Review

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Future MICROBIOLOGY

Group A *Streptococcus* encounters with host macrophages

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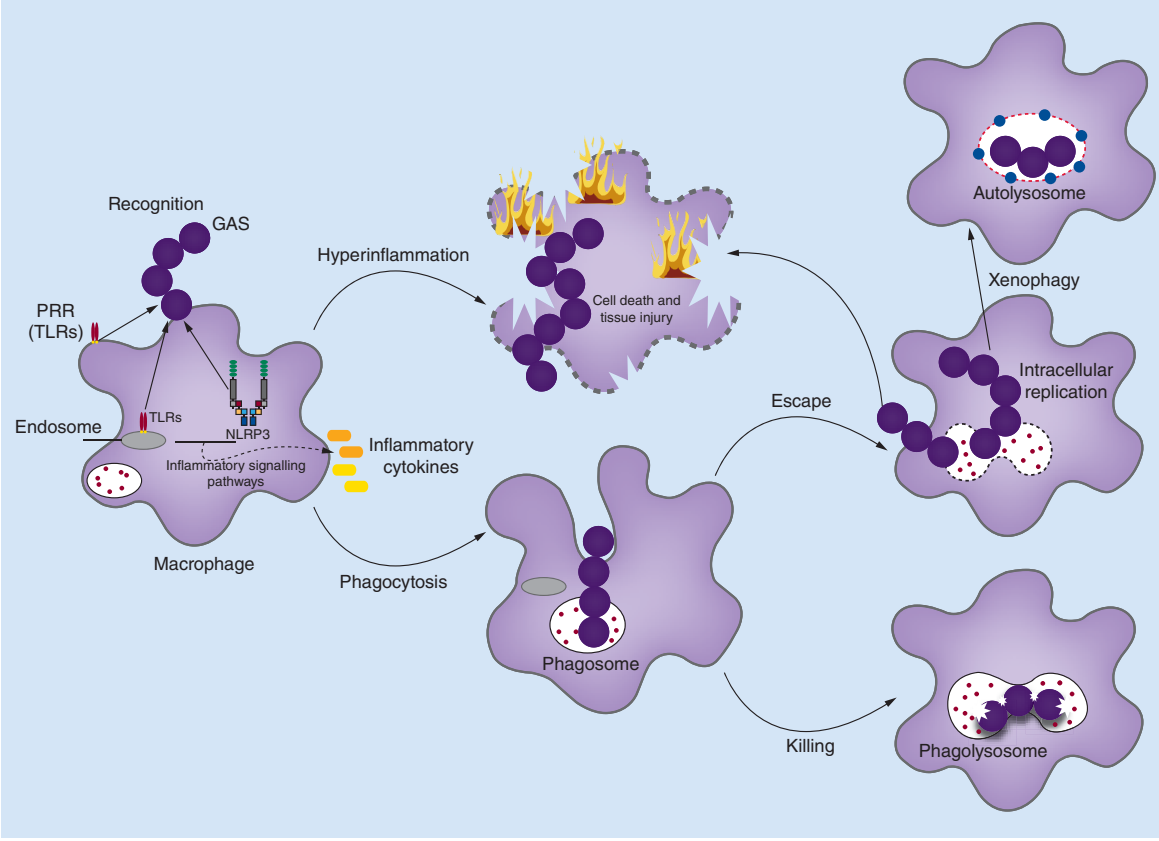
Group A *Streptococcus* (GAS) is a leading human bacterial pathogen with diverse clinical manifestations. Macrophages constitute a critical first line of host defense against GAS infection, using numerous surface and intracellular receptors such as Toll-like receptors and inflammasomes for pathogen recognition and activation of inflammatory signaling pathways. Depending on the intensity of the GAS infection, activation of these signaling cascades may provide a beneficial early alarm for effective immune clearance, or conversely, may cause hyperinflammation and tissue injury during severe invasive infection. Although traditionally considered an extracellular pathogen, GAS can invade and replicate within macrophages using specific molecular mechanisms to resist phagolysosomal and xenophagic killing. Unraveling GAS–macrophage encounters may reveal new treatment options for this leading agent of infection-associated mortality.



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The Gram-positive bacterium *Streptococcus pyogenes,* or group A *Streptococcus* (GAS), is a leading human pathogen, responsible for disease conditions ranging from mild infections, such as pharyngitis and impetigo, to invasive diseases, including necrotizing fasciitis (NF) and streptococcal toxic-shock syndrome. Serious immune sequelae may be triggered after repeated GAS infections, including acute glomerulonephritis and rheumatic heart disease [1,2]. The estimated number of people suffering from serious GAS disease includes 1.8 million new cases each year, accounting for 517,000 deaths annually, placing GAS among the 10 major causes of human infection-associated mortality [3,4]. During recent decades, significant progress has been made in understanding the molecular mechanisms of GAS disease pathogenesis. GAS expresses a multitude of surface-bound and secreted virulence factors that are variably expressed among strains and underlie differential host susceptibilities [2,5–6].

The host's initial barrier against GAS infection involves the physical integrity of mucosal or skin epithelium and the beneficial antagonism of commensal microflora. However, once GAS has gained a foothold in the host and is poised to breach the epithelial barrier, the innate immune system becomes central in defense against invasive infection. Front line effectors of innate immunity are 'professional' phagocytic cells, including macrophages (MΦs), dendritic cells (DCs) and monocytes, along with granulocytic cells types such as neutrophils and mast cells. These leukocytes are distinguished from 'nonprofessional' phagocytes by their effectiveness in internalizing large particles, such as cellular debris, apoptotic cells and microbial pathogens, into phagosomes (Figure 1) [7]. The phagocytic process is initiated upon recognition of a particle ligand by cell surface receptors. Such receptor–ligand recognition events launch signaling pathways that induce actin cytoskeleton remodeling and extensions of membrane protrusions to surround the ligand, forming a phagocytic cup and ultimately a phagosome after fusion and fission events with vesicles of the endocytic compartment. The nascent phagosome interacts with different types of endosomes to gradually mature from its 'early' to 'late' forms. Ultimately, phagosome fusion with lysosomal compartments forms the phagolysosome (Figure 1) [8].

MΦs comprise complex populations of self-renewing tissue-resident cells supplemented by additional numbers derived from circulating monocytes in response to physiological stimuli [9–11]. MΦs are strategically distributed throughout host tissues where they can encounter, ingest and process microorganisms, dead cells and debris, and generate inflammatory signals that recruit or modulate the activity of additional MΦs or immune effector cells, including neutrophils. MΦs are dynamic and heterogeneous, due to diverse mechanisms governing their differentiation, tissue distribution, microenvironment and responsiveness to stimuli [12–14]. Compared to other professional phagocytic cells, MΦ phagosomes acidify strongly and rapidly, inhibiting bacterial growth, while activating resident proteases in an attempt to destroy the entire phagocytic cargo [15]. Efficient phagocytose and killing of GAS by resident MΦs follows *in vivo* infection [16]. MΦ depletion by carrageenan treatment or blockade of MΦs phagocytic functions using gadolinium (III) chloride in mice leads to increased mortality following intravenous GAS challenge, showing their important role in GAS bloodstream clearance [16]. Similarly, mice depleted of MΦs by clodronate showed increased GAS dissemination from an initial soft tissue focus of infection into blood and internal organs [17].

A large body of literature explores the arsenal of individual GAS virulence factors and their pathogenic mechanisms [2,5]. Yes, despite the prominent role of MΦs in innate defense against the bacterium, molecular mechanisms of GAS–MΦ interaction remain incompletely understood. This review integrates the latest studies on how GAS and its virulence determinants are recognized by MΦs, highlighting the particular importance of Toll-like receptor (TLR) and inflammasome pathways. We also examine GAS strategies for survival and replication within MΦs, and the functional and immune signaling responses of MΦs to the pathogen encounter.

Macrophage recognition of GAS

Recognition through TLR signaling

Multiple receptors on the phagocyte cell surface function to detect signals not normally present in healthy tissues [18]. These molecules, collectively referred to as pattern recognition receptors (PRRs), sense conserved microbial structures called pathogen-associated molecular patterns (PAMPs), and also recognize endogenous molecules released from injured cells, termed damage-associated molecular patterns (DAMPs). Among PRRs, the TLR family is the earliest and best characterized, and TLRs are expressed on most innate immune cells. To date, ten TLRs have been identified in humans and 13 in mice [19]. TLRs sense invading pathogens both outside the cell and in intracellular

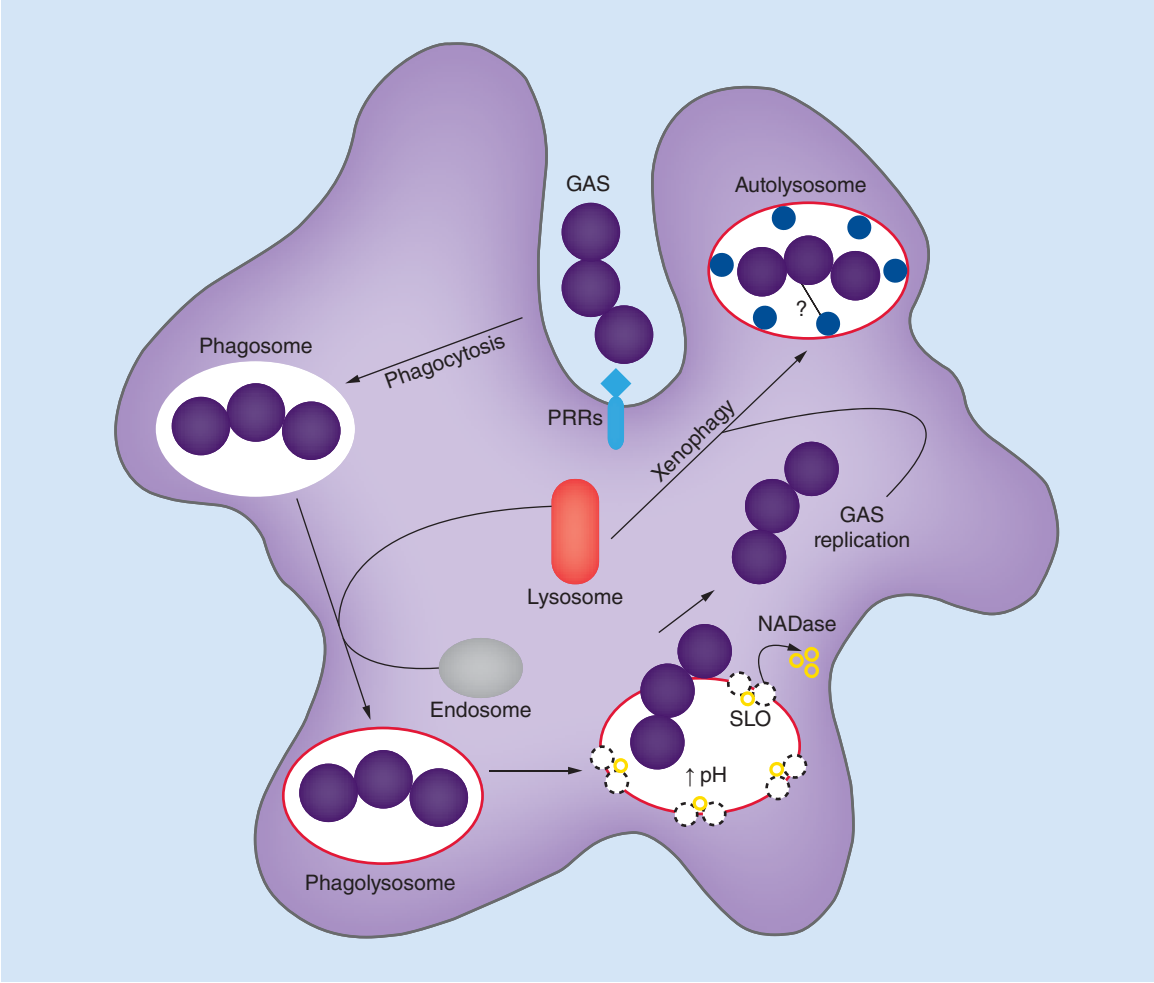

Figure 1. Group A *Streptococcus*-induced phagocytosis and xenophagy in macrophages. The phagocytic process is initiated by the recognition of group A *Streptococcus* (GAS) by macrophage (MΦ) surface pattern recognition receptors. Receptor recognition activates signaling pathways that induce remodeling of the actin cytoskeleton to form a phagosome in a process that involves fusion and fission events with vesicles of the endocytic compartment. The formed phagosome interacts with different types of endosomes and lysosomes, leading to development of phagolysosomes. GAS survival within the MΦ is mediated by streptolysin O-induced pore formation, which prevents acidification of the GAS-containing phagolysosome and allows the delivery of its co-toxin NAD–glycohydrolase (NADase) from the phagosome into the MΦ cytosol, increasing streptolysin O cytotoxicity and promoting GAS replication. Intracellular GAS can then be targeted by the autophagy machinery in a process called xenophagy, and ultimately delivered to the autophagosome, although the GAS-derived specific molecules recognized during xenophagy remain unknown **(?)**.
PRRs: Pattern recognition receptors.

compartments, such as endosomes and lysosomes [20]. Subcellular localization of TLRs is important for recognition of specific PAMPs and DAMPs [21]. TLR3, TLR7, TLR8 and TLR9 are localized in the endosomal compartments whereas the remaining TLRs signal from the cell membrane [22]. TLR-mediated signaling pathways are initiated commonly via the adaptor protein MyD88 [23], but may also involve TIR domain-containing adapter-inducing interferon-β (TRIF) [24] or TIR domain-containing adaptor protein [25,26]. MyD88 mediates activation of the key transcription factor NFκB and subsequent inflammatory cytokine gene expression for all TLRs except for TLR3. TLR3 employs TRIF, which is also used by TLR4 together with MyD88, to trigger activation of IRF3 and NFκB transcriptional factors and subsequent induction of IFN-β [27]. In addition to TLR-mediated intracellular recognition, pathogen-derivative products can also be detected once internalized into the cell in a MyD88-independent manner by other cytosolic PRRs, such as nucleotide-binding (NOD)-like and oligomerization domain (NLRs

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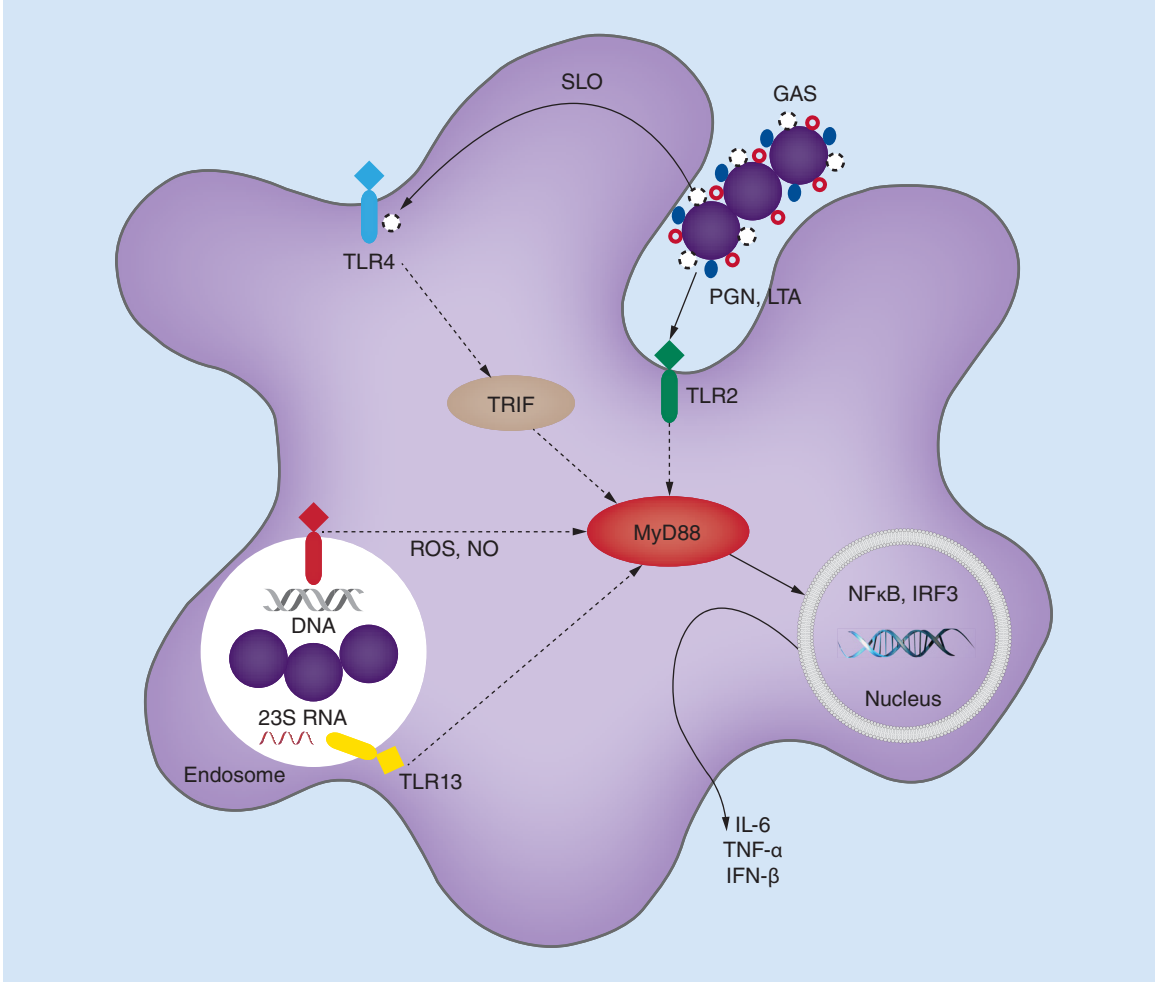

Figure 2. Toll-like receptor-mediated group A *Streptococcus* recognition in macrophages. Macrophages express all Toll-like receptors (TLRs), which are responsible for sensing invading pathogens, including group A *Streptococcus* (GAS), both from the cell surface and in intracellular endosomes and lysosomes. Specific GAS-derived molecules activate the TLR-mediated signaling cascades, which require the adaptor MyD88, although some TLRs such as TLR4 use both MyD88 and TRIF adaptors. MyD88 and TRIF mediate the activation of IRF3 and NFκB transcriptional factors and subsequent induction of IFN-β and proinflammatory cytokines, such as TNF-α and IL-6. TLR2 recognizes GAS lipoteichoic acid and peptidoglycan. TLR4, a receptor for lipopolysaccharide of Gram-negative bacteria, has also been shown to have an effect in the recognition of the GAS pore-forming toxin streptolysin O. TLR9 recognizes unmethylated CpG-rich DNA motifs and plays an important role in host defense against GAS by stimulating the production of reactive oxygen species and nitric oxide (NO). Bacterial 23S RNA, including GAS rRNA, is also recognized by TLR signaling, specifically by murine TLR13. Recognition of GAS RNA by TLR13 is dependent on phagocytosis and endosomal GAS recognition.

receptors) [19,20]. Since MΦs express all TLR signaling pathway components, these cells are critical for recognition of GAS virulence determinants both intra- and extra-cellularly (Figure 2).

MyD88-deficient patients have impaired responses to most TLR ligands and display a narrow but potentially life-threatening predisposition to pyogenic bacterial diseases [28,29]. GAS activation of NFκB and MAPK in macrophages *in vitro* leads to production of proinflammatory cytokines TNFα and IL-6 in a fully MyD88-dependent manner [30]. Concurrently, there is an absolute requirement of MyD88 for upregulation of DC maturation markers, such as CD40, CD80 and CD86, and DC production of proinflammatory cytokines IL-12, IL-6 and TNFα, upon GAS infection [31]. In experimental GAS skin infection in mice [32], MyD88 signaling triggered a rapid innate response operating at two levels: upregulation of inflammatory mediators for neutrophil and MΦ recruitment; and expression of regulatory chemokines, such as CXCL9 and CXCL10, inhibiting recruitment of other immune cell types with potential detrimental effects [32]. TNF-α deficient mice are also highly susceptible to GAS soft tissue infections with

a specific defect in MΦ recruitment [17]. However, GAS recognition by TLR receptors upstream of MyD88 remains a subject of some controversy. In mouse MΦs infected with GAS *ex vivo*, neither activation of MAPK and NFκB nor production of MyD88-dependent cytokines required TLR4, TLR9 or TLR2 as corroborated in single and triple TLR2/4/9 deficient cells [30], despite an abundance of peptidoglycan and lipoteichoic acid (LTA) in the GAS cell wall, two well characterized TLR2 ligands (Figure 2) [33]. Moreover, TLR2-deficient mice are not hypersusceptible to GAS systemic infection *in vivo* [17]. These contrasting results might be reconciled in two different ways; from the host side, TLR signaling can be functionally redundant with different TLRs and other PRRs such as scavenger receptors (SR) recognizing the same GAS PAMPs; indeed SR can trigger MΦ MyD88 signaling in MΦ [34]. M protein, the major GAS surface-associated virulence factor, had been proposed in two independent studies using monocytes and MΦs to be recognized by TLR2 [35] and SR-A [36], respectively. A recent study, however, suggested that M protein alone may not be a true TLR2 agonist, but rather when released may remain bound to other GAS cell wall-derived molecules (e.g., peptidoglycan or LTA) that provoke TLR2-dependent cytokine production in the host [37]. From the pathogen perspective, many GAS virulence factors are multifaceted in nature, and inactivation of any particular virulence determinant may be compensated by another that elicits a similar immune response. Fieber and Kovarik [38] also hypothesized that GAS TLR2 agonists such as LTA and peptidoglycan are masked by its hyaluronic acid capsule, a critical evasion factor present in most GAS strains [39], to be recognized only after phagocytosis and breakdown of capsular integrity. The phagosome-specific configuration of TLR family members may preclude substantial TLR2 responses and allow other TLRs to be triggered by GAS ligands [38]. For example, TLR4 is considered a specific receptor for lipopolysaccharide of Gram-negative bacteria; however, TLR4-deficient murine bone marrow-derived MΦs (BMDMs) have reduced IL-6 and TNF-α expression after challenge with GAS pore-forming toxin streptolysin O (SLO) (Figure 2) and other cholesterol-dependent bacterial cytolysins [40].

Two independent studies in primary human MΦs [41] and BMDMs [30] showed that GAS also elicits type I interferon signaling and interferon-driven interferon β (IFN-β) production through signaling from endosomal TLRs, for example, TLR3, TLR7, TLR9. Cell surface-localized TLR2 and TLR4 can also become IFN-β inducers after ligand-triggered internalization of the receptors [42,43]. IFN signaling is key to host defense against GAS, and mice lacking type I interferon receptor (IFNAR-1) are hyper-susceptible to lethal invasive GAS infection [44]. BMDM IFN-β production is independent of GAS cytolysins SLO and SLS [30], in contrast to MΦ responses to other Gram-positive pathogens, where cytolysin-mediated escape from phagocytic vesicles is implicated in interferon induction [40,45–47]. GAS-derived DNA is the PAMP inducing IFN-β in BMDMs, involving both MyD88-dependent and -independent pathways, with full IRF3 control in the absence of known IFN-β-inducing PRRs NOD1, NOD2, TLR3, TLR7 and TLR9 [44]. TLR9 recognizes unmethylated CpG-rich DNA motifs and plays important role in host defense against GAS, improving MΦ killing of the pathogen by inducing transcription factor HIF-1α and increasing bactericidal reactive oxygen species and nitrogen oxide (NO) generation (Figure 2) [48]. However, in invasive M1 GAS strains, the potent secreted DNAse Sda1 [49] can degrade the bacterium's own CpG-rich DNA to block TLR9-mediated IFN-α and TNF-α production, blunting bactericidal activity [50]. GAS 23S rRNA is also recognized by TLR13 [51–53] following phagocytosis in murine MΦs (Figure 2) [54]. However, while expressed in all kingdoms, only a few mammals possess TLR13, with humans lacking the receptor. Instead, GAS 23S rRNA is detected by TLR8 in human monocyte-derived MΦs [55]. In summary, recognition of GAS nucleic acids is an important alarm likely to play a significant role in immune susceptibility to the pathogen.

Inflammasome recognition & IL-1β signaling

Inflammasomes are macromolecular protein assemblies that impact inflammatory responses to environmental stimuli and serve a key function in innate defense against diverse pathogens [56]. Dysregulated inflammasome activation, however, can predispose the host to chronic autoimmune and inflammatory pathologies, cancer or neurodegenerative diseases. Thus, inflammasome assembly and downstream signaling must be finely tuned to simultaneously promote rapid antimicrobial or inflammatory responses while limiting unwanted tissue injury [57].

Five sensor proteins have been proven to assemble inflammasomes in response to PAMPs and/or DAMPs, including the nucleotide-binding oligomerization domain (NOD), leucine-rich repeat containing receptor (NLR) family members NLRP1, NLRP3 and NLRC4 and the proteins absent in melanoma-2 (AIM2) and pyrin. Recognition of the correct inflammatory stimulus leads to sensor activation, oligomerization and recruitment of the adaptor protein ASC, and activation of cysteine protease caspase-1, leading to proteolytic processing of inactive pro-IL-1β and pro-IL-18 into their mature forms, IL-1β and IL-18-all hallmarks of inflammasome activation [58,59].

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IL-1β signaling is critical for defense against several streptococcal species [60], including GAS [61,62]. Knockout mice lacking the IL-1 receptor (IL-1R) and therefore unresponsive to IL-1β signaling are more susceptible to GAS infections, in part due to the contribution of IL-1β to neutrophil influx at the site of infection [61]. US FDA-approved IL-1β inhibitor Anakinra reduces MΦ killing of GAS [63] and facilitates GAS replication and dissemination in infected mice [62,63].

Most of the known inflammasomes respond to specific microbial stimuli. Lethal toxin of *Bacillus anthracis* and *Toxoplasma gondii* infection activates NLRP1 [64–66], AIM2 recognizes double-stranded DNA from lysed intracellular bacteria [67,68], NLRC4 responds to bacterial flagellin and type 3 secretion systems [69,70] while pyrin senses Rho GTPase modifications induced by bacterial toxins [71]. A role of these inflammasomes in GAS recognition has yet to be described. In contrast, the NLRP3 inflammasome responds to a surprisingly diverse set of PAMPs and DAMPs [72], and is a relevant contributor to MΦ detection of GAS; IL-1β production is impaired in NLRP3 [-/-] deficient mice infected with the pathogen [73].

Two GAS proteins were initially described to be detected by the canonical NLRP3 inflammasome, the pore-forming toxin SLO [73] and the ADP–ribosyltransferase SpyA toxin (Figure 3) [62]. In addition to activation of IL-1β signaling, these two GAS virulence factors induce a rapid, necrotic and inflammatory programmed cell death termed 'pyroptosis' [74]. Bacterial pore-forming toxins are often detected by the NLRP3 inflammasome complex [75], and SLO promotes immune evasion by accelerating cell death [76,77]. SLO is released as a monomer that binds cholesterol in cell membranes and oligomerizes to form large transmembrane pores [78] while concurrently activating IL-1β and pyroptosis through NLRP3 [73,79]. Activation of the NLRP3 inflammasome depends on two signals: a priming signal for upregulation of NLRP3 and pro-IL-1β, and a second signal that triggers assembly of the inflammasome complex (Figure 3) [80,81]. Consistent with this feature, co-stimulation of MΦs with recombinant SLO and certain TLR ligands, but not each stimulus alone, triggered caspase-1 activation through the NLRP3 inflammasome [82]. MΦ cell death ultimately provoked by SLO reduces IL-1β secretion, [76] and experiments in which MΦs were treated with modified SLO derivative that produce strands and altered pores could yield a more robust adjuvant effect through enhanced IL-1β secretion [79]. The precise mechanism of SLO detection by NLRP3 is not clear, although NLRP3 does not appear to directly sense GAS pore-forming toxins by direct binding [60]. Similar to other bacterial pore-forming toxins, SLO can activate other MΦ cell death pathways that have features of osmotic lysis, apoptosis and oncosis [76,83]. However, since pyroptosis occurs more rapidly, it may predominate over other cell death processes during many GAS–MΦ encounters in conjunction with SLO-mediated IL-1β signaling.

The NLRP3 inflammasome is activated by SpyA, a membrane-bound C3-like ADP–ribosyltransferase that contributes to GAS pathogenesis in mouse cutaneous infection models [84–86]. However, in systemic infection, SpyA-deficient GAS evade MΦ killing and cause higher mortality and bacterial burdens, suggesting that recognition of SpyA is important for host defense against GAS [62]. Anakinra treatment exaggerates these SpyA-dependent virulence phenotypes, highlighting the importance of IL-1β signaling for limiting GAS replication. SpyA triggers MΦ cell death along with NLRP3 and caspase-1 dependent IL-1β secretion, similarly to other ADP-ribosylating toxins from *Pseudomonas aeruginosa* [62] and *Mycoplasma pneumonia* [87]. However, the putative PRRs involved in the recognition of SpyA and its homologs upstream of NLRP3 activation still require elucidation. A single common receptor for this family of bacterial toxins is unlikely, however, since an ADP–ribosyltransferase toxin from *Clostridium botulinum* activates IL-1β signaling via the pyrin inflammasome [71].

In addition to caspase-1 activation and pyroptosis, SLO and SpyA also activate caspase-3 in MΦ [62,76], a phenomenon traditionally linked to apoptosis. Pyroptosis induction was recently shown to require gasdermin D (GSDMD), a member of the enigmatic gasdermin protein family [88–90]. GSDMD is cleaved by inflammatory caspases, resulting in the generation of an N-terminal fragment that leads to pyroptotic cell death. However, recent studies have shown that in the absence of GSDMD, caspase-1 activates caspase-3 and -7 to induce apoptosis. Conversely, during apoptosis, caspase-3/-7 specifically blocks pyroptosis by cleaving GSDMD at a distinct site from the inflammatory caspase-1, resulting in its inactivation and revealing a bidirectional crosstalk between apoptosis and pyroptosis in monocytes and MΦs [91].

In very recent work from our laboratory [37], we found that M protein, the most abundant protein on the GAS surface [92] and one of the best studied of all Gram-positive bacterial virulence factors [93], makes an independent and important contribution to activation of the NLRP3 inflammasome. GAS strains are immunologically classified into >220 serotypes based on M protein variation [94]. However, only a limited number of M protein/*emm* types are widespread and associated with severe invasive infections, with serotype M1/*emm1* being the most prevalent cause of severe invasive GAS infections in recent decades [95–97]. M proteins can be released during infection by the

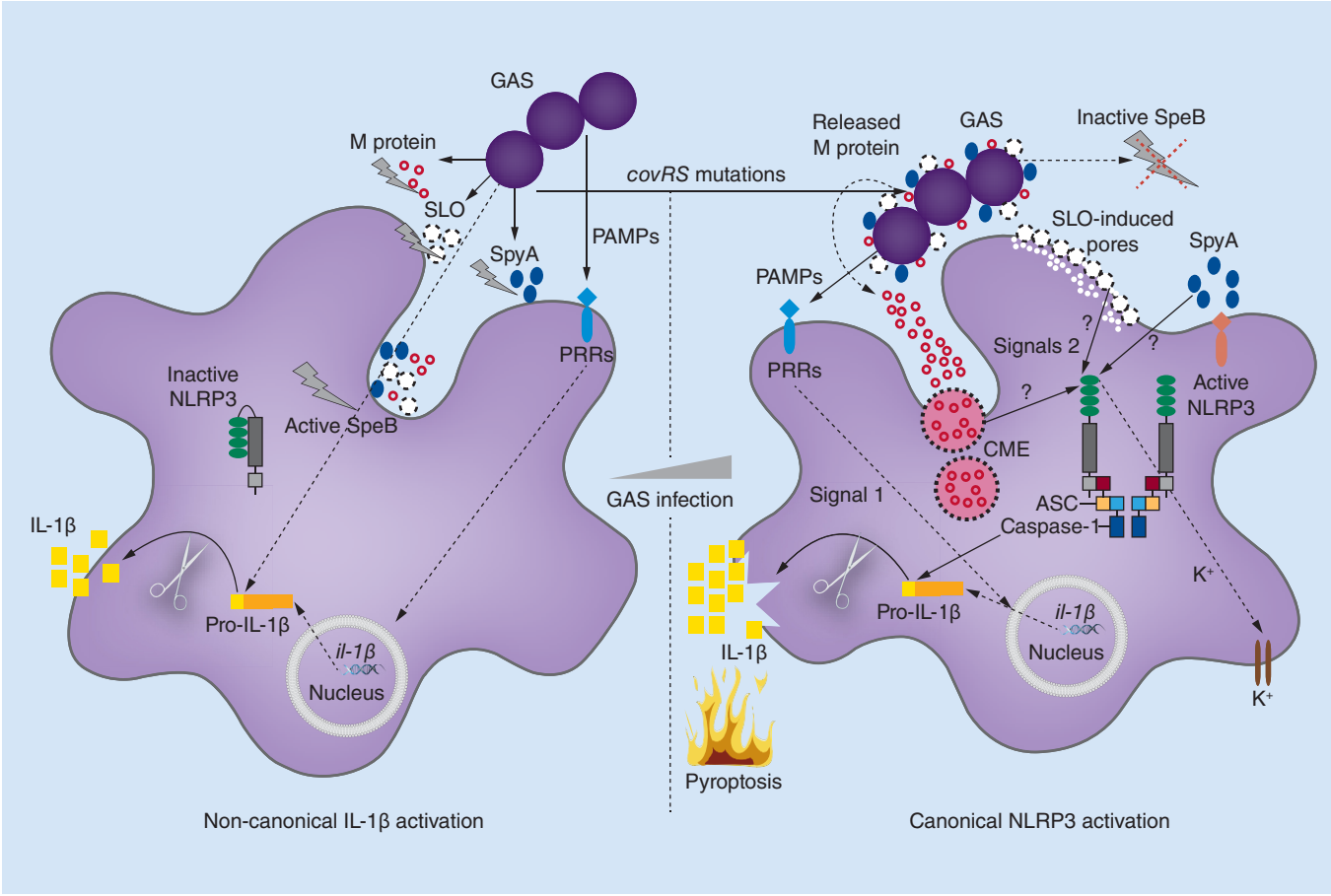

Figure 3. Comparison between group A *Streptococcus*-induced noncanonical and NLRP3 canonical IL-1β signaling activation. During early stages of group A *Streptococcus* (GAS) infection, the proteolytic activity of the GAS cysteine protease SpeB hastens degradation of all known GAS canonical NLRP3 activators: streptolysin O (SLO), SpyA and M protein. SpeB also elicits the maturation of the proinflammatory cytokine pro-IL-1β by direct cleavage to its mature form IL-1β, overriding the contribution of the NLRP3 inflammasome. During the progress from mild to severe invasive GAS infections, mutations of *covR/S* take place, inactivating SpeB, while accelerating the expression and release of SLO, SpyA and M protein. Canonical NLRP3 inflammasome activation depends on two signals. Pathogen-associated molecular patterns are recognized by specific pattern recognition receptors, to induce the transcriptional activation of *il-1β* and other inflammasome components (signal 1). SLO pore formation, uptake of soluble M protein by clathrin-mediated endocytosis (CME) and SpyA make an independent contribution to the activation and assembly of the NLRP3 inflammasome and trigger potassium efflux (K [+] efflux) through an unknown mechanism **(?)**. The assembly of the inflammasome leads to the caspase-1-dependent processing of pro-IL-1β to IL-1β, and resulting in DNA damage and pyroptotic cell death in macrophages.
PAMPs: Pathogen-associated molecular patterns; PRRs: Pattern recognition receptors.

action of neutrophil-derived granule proteases and exert proinflammatory effects through their interaction with host factors [98,99]. Released M1 protein and of itself triggers tissue injury and vascular leakage *in vivo*, similar to that observed in severe GAS NF and streptococcal toxic-shock syndrome [98,100–101]. Under physiological conditions, M1 protein is also released from the GAS surface and detected extracellularly in high concentrations [102]. M1, through its structurally dynamic B-repeat domain, serves as a second signal for caspase-1-dependent NLRP3 inflammasome activation, IL-1β maturation and MΦ pyroptosis. M1-induced MΦ IL-1β signaling involves K [+] efflux, a common step in NLRP3 inflammasome activation [103], and M1 internalization by clathrin-mediated endocytosis (Figure 3) [37]. GAS-infected mice produced more IL-1β *in vivo* than GAS lacking M1-infected mice. Indeed, soluble M1 was itself sufficient and specific for IL-1β activation *in vitro* and *in vivo* [37]. GAS-mediated canonical NLRP3 and IL-1β activation through the independent contributions of M1, SLO and SpyA exemplifies the complexity of GAS virulence factors and how the host applies a multifaceted approach to pathogen detection.

In a discovery that changed conventional dogma regarding IL-1β signaling in innate immunity, GAS was found to be capable of noncanonical, inflammasome-independent IL-1β activation, via direct cleavage of pro-IL1β by its

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broad-spectrum cysteine protease, SpeB. The most abundant secreted protein in GAS culture supernatants, SpeB also cleaves several GAS surface-associated and secreted virulence factors (Figure 3) [104,105]. In this fashion, IL-1β itself acts as a sensor to directly respond to pathogen-associated proteolysis through an independent pathway operating in parallel to inflammasomes [63]. Both canonical NLRP3 inflammasome and noncanonical IL-1β signaling occur in MΦs responding to a GAS encounter, and likely depend on the site, stage and magnitude of infection, from initial colonization to systemic disease. Of note, SpeB itself degrades the canonical inflammasome-activating GAS virulence factors SLO, SpyA and M protein [63,104,106], suggesting that noncanonical IL-1β signaling may predominate whenever SpeB expression is activated. However, in M1T1 GAS and certain other invasive serotypes, naturally occurring mutations in the two-component regulatory system CovR/CovS (encoded by *covRS* genes) can arise *in vivo*, leading to upregulation of several virulence factors, such as, SLO and SpyA, while suppressing expression of SpeB. Absence of SpeB spares the GAS canonical NLRP3 activators from proteolytic degradation and increases the levels of their soluble forms [5,104,107–108]. Epidemiological studies have found that SpeB expression and activity are significantly higher in GAS serotype M1T1 isolates from nonsevere invasive infections than isolates from severe cases [106,109–110]. Based on this evidence, we propose a model (Figure 3) wherein during early stages of GAS infection, all known GAS canonical NLRP3 activators are partially degraded by active SpeB proteolysis, which also directly processes pro-IL-1β to the active cytokine, providing an early signal to activate host immunity against the pathogen. During the progression of GAS infection *in vivo*, mutations of *covR/S* take place, silencing SpeB expression and eliminating the noncanonical IL-1β signaling response that helps guard against invasive infection. As the organism gains a foothold deep in the body or the bloodstream, expression and release of SLO, SpyA and M protein are upregulated and these factors are no longer degraded by SpeB. In later stages of infection, canonical NLRP3 inflammasome activation in response to these virulence determinants may be associated with hyperinflammation, aggravating tissue injury and toxic-shock syndrome.

Survival & replication of GAS in macrophages

Although GAS has traditionally been considered an extracellular pathogen, it can invade host cells and persist in an intracellular environment [111]. Intracellular reservoirs of viable GAS have been identified in different cell types, including epithelial cells [112,113], neutrophils [114] and MΦs [115–117]. GAS intracellular survival may promote persistent colonization and dissemination by shielding the bacterium from immune effectors and antibiotics. For many intracellular pathogens, survival within MΦs may be achieved by transitioning the host cell to an anti-inflammatory, immunomodulatory ('alternatively activated') M2 phenotype, in contrast to the proinflammatory, microbicidal ('classically activated') M1 phenotype commonly associated with infection [118]. In this context, human and murine MΦs differ in their responses to GAS. In humans, GAS induces a M1 profile characterized by enhanced mRNA expression of inflammatory chemokines, such as, CCL2, CCL5, CXCL8 and CXCL10 [119], whereas in mice, the pathogen stimulates an unusual activation program that combines M1 and M2 profiles [120]. The two MΦ phenotypes are defined by their cytokine/chemokine receptor profiles, and NFκB signaling drives their polarization [121]. Viable GAS has been recovered from biopsies of patients with soft tissue infections, and bacteria are present both extracellularly and intracellularly within phagocytic cells, primarily within MΦs [117]. In these studies, intracellular GAS was predominantly found in biopsies characterized by lower inflammation and bacterial load, whereas purely extracellular GAS or a combination of intra- and extra-cellular GAS dominated in severely inflamed tissue despite intravenous antibiotic therapy, suggesting that internalization can promote the spread of GAS within the tissue [117]. Different intracellular survival strategies of the pathogen have been proposed, including SLO-dependent evasion of lysosomal killing in epithelial cells [122] and GAS M1 protein-mediated inhibition of granule fusion with the phagosomes of neutrophils [123]. Internalization of GAS in host cells could provide a safe haven from immune cell and antibiotic-mediated killing *in vivo* [112,124]. In primary human monocyte-derived MΦs, GAS resides within spacious phagocytic vacuoles surrounded by an electron-dense membrane, and intracellular bacteria remain within these vacuoles up to 12 h [116]. An initial study showed *in vitro* evidence of a relationship between GAS survival and M1 protein-dependent intracellular trafficking in the phagosomal–lysosomal pathway, which resulted in impaired fusion with lysosomes [116]. However, another study found the partnership between SLO and its intimately associated co-toxin NAD-glycohydrolase (NADase) to drive GAS intracellular survival during infection in MΦs by two mechanisms [115]. SLO-induced pore formation prevents acidification of the GAS-containing phagolysosome, thereby impairing effective bacterial killing. SLO-mediated translocation delivers NADase from the phagosome into the MΦ cytosol, where NADase compounds SLO cytotoxicity by inhibiting cellular repair mechanisms (Figure 1) [115]. In epithelial cells by contrast, both toxins inhibit transport of the GAS-

containing vacuole to a functional bactericidal compartment by inhibiting fusion with lysosomes [122,125–126]. GAS resistance to phagocytosis and escape from the phagolysosome machinery may be exaggerated in invasive infections, when *covRS* spontaneous mutations markedly upregulate both SLO and NADase [127–129]. Single cell and time-lapse microscopy analysis demonstrated that GAS not only survived in MΦs, but replicated intracellularly to form strikingly long chains (Figure 1) [130], reminiscent of chain elongation seen in response to other environmental stresses such as reduced pH and nutrient limitation. SLO expression, but not M protein, is also required for replication of GAS within the MΦ [130]. SLO-dependent phagosomal rupture leads to a significant proportion of cytosolic GAS. These recent findings support the idea that the intracellular compartment is a place of silent sequestration for the pathogen [111].

Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome and is mediated by a unique organelle, the autophagosome [131–134]. Distinct from endocytosis-mediated lysosomal degradation [133], the autophagic response against intracellular pathogens is named xenophagy, involving selective uptake of invading microorganisms via signals, autophagic adaptors and receptors, and delivery of the cargo to autophagosomes [135,136]. In autophagy-deficient cells, GAS shows enhanced survival and proliferation [137,138], whereas in cells with enhanced baseline autophagy, GAS is more rapidly cleared [139]. After escaping from endosomes into the cytoplasm, GAS is targeted for autophagic degradation via ubiquitination and LC3 labeling, a critical marker in the autophagy pathway, resulting in the formation of GAS-containing, autophagosome-like vacuoles (GcAVs), wherein the pathogen is subsequently killed upon fusion of these compartments with lysosomes (Figure 1) [138]. GAS can also directly induce autophagy upon recognition by the cell surface receptor CD46, without any further delay [137]. Host small G proteins, including Rab5 and Rab7, are associated with autophagosome formation and the fate of intracellular GAS. Rab5 is involved in bacterial invasion and endosome fusion, while Rab7 is multifunctional, contributing to bacterial invasion, endosome maturation and autophagosome formation [140,141]. Other small GTPases, such as Rab9A and Rab23, also play roles in xenophagic clearance of GAS [142].

Successful activation of the autophagic machinery and elimination of GAS by the cell depends on the GAS strain and its repertoire of virulence determinants. Certain strains from serotypes not commonly associated with human invasive disease epidemiology, including M6 [137–138,140], M49 [137] and M89 [143] are efficiently targeted to autophagy and cleared, whereas globally disseminated M1T1 clone of GAS replicates efficiently in the cytosol of infected epithelial cells. M1T1 GAS evades autophagy through SpeB-mediated degradation of the ubiquitin-autophagy adaptor proteins p62, NDP52 and NBR1 [144]. However, the phenomenon wherein SpeB impairs GAS-induced autophagy in epithelial cells is less prominent in MΦs, since both M1T1 5448 and M49 NZ131 GAS strains, which harbor active SpeB, are recognized by ubiquitin and p62 and associated with LC3 [130]. Not all cytosolic bacteria are targeted to the xenophagy pathway in MΦs, indicating a subpopulation of cytosolic GAS that is not recognized or evades autophagy-mediated growth attenuation following GcAV rupture [130]. The molecular basis for replication of GAS within the MΦ cytosol, and whether specific bacterial virulence determinants are targeting the autophagic machinery of these professional phagocytic cells, still need to be elucidated (Figure 1). Future comparisons of the stage and efficiency of the phagosomal–lysosomal versus autophagy pathways will lead to a better understanding of the intracellular GAS fate, and perhaps identify additional autophagic adaptors and regulatory mechanisms that specifically target, attack and degrade the pathogen. In this sense, autophagy and phagocytosis may overlap mechanistically. In *Mycobacterium*, activation of TLR signaling enhances phagosome maturation and at the same time, increases recruitment of the bacteria into autophagosomes [145]. Accordingly, LC3, the critical component in the autophagy pathway, can be recruited to phagosomes following the exposure of MΦs to TLR agonist-coated beads or zymosan [146]. New evidence highlights an interaction between autophagy and phagocytosis, as autophagy-deficient MΦs have increased levels of class A scavenger receptors, leading to higher phagocytic uptake [147].

Conclusion & future perspective

MΦs are essential in the host response to GAS, not just as professional detectors of the bacteria, resulting in the release of inflammatory alarms, but also as direct killers through phagocytosis and xenophagy. However, survival and replication versus recognition, inflammatory response and killing mechanisms, cannot be considered as single events, since many of them overlap and are dependent on each other. This review has highlighted events that occur during the different stages of GAS–MΦ interaction, and the various virulence strategies that GAS deploys to coordinate its escape from phagocytic cell clearance. Further efforts to identify in more detail the specific interactions

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and the integration of both bacteria and cell strategies for survival may allow the development of new virulence inhibitors or host-directed therapeutic options against this important human pathogen.

Executive summary

Group A *Streptococcus* in human infection

- Group A *Streptococcus* (GAS) is a leading human pathogen on a global scale, responsible for several diseases ranging from mild to severe infections, and potential immune sequelae after repeated GAS exposures.
- Nearly 2 million new cases of serious GAS disease occur each year, accounting for 517,000 deaths annually, placing GAS among the top 10 causes of human infectious disease mortality.
- GAS is protected by a multitude of surface-bound and secreted virulence factors that are variably expressed among GAS strains and from different host susceptibilities.

Macrophages (MΦs) play a critical role during GAS infections

- MΦs are strategically located throughout the body tissues where they ingest and process microorganisms.
- MΦs are highly effective at phagocytosis because of the strong and rapid acidification of their phagosomes, activating proteases and peptides that inhibit bacteria growth or destroy the entire phagocytic cargo.
- Efficient MΦ phagocytosis and killing of GAS has been demonstrated *in vitro*, and resident MΦs aid in GAS clearance during *in vivo* infection.
- Depletion of MΦs or inhibition of their phagocytic properties leads to a substantial increase in GAS dissemination from tissues into blood and internal organs, supporting the key role of this immune cell type in the control of GAS virulence.

Toll-like receptors mediated MΦ recognition of GAS

- MΦ express a multitude of pattern recognition receptors to detect signals that are not normally found in healthy tissues.
- Toll-like receptor (TLR) family is the best characterized of pattern recognition receptors and is responsible for sensing GAS outside of the cell and in intracellular endosomes and lysosomes.
- GAS-induced TLR signaling pathways result in the production of inflammatory cytokines and IFN-β, in a manner completely dependent on adaptor MyD88.
- Although the upstream recognition of GAS by TLRs is incompletely understood, different GAS molecules have been reported to be recognized by specific TLRs.

Inflammasome-mediated recognition of GAS & IL-1β signaling

- Inflammasomes are key elements of innate immunity recognized to control the inflammatory response to various stimuli and for their crucial role in host defense against pathogens.
- Activation of IL-1β signaling is critical in defense against several streptococcal species, including GAS.
- NLRP3 inflammasomes respond to a surprisingly diverse set of pathogen-associated molecular patterns and damage-associated molecular patterns, and make a significant contribution to MΦs detection of GAS.
- Three GAS virulence factors – pore-forming toxin streptolysin O (SLO), ADP–ribosyltransferase SpyA and M protein – are detected by NLRP3, each making an independent contribution to IL-1β signaling.
- As a consequence of canonical NLRP3 activation, SLO, SpyA and M protein induce pyroptotic cell death in macrophages.
- GAS also induces noncanonical IL-1β activation via direct cleavage of the cytokine precursor by SpeB, a broad-spectrum, GAS-secreted protease.

Survival & replication of GAS in macrophages

- Although traditionally considered an extracellular pathogen, GAS invades host cells and persists in their intracellular environment.
- GAS intracellular survival may promote persistence, pathogen colonization and dissemination of the infection *in vivo*.
- Once inside the MΦs, GAS resides within spacious phagocytic vacuoles surrounded by an electron-dense membrane; intracellular GAS remains within these vacuoles up to 12 h.
- SLO and its intimately associated co-toxin NAD-glycohydrolase (NADase) are a key GAS virulence mechanism driving GAS intracellular survival and resistance to MΦ killing.
- Surviving intracellular GAS can replicate and even form long chains within human MΦs.
- GAS replication occurs in the macrophage cytosol after SLO-dependent phagosomal rupture, leading to a significant proportion of cytosolic bacteria.
- After escaping from endosomes into the cytoplasm, GAS is targeted by the autophagy machinery (xenophagy), which can deliver bacteria to be eliminated in autophagosomes.

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