop-2 also markedly decreased LDH release and cytotoxicity in primed BMDMs exposed to recombinant M1 (Fig. 4g), confirming that CME helps to activate the NLRP3 inflammasome and pyroptosis.

Contribution of M1 expressed by GAS to IL-1β production and pyroptosis. As soluble M1 was sufficient to induce IL-1β release, we examined whether M1 protein expressed by live GAS contributes to inflammasome activation and pyroptosis. When incubated with THP-1 Mφ, a WT M1T1 GAS bacterial strain induced significantly more cytokysis (Fig. 5a) and IL-1β release (Fig. 5b), at multiplicities of infection (MOIs) ranging from 1 to 20, than an isogenic M1-protein deficient (ΔM1) mutant strain. Correspondingly, supernatants from WT M1T1 GAS-infected macrophages contained higher levels of processed IL-1β, as detected by IL-1β reporter cells,
Macrophages infected with the three isogenic mutant strains had significantly lower IL-1β production than those infected with the WT strain, but IL-1β release was most attenuated in macrophages infected with ΔSLO or ΔM1 strains. Natively expressed M1 protein thus plays an important role in stimulating IL-1β processing and pyroptosis in macrophages.

**M1 stimulates release of IL-1β in vivo.** To corroborate our in vitro findings, we compared the induction of IL-1β by GAS WT and isogenic ΔM1 and ΔSLO mutant strains during intraperitoneal infection of mice. Although the production of IL-1β from ΔSLO-infected mice did not differ from WT-infected animals, the ΔM1 strain induced significantly less IL-1β (Fig. 5c). Of note, there was a significant decrease in bacterial load recovered from the peritoneal fluid of mice infected with GAS ΔM1 mutant strain compared to supernatants from ΔM1 GAS-infected macrophages (Fig. 5c).

Compared to supernatants from ΔM1 GAS-infected macrophages (Fig. 5c), restoring M1 expression by plasmid complementation of the ΔM1 mutant (GASΔM1comp) yielded a very similar profile of cytokysis (Fig. 5a), IL-1β production (Fig. 5b) and IL-1β processing (Fig. 5c) to the WT parent strain across the different MOIs tested. Flow cytometry experiments of macrophages infected with FITC-labelled M1T1 GAS WT or isogenic mutant ΔM1 strains showed a very minor reduction in bacterial invasion of macrophages infected with the latter (Supplementary Fig. 8).

GAS expresses at least two other protein virulence factors that activate the NLRP3 inflammasome: SLO and SpyA. We compared the release of IL-1β from macrophages infected with GAS M1T1 WT or isogenic ΔM1, ΔSLO or ΔSpyA mutants (Fig. 5d). Macrophages infected with the three isogenic mutant strains had significantly lower IL-1β production than those infected with the WT strain, but IL-1β release was most attenuated in macrophages infected with ΔSLO or ΔM1 strains. Natively expressed M1 protein thus plays an important role in stimulating IL-1β processing and pyroptosis in macrophages.

**Fig. 4 | M1 uptake is required for inflammasome activation.**

- **a.** Production of IL-1β measured by ELISA from THP1-Mφ that were untreated (control) or pretreated with wortmannin or methyl-β-cyclodextrin for 30 min or with Pitstop-2 (Pitstop2) for 10 min before stimulation with M1 protein (2 μM) for 1 h. **b.** Production of processed IL-1β in THP1-Mφ that were either untreated or pretreated with Pitstop-2 for 10 min and then treated with M1 (2 μM) for 1 h. **c.** Confocal microscopy of BMDMs that were untreated (−M1) or incubated with M1-mCherry protein (2 μM) for the indicated times. DAPI (blue) and M1-mCherry (red) merged images are shown on the left and merged fluorescence and phase-contrast images on the right. Scale bars, 10 μm.

**d.** Confocal microscopy images of BMDMs incubated for 10 min with M1-mCherry protein (2 μM) and then fixed and stained with anti-EEA1 antibody. The co-localization of M1-mCherry (red) and EEA1 (green) is indicated by white arrows in the merged image. Bottom: magnification of the dashed boxed region in the merged image. Scale bars, 10 μm.

**e.** Confocal microscopy images of BMDMs treated with M1-mCherry for 30 min in the presence (+Pitstop) or absence (−Pitstop) of Pitstop-2. DAPI (blue) and M1-mCherry (red) merged images are shown. Scale bars, 10 μm.

**f.** Quantification of M1-mCherry fluorescence (intensity of M1-mCherry fluorescence of each treatment group and shown as relative fluorescence in arbitrary units). **g.** LDH release from BMDMs pretreated (+priming) or untreated (−priming) with LPS for 4 h and incubated without (−M1) or with (+M1) M1-mCherry (2 μM) in the presence (+Pit2) or absence (−Pit2) of Pitstop-2 (100% represents total cytokysis). Data in a, b and g are plotted as mean ± s.e.m. from three independent experiments performed in triplicate. Panels c, e and f show representative images of three and two independent experiments, respectively. Data in f are plotted as mean ± s.e.m., representing the fluorescence of at least 100 cells from two independent experiments. Data in a, b, f and g were analysed by Student’s t-test. NS, not significant (P > 0.05); **P < 0.01, ***P < 0.001.
**Fig. 5** | M1 action as natively expressed on GAS in vitro and in vivo and as soluble protein in vivo. a–c, THP1-Mφ were co-incubated with WT (GAS), isogenic Δemm1 mutant (GASΔM1) or M1-complemented (GASΔM1comp) strains at different MOIs. After 2 h of infection, macrophage supernatants were collected and analysed for detection of LDH (a), total IL-1β (b) and mature IL-1β (c). d, Comparison of IL-1β secretion from THP1-Mφ co-incubated for 2 h with WT GAS or isogenic Δemm1 (GASΔM1), Δslo (GASΔSLO) and ΔspyA (GASΔSpyA) mutant strains. Uninfected macrophages were used as negative control (UI). IL-1β production was analysed by ELISA and the presence of mature IL-1β by HEK-Blue IL-1R reporter cells. e, f, IL-1β detection (e) and bacteria recovered (c.f.u.) (f) from peritoneal lavage fluid of WT C57BL/6 mice 6 h after i.p. infection with 1 × 10^9 c.f.u. of WT or isogenic ΔM1 or ΔSLO mutant strains. Control group mice were injected with PBS and used as negative control (UI). g, i, Detection of IL-1β (g) and IL-6 (i) from peritoneal lavage fluid of WT C57BL/6 mice, 4 h after infections with different concentrations of M1 protein (g) and with 150 μg of purified M1 or ΔB1ΔB2 proteins (h, i). Control group animals were injected with PBS. Cytokine quantification was performed by ELISA. Data are plotted as mean ± s.e.m. Data in a–d represent three independent experiments performed in triplicate. In e and f N = 7, in g N = 6 and in h and i N = 10, and results represent the combination of two independent experiments. Results in a–c were analysed by two-way ANOVA multiple comparisons and results in d–i were analysed by Student’s t-test. NS, not significant (P > 0.05); *P < 0.05; **P < 0.01; ***P < 0.001.

GAS WT- or ΔSLO-infected mice (Fig. 5f). These results support a crucial role of M1 protein for full GAS virulence and its contribution to stimulation of IL-1β signalling in vivo.

We next injected increasing concentrations of M1 into the peritoneum of WT C57BL/6 mice and quantified IL-1β levels in the peritoneal fluid 4 h later. M1 indeed triggered IL-1β production in vivo in a dose-dependent manner (Fig. 5g). Consistent with our findings using cultured macrophages, removal of the B1 and B2 repeat domains (ΔB1ΔB2) from the M1 protein eliminated its ability to stimulate IL-1β in vivo following peritoneal injection (Fig. 5h). Moreover, M28 protein, which did not induce pyroptosis in vitro (Fig. 1b,c), did not stimulate production of IL-1β in mice when injected as a recombinant protein (Supplementary Fig. 9). Injection of intact M1 or ΔB1ΔB2 mutant proteins did not stimulate release of inflammasome-independent cytokine IL-6 (Fig. 5i), in contrast to the high levels of IL-6 produced upon injection of TLR agonist LPS (Supplementary Fig. 10). Together, these results indicate a specific role for M1 protein in vivo as a second signal for inflammasome activation.

**Discussion**

IL-1β signalling and inflammasome activation are important in the complex suite of innate immune responses elicited during GAS infection, wherein robust tissue inflammation is a hallmark of tissue invasion^{21}. Both canonical NLRP3 inflammasome and non-canonical (bacterial protease-induced) IL-1β signalling occur in macrophages responding to a GAS encounter^{22}. Our studies indicate that the classical GAS virulence factor M1 protein makes an independent and important contribution to GAS NLRP3 inflammasome activation, IL-1β processing and macrophage pyroptosis, thereby joining pore-forming toxin SLO^{23} and ADP-ribosylating toxin SpyA^{24} as known inflammasome activators produced by the pathogen.

Because interleukin-1 receptor (IL-1R) deficiency in mice^{46} and treatment with IL-1R antagonist Anakinra in mice or humans^{55} increases susceptibility to severe GAS infection, NLRP3 detection of M1 protein and other GAS factors appears on balance to serve a critical role in host defence against the organism. However, during advanced stages of systemic infection with invasive M1 GAS, IL-1β release and pyroptosis elicited by M1 may contribute to hyperinflammation and associated pathologies^{45,60,61}. During invasive GAS M1T1 disease, pathoadaptive covR/S mutations that shut off SpeB expression may be selected for and are associated with hypervirulence^{62,63}. Clinical epidemiological studies found that SpeB expression and activity were significantly higher in GAS serotype M1T1 isolates from non-severe invasive infections than isolates from severe cases^{60}. Silencing of SpeB activity may allow for an increased presence of soluble, released M1 protein during severe GAS infections. Indeed, the presence of soluble, released M1 has been documented in biopsies of patients with NF and STSS^{57}. 
Our in vitro experiments examining macrophages infected with live WT or M1-deficient GAS bacterial strains show a significant contribution of natively expressed M1 protein in the induction of IL-1β and pyroptosis. In our studies, the presence of M1 was not a strong factor in establishing initial interaction with macrophages compared to its known role in adhesion or invasion of epithelial cell types, emphasizing the dominance of M1 action on the inflammasome as compared to other known M1 properties in our assays. Production of IL-1β during GAS infection in vivo was greater when M1 was present, although the bacterial load was significantly lower in the absence of the protein. However, purified M1 protein alone, injected in vivo, was sufficient to produce the induction of IL-1β, suggesting that soluble M1 induces IL-1β signalling independently of its other well-known virulence properties.

In contrast to the two signals required in vitro for M1-induction of IL-1β, M1 alone was sufficient to stimulate the IL-1β response in vivo. We hypothesize that in the latter case, macrophages are intrinsically stimulated by cellular processes such as metabolic activation elicited by the thioglycollate pre-treatment in our in vivo experiments, or alternatively that M1 may activate host-derived pro-inflammatory factors facilitating signal 1 priming. The latter mechanism is consistent with recent studies, which have shown that keratinocytes recognize soluble M1 as a PAMP to release interleukin-8, growth-related oncogene-alpha, migration inhibitory factor and other inflammatory response alarms. Another study reported that M1 protein can synergize with heparin-binding protein to interact with TLR2 on monocytes and promote release of IL-1β and IL-6, which differs somewhat from our findings showing no effect of M1 protein on IL-6 production in vitro or in vivo. This difference probably reflects the source of the protein in each study: purified recombinant M1 protein in the present study versus M1 protein purified from GAS supernatants in the aforementioned study. Our studies suggest that M1, per se, is not a direct TLR agonist as proposed earlier; however, during GAS infection, release of M1 protein complexed with peptidoglycan (PGN) or lipoteichoic acid (LTA) to serve as PAMPs may contribute to the activation of TLRs and consequent accelerated cytokine production.

CME, which constitutes the major and best-characterized endocytic pathway, carries out the continuous uptake of essential nutrients, antigens, growth factors and pathogens. Our study demonstrates that CME contributes to M1 uptake and that a specific clathrin inhibitor blocks M1 uptake to reduce NLRP3 activation, IL-1β production and pyroptosis. Human immunodeficiency virus and hepatitis C virus were recently shown to activate the inflammasome by CME-dependent mechanisms. Furthermore, muramyl dipeptide, a bacterially derived agonist of the NOD2 receptor that induces caspase-1 activation through the NLRP3 inflammasome, is internalized by macrophages through CME. The mechanism by which M1 is targeted into clathrin-coated pits is at present unclear, from our findings showing no effect of M1 protein on IL-6 production in vitro or in vivo. This mechanism is consistent with recent studies, which have shown that keratinocytes recognize soluble M1 as a PAMP to release interleukin-8, growth-related oncogene-alpha, migration inhibitory factor and other inflammatory response alarms. Another study reported that M1 protein can synergize with heparin-binding protein to interact with TLR2 on monocytes and promote release of IL-1β and IL-6, which differs somewhat from our findings showing no effect of M1 protein on IL-6 production in vitro or in vivo. This difference probably reflects the source of the protein in each study: purified recombinant M1 protein in the present study versus M1 protein purified from GAS supernatants in the aforementioned study. Our studies suggest that M1, per se, is not a direct TLR agonist as proposed earlier; however, during GAS infection, release of M1 protein complexed with peptidoglycan (PGN) or lipoteichoic acid (LTA) to serve as PAMPs may contribute to the activation of TLRs and consequent accelerated cytokine production.

The M1 B-repeat region serves an essential function in GAS pathogenesis, as it is responsible for M1 binding to fibrinogen (Fg) and the formation of supramolecular M1–Fg complexes that induce neutrophil activation and transition to a proinflammatory state. Based on these previous studies and taking into account our new findings, a potential scenario during GAS invasive infection is that released, soluble M1 protein first activates infiltrating neutrophils through the M1–Fg supramolecular complex, resulting in the recruitment and influx of macrophages to the site. Released, soluble M1 would then act in a Fg-independent manner on these macrophages, causing pyroptotic cell death. Our studies provide evidence that the M1 B-repeat region is a multifunctional domain that plays an essential role in M1-induced inflammation.

Comparison of the inflammasome-activating effect of M1 to other M proteins suggests that the activation of NLRP3 and the consequent cell death is not unique to the M1 serotype, but is not a general property of all M protein serotypes. Due to the pronounced hypervariability of the N-terminal domain (B-repeats included) across M proteins, a single common pattern for the M proteins that activates IL-1β is unlikely. However, it has recently been recognized by X-ray crystallography that the three-dimensional structure of different M proteins in complex with host factors may reveal conserved sequence patterns hidden within hypervariability. K⁺ efflux is a common mechanism required for NLRP3 activation, and we confirmed that M1-induced inflammasome activation also requires K⁺ efflux. Further studies are required to understand the exact molecular mechanism that triggers K⁺ efflux and downstream mechanisms of NLRP3 activation upon M1 uptake. Components or by-products from the depolymerization of clathrin-coated pits, the subsequent transition from early to late endosomes, or the final fusion with recycling lysosomes are potentially important for the molecular mechanism of M1-induced NLRP3 activation. Indeed, the NLRP3 inflammasome may detect internal membrane perturbations and thereby respond to many different stimuli, which all have in common the ability to induce lysosomal destabilization.
In summary, we propose a model for GAS M1-dependent inflammasome activation and pyroptosis in macrophages (Fig. 6). Upon GAS tissue invasion, bacteria-derived PAMPs (for example, PGN or LTA) or host-derived DAMPs generated upon tissue injury are recognized by the macrophage through different mechanisms, including TLRs. These first signals activate the transcription of genes encoding NLRP3 inflammasome components and pro-IL-1β. M1 may be released from the GAS surface by cell wall turnover, host neutrophil proteases, or both, and is taken up by macrophages via CME to serve as a second signal required for NLRP3 activation. Internalization of M1 activates NLRP3 through an unknown subcellular molecular intermediate or event, for example, lysosomal destabilization, triggering K⁺ efflux and promoting inflammasome assembly by recruitment of NLRP3, its well-characterized apoptotic-si-associated speck-like accessory protein (ASC) and caspase-1, resulting in the maturation and secretion of the pro-inflammasome cytokine IL-1β, DNA damage, membrane disruption and pyroptosis in macrophages. This work reveals an unexpected function in GAS molecular pathogenesis for the M protein, the most abundant protein on the GAS surface, with multifaceted roles in virulence and as a target of host immunity.

**Methods**

**Reagents and inhibitors.** Lipopolysaccharide from E. coli serotype EH100(ΔlacZ) was purchased from Enzo. Lipotechoic acid from Bacillus subtilis, peptidoglycan from Staphylococcus aureus, R848 and Pam3CSK4 were purchased from Invivogen. Ac-YVAD-CMK (inhibitor of caspase-1) and CRID 3 (inhibitor of NLRP3) were purchased from Enzo and Tocris, respectively. Recombinant mCherry was purchased from BioVision. Pitstop-2 was purchased from Abcam. Wortmannin, methyl β-cyclodextrin, ATR nigericin and human fibrinogen were purchased from Sigma.

**Cell isolation and culture.** BMDMs were generated as previously described14 from the femurs and tibias of 8- to 12-week-old male or female WT C57BL/6 (Jackson Laboratory), female BALB/c (Harlan Laboratories), or nfLPR (provided by J. Bertin) mice. The human THP-1 monocytes cell line was provided and authenticated by ATCC and stored at the UCSD cell culture facility, which routinely carried out surveillance mycoplasma testing of its facility. THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, 0.2% d-glucose, and 25 mM HEPES, with 10% vol/vol FBS. When needed, cells were cultured in medium (serum-free buffer with 10 mM HEPES, 150 mM NaCl, 5 mM KH2PO4, 1 mM MgCl2, 1 mM CaCl2, 1% BSA, pH 7.4) or in Na+ medium (serum-free buffer with 10 mM HEPES, 150 mM NaCl, 5 mM KH2PO4, 1 mM MgCl2, 1 mM CaCl2, 1% BSA, pH 7.4).

**Animal experiments.** The UCSD Institutional Animal Care and Use Committee approved all animal use and procedures. In compliance with ethical guidelines, to minimize the number of animals, we used a minimum of five mice for each data point (except where indicated in the figure legends) to ensure statistical power. All mice were randomly distributed into the different groups as indicated in the corresponding figure legend. No blinding was performed in the animal experiments, 5 mice for each treatment group. macropathogens. At 72 h post-treatment, mice were injected with different amounts of M protein in 100 μl (0.2% FBS) for 6 h. At 5 min after M protein treatment, mice were euthanized with isoflurane followed by cervical dislocation. Post-mortem, peritoneal fluid was collected by lavage with 3 ml sterile PBS, followed by massage. Cytokines and bacterial counts in the peritoneal fluid collected were quantified by ELISA and c.f.u., respectively.

**Bacterial strains and culture conditions.** GAS M1T1 5448 was originally isolated from a patient with necrotizing fasciitis and toxic shock15. The isogenic in-frame alleles exchange knockout mutant 5448 Δemm1 (ΔM1) and its complemented strain have been described previously16 as well as the knockout mutants 5448 Δsto (ΔSLO)17 and 5448 ΔpyrA (ΔSpyA)18. All GAS strains were routinely propagated at 37°C on Todd–Hewitt agar (Difco) or in static liquid cultures of Todd–Hewitt broth. Where appropriate, strains were grown in medium supplemented with 5 μg/ml streptomycin or 2 μg/ml chloramphenicol.

**Infection of macrophages with GAS.** THP-1 Mφ were seeded at 5 × 10⁴ cells in 600 μl of RPMI–2% FBS in a 12-well plate. Macrophages were infected with overnight bacterial cultures diluted in 100 μl of RPMI–2% FBS at MOIs of 1, 5, 10 or 20. Plates were centrifuged at 600 x g for 5 min to facilitate bacterial contact with macrophages. Cells were incubated at 37 °C for 2 h. Supernatants were collected by centrifugation (600 x g for 5 min at room temperature) and analysed for LDH release, total IL-1β production and IL-1β signaling. For GAS invasion experiments, stationary phase bacteria were labelled with FITC through 30 min incubation with 0.2 μg/ml FITC (containing 0.05% Tween 80) covered on ice, and washed twice with PBS. A direct-activated THP-1 Mφ were co-incubated with FITC-labelled bacteria at an MOI of 10 in RPMI containing 2% FBS for 2 h. Macrophages were dissociated with trypsin–EDTA, washed in PBS supplemented with 0.5% FBS, and immediately run on FACScalibur (BD Biosciences). Ten thousand events were collected, and samples were gated for live macrophages based on unlabelled bacteria-infected controls. Flow cytometry data were analysed using FlowJo v9.x.10 (Tree Star).

**LDH assay and ELISA.** Cell culture supernatants were collected by centrifugation at 500 g for 5 min at 20°C and analysed with a Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega). Procedures and calculations of LDH release paralleled percentage (%LDH) were performed according to the manufacturer’s instructions. For LDH assay, cell supernatants were assayed for secreted alkaline phosphatase activity by the addition of 50 μl HEK-Blue IL-1β reporter cell supernatants onto 150 μl of Quanti-Blue reagent (Invivogen) and monitoring the optical density at 620 nm with an EnSpire plate reader (PerkinElmer).

**Confocal and fluorescence microscopy.** BMDMs were seeded on coverslips in 24-well plates. Cells were primed with LPS (10 ng·ml⁻¹) for 4 h. As appropriate, cells were treated with Pitstop-2 (25 μM) or with vehicle control (dimethyl sulfoxide, DMSO) for 10 min and then stimulated with M1- or M2-polarizing media (2 μg/ml) for 10, 30 or 60 min. After these treatments, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. After fixation, cells were washed three times with PBS and coverslips were mounted with ProLong Gold antifade reagent containing DAPI (Life Technologies). To quantify the macrophage phenotype, in-focus planes were acquired with ImageXpress (v.2.0.0, NIH) as previously described13. An outline of the cell was drawn and measurements of mean fluorescence and integrated density were acquired. In experiments with 10% CO₂, stationary phase bacteria were labelled with FITC through 30 min incubation with 0.2 μg/ml FITC (containing 0.05% Tween 80) covered on ice, and washed twice with PBS. A direct-activated THP-1 Mφ were co-incubated with FITC-labelled bacteria at an MOI of 10 in RPMI containing 2% FBS for 2 h. Macrophages were dissociated with trypsin–EDTA, washed in PBS supplemented with 0.5% FBS, and immediately run on FACScalibur (BD Biosciences). Ten thousand events were collected, and samples were gated for live macrophages based on unlabelled bacteria-infected controls. Flow cytometry data were analysed using FlowJo v9.x.10 (Tree Star).

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**Propidium iodide uptake assay.** THP-1 Mφ were incubated in the presence or absence of purified M1 protein (2 μM) for 2 h in RPMI containing 2% FBS. Cells were washed three times with PBS, incubated in 200 μl PI staining solution (1% BSA and 3 μM PI, Invitrogen) and were visualized using a Zeiss Axio Observer.D1 fluorescence microscope.

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Mean fluorescence intensity was calculated from a total of 10,000 cells, and macrophages treated with PBS alone were used as negative control. Flow cytometry data were analysed with FlowJo v.9.4.10 (Tree Star).

**TUNEL assay.** THP-1 macrophages were incubated for 4 h in the presence or absence of purified M1 protein (0.2 μM or 2 μM). Cells were detached from the well by incubation for 5 min at 37°C with cold PBS containing 5 mM EDTA, washed once with PBS, and assayed for the percentage of cells with DNA fragmented by the TUNEL technique, as per the Roche TUNEL assay kit, In Situ Prime (Roche). According to the manufacturer’s instructions, DNA fragmentation—fluorescence intensity was measured by flow cytometry for a total of 10,000 cells. Macrophages treated with PBS were used as a negative control. Flow cytometry data were analysed with FlowJo v.9.4.10 (Tree Star).

**Protein expression and purification.** Procedures for the construction and expression of M1 protein, truncated variants of M1 protein and other M protein types have been described previously44,45, except for M1-cherry and M1ΔB1ΔB2-mCherry proteins. For M1-cherry, the coding sequence of intact M1 protein (residues 1–236) was cloned into the pET28-M1 vector. The PCR product was amplified with primers 5′-XhoIm-Cherry and 3′-M1dB1B2-cherry (EcoRI) (5′-3′) of M1. For M1ΔmCherry and the corresponding M1ΔB1ΔB2-M1 vector, Protein expression constructs were expressed in *E. coli* BL21 (DE3) cells. Bacteria were grown in LB containing 34 mg ml−1 kanamycin at 37°C until mid-logarithmic phase and then induced at room temperature with 1 mM isopropyl β-D-1-thiogalactopyranoside and grown for a further 18 h. Bacteria were collected by centrifugation and resuspended in lysis buffer (300 mM NaCl, 100 mM Tris-HCl, pH 8, 10 mM imidazole) with protease inhibitors (Complete Tablet, Roche). Bacteria were lysed using an EmulsiFlex-C5 (Avestin; 20,000 p.s.i. with 3′-5′). Blends were incubated for 4 h in the presence or absence of purified M1 protein (0.2 μM or 2 μM). Cells were detached from the well by incubation for 5 min at 37°C with cold PBS containing 5 mM EDTA, washed once with PBS, and assayed for the percentage of cells with DNA fragmented by the TUNEL technique, as per the Roche TUNEL assay kit, In Situ Prime (Roche). According to the manufacturer’s instructions, DNA fragmentation—fluorescence intensity was measured by flow cytometry for a total of 10,000 cells. Macrophages treated with PBS were used as a negative control. Flow cytometry data were analysed with FlowJo v.9.4.10 (Tree Star).

**References**

4. Phillips, G. N., Flicker, P. F., Cohen, C., Manjula, B. N. & Akesson, P. M1 protein and protein types have been described previously44,45, except for M1-cherry and M1ΔB1ΔB2-mCherry proteins. For M1-cherry, the coding sequence of intact M1 protein (residues 1–236) was cloned into the pET28-M1 vector. The PCR product was amplified with primers 5′-XhoIm-Cherry and 3′-M1dB1B2-cherry (EcoRI) (5′-3′) of M1. For M1ΔmCherry and the corresponding M1ΔB1ΔB2-M1 vector, Protein expression constructs were expressed in *E. coli* BL21 (DE3) cells. Bacteria were grown in LB containing 34 mg ml−1 kanamycin at 37°C until mid-logarithmic phase and then induced at room temperature with 1 mM isopropyl β-D-1-thiogalactopyranoside and grown for a further 18 h. Bacteria were collected by centrifugation and resuspended in lysis buffer (300 mM NaCl, 100 mM Tris-HCl, pH 8, 10 mM imidazole) with protease inhibitors (Complete Tablet, Roche). Bacteria were lysed using an EmulsiFlex-C5 (Avestin; 20,000 p.s.i. with 3′-5′). Blends were incubated for 4 h in the presence or absence of purified M1 protein (0.2 μM or 2 μM). Cells were detached from the well by incubation for 5 min at 37°C with cold PBS containing 5 mM EDTA, washed once with PBS, and assayed for the percentage of cells with DNA fragmented by the TUNEL technique, as per the Roche TUNEL assay kit, In Situ Prime (Roche). According to the manufacturer’s instructions, DNA fragmentation—fluorescence intensity was measured by flow cytometry for a total of 10,000 cells. Macrophages treated with PBS were used as a negative control. Flow cytometry data were analysed with FlowJo v.9.4.10 (Tree Star).

41. Stewart, C. et al. Coiled-coil destabilizing residues in the group A
Streptococcus M1 protein are required for functional interaction.
42. Buffalo, C. Z. et al. Conserved patterns hidden within group A Streptococcus
M protein hypervariability recognize human C4b-binding protein.
43. Hornung, V. et al. Silica crystals and aluminum salts activate the NALP3
inflammasome through phagosomal destabilization. Nat. Immunol. 9,
44. Chatellier, S. et al. Genetic relatedness and superantigen expression in group
A Streptococcus serotype M1 isolates from patients with severe and nonsevere
45. Lauth, X. et al. M1 protein allows Group A streptococcal survival in
46. Timmer, A. M. et al. Streptolysin O promotes group A Streptococcus
immune evasion by accelerated macrophage apoptosis. J. Biol. Chem. 284,
47. McCloy, R. A. et al. Partial inhibition of Cdk1 in G2 phase overrides the

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Author contributions
J.A.V., P.G. and V.N. formulated the original hypothesis, designed the study and
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wrote the manuscript and all authors reviewed the manuscript, data and conclusions
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Competing interests
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Group A streptococcal M protein activates the NLRP3 inflammasome

J. Andrés Valderrama¹², Angelica M. Riestra¹, Nina J. Gao¹, Christopher N. LaRock¹, Naveen Gupta³, Syed Raza Ali¹, Hal M. Hoffman¹³, Partho Ghosh²* and Victor Nizet¹⁴*

¹Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093, USA. ²Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093, USA. ³Department of Medicine, University of California, San Diego, La Jolla, CA 92093, USA. ⁴Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093, USA. *e-mail: pghosh@ucsd.edu; vnizet@ucsd.edu
Supplementary Information

Group A Streptococcal M Protein Activates the NLRP3 Inflammasome

J. Andrés Valderrama$^{1,2}$, Angelica M. Riestra$^1$, Nina J. Gao$^1$, Christopher N. LaRock$^1$, Naveen Gupta$^3$, Syed Raza Ali$^1$, Hal M. Hoffman$^{1,3}$, Partho Ghosh$^2$* and Victor Nizet$^{1,4}$*

$^1$Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093, USA.
$^2$Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093, USA.
$^3$Department of Medicine, University of California, San Diego, La Jolla, CA 92093, USA.
$^4$Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093, USA.

Contents: Supplementary Figures 1-10 and corresponding legends.
Supplementary Figure 1. M1-induced cell death is fibrinogen-independent in macrophages and does not occur in red blood cells. a, Percentage of LDH released from THP1-Mφ after 2 h treatment in the presence of M1 (2 µM), human fibrinogen (Fg, 1 mg ml⁻¹) or M1+Fg (M1, 2 µM + Fg, 1 mg ml⁻¹). b, Percentage of hemoglobin released (Hb % release) from human erythrocytes after 2 h treatment in the presence of M1 (2 µM). PBS and triton 0.1 % were used as the minimal or maximal inducers of Hb release, respectively. Data are plotted as the mean ± SEM and represent two independent experiments performed in duplicate and analyzed by Student’s t-test. NS = not significant (P>0.05).
Supplementary Figure 2. Macrophage viability upon treatment with M1 or various TLR agonists. Percentage of LDH released from THP1-Mϕ after 15 h stimulation with M1 (0.02 µM), lipopolysaccharide (LPS, 100 ng ml⁻¹), peptidoglycan (PGN, 1 µg ml⁻¹), or lipoteichoic acid (LTA, 1 µg ml⁻¹). Data are plotted as the mean ± SEM and represent three independent experiments performed in triplicate.
Supplementary Figure 3. PMA triggers signal 1 in THP-1 differentiated macrophages. qPCR analysis of IL-1β gene expression from undifferentiated THP-1 cells (THP1 Mo, - PMA) or THP-1 differentiated to macrophages (THP1-Mφ, + PMA). The qPCR results were calculated by Delta-Delta CT method with the *gadph* gene as internal control (housekeeping gene). Data are plotted as the mean ± SEM and represent three independent experiments performed in triplicate and analyzed by Student’s *t*-test. *NS* = not significant (P>0.05), ***P<0.001).
Supplementary Figure 4. M1 uptake occurs independently of inflammasome signal one. Confocal microscopy images of BMDMs primed (left) or unprimed (right) cells with LPS (10 ng ml⁻¹) for 4 h and then incubated with 2 µM of M1-mCherry for 30 minutes. DAPI (blue) and M1-mCherry (red) merge of fluorescence and phase contrast images are shown; scale bar = 10µm. Images are representative of two independent experiments.
Supplementary Figure 5. mCherry is not taken up by macrophages. Fluorescence microscopy of BMDMs incubated with 2 µM of M1-mCherry (left) or 2 µM of mCherry (right) for 10 (top) or 30 min (bottom). DAPI (blue) and M1-mCherry (red) merged images are shown; scale bar = 30µm. Images are representative of two independent experiments.
Supplementary Figure 6. M1 B repeats are not required for M1 uptake. Confocal microscopy images of BMDMs incubated with 2 μM of M1-mCherry (left) or 2 μM of M1ΔB1ΔB2-mCherry (right) for 10 min. DAPI (blue) and M1-mCherry (red) merged images are shown. Insets are magnifications of the dashed boxed regions in the merged images; scale bar = 30μm. Images are representative of two independent experiments.
Supplementary Figure 7. Quantification of colocalization between M1-mCherry and the early endosomal marker EEA1. Thresholded Mander’s correlation coefficients tM1 and tM2 represent the fraction of overlapped signal of each channel. Data are plotted as the mean ± SEM and show quantification in 15 representative cells shown in Figure 4d.
Supplementary Figure 8. Binding of GAS to macrophages is not strongly affected by M1. THP1-Mφ infected with FITC-labeled GAS or GASΔM1 bacteria at MOI 10. Macrophage-bacteria interactions were examined 2 h post-infection by flow cytometry. Events were gated in the population of macrophages. Uninfected THP1-Mφ served as a negative control. A representative histogram of FITC fluorescence is shown in panel a and panel b shows the geometric mean of fluorescence intensity ± SEM and represent three independent experiments performed in triplicate and analyzed by Student’s t-test. NS = not significant (P>0.05), *P<0.05, ***P<0.001.
Supplementary Figure 9. Comparison of M1 and M28 proteins induction of IL-1β in vivo. Detection of IL-1β from peritoneal lavage fluid of wild type C57BL/6 mice, 4 h after injection with 150 µg purified M1 or M28 protein. Control group animals were injected with PBS. Cytokine quantification was performed by ELISA. Data are plotted as the mean ± SEM, N=5 and were analyzed by Student’s t-test. NS = not significant (P>0.05) and *P<0.05).
Supplementary Figure 10. Detection of IL-6 \textit{in vivo}. Detection of IL-6 from peritoneal lavage fluid of wild type C57BL/6 mice, 4 h after injection with 10 mg/kg LPS. Control group animals were injected with PBS. Cytokine quantification was performed by ELISA. Data are plotted as the mean ± SEM, N=4 and were analyzed by Student’s $t$-test (***(P<0.001).