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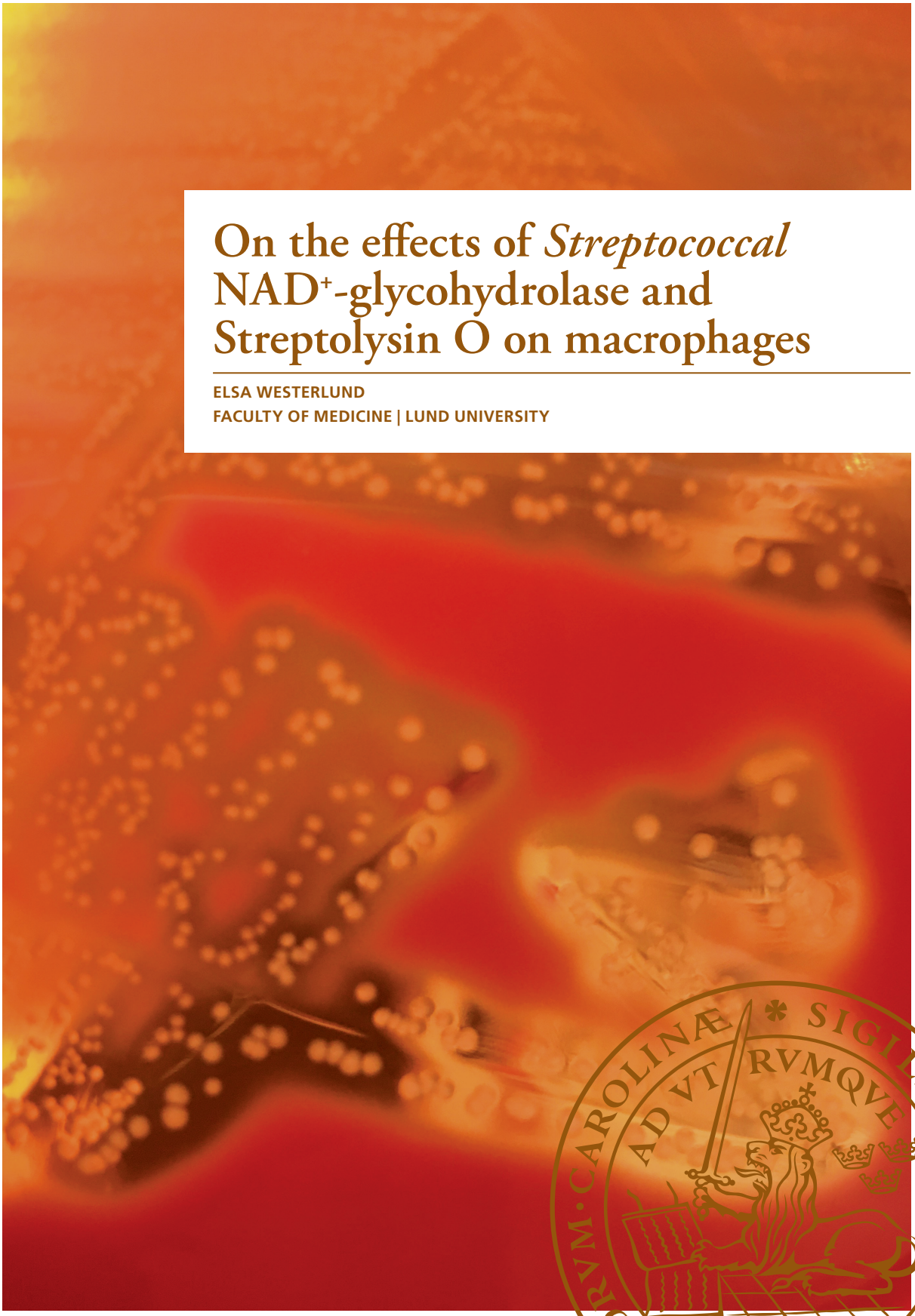

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On the effects of *Streptococcal* NAD^+-glycohydrolase and Streptolysin O on macrophages

Elsa Westerlund

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Title

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Summary

The human pathogen *Streptococcus pyogenes* (GAS) causes both superficial infections, such as strep throat, and invasive infections, such as necrotizing fasciitis, and is responsible for about half a million deaths each year. The number of GAS infections have increased since the 1980's, due to the emergence of a M1T1 strain that has become widely disseminated. Increased expression of Streptolysin O (SLO) and NAD^+-glycohydrolase (NADase) has been correlated with the emergence of this strain. This thesis has focused on how these two virulence factors affect cytokine release from macrophages, an innate immune cell important for the host's defense against GAS.

In Paper I we describe a novel function for NADase: inhibition of IL-1β release after NLRP3 inflammasome activation. This inhibitory effect was mediated by extracellularly located NADase, which represents a novel functional niche for this enzyme.

In Paper II, we explore this effect further and show that NADase inhibits an unconventional IL-1β release pathway that is dependent on the P2X7 receptor and membrane permeabilization. Interestingly, we see that IL-1β release in response to GAS is independent of the pore-forming protein GSDMD.

In Paper III we show that pro-IL-1β is ubiquitinated and degraded during GAS infection. Pro-IL-1β ubiquitination requires the presence of SLO and results in heterotypic linkage types. The broad PI3K inhibitor 3-MA rescues pro-IL-1β degradation.

Paper IV focuses on NADase and the observation that NADase binds macrophages in the absence of SLO, in contrast to what has been shown previously. Recombinant NADase binds macrophages in the absence of other bacterial proteins and induces cytokine release that requires TLR4 and CD14 and is dependent on MyD88 and TRIF.

Key words

Innate immunity, macrophage, *Streptococcus pyogenes*, IL-1β, NAD^+-glycohydrolase

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Cover photo: *Streptococcus pyogenes* colonies on a blood agar plate, taken by Elsa Westerlund

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Abbreviations

3-MA	3-methyladenine
ABC	ATP-binding cassette
ADP	Adenosine di-phosphate
ADP-r	ADP-ribose
AIM2	Absent in melanoma 2
AMP	Anti-microbial peptide
ALR	AIM2-like receptor
APC	Antigen-presenting cell
ASC	Apoptosis speck-like protein containing a CARD
ATP	Adenosine tri-phosphate
BCR	B cell receptor
CARD	Caspase activation and recruitment domain
CD	Center of differentiation
CDC	Cholesterol-dependent cytolysin
cGAS	cyclic GMP-AMP synthase
CLR	C-type lectin receptor
CMT	Cytolysin-mediated translocation
CovRS	Control of virulence regulatory system
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DUB	Deubiquitinase enzyme
FADD	Fas-associated protein with a death domain
FGF-2	Fibroblast growth factor 2

GAC	Group A carbohydrate
GAS	Group A Streptocococcus (*Streptococcus pyogenes*)
GSDMD	Gasdermin D
GSK	GlaxoSmithKline
HAMP	Homeostasis-altering molecular process
HGT	Horizontal gene transfer
IFS	Immunity factor for *Streptococcus pyogenes* NADase
IKK	IκB kinase
IL	Interleukin
IL-1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist
IFN	Interferon
IRAK	IL-1 receptor associated kinase
IRF	IFN regulatory factor
K	Lysine
K^+	Potassium
LBP	LPS-binding protein
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
Mg^{2+}	Magnesium
MHC-II	Major histocompatibility complex II
MMP	Matrix metalloproteinase
MVB	Multivesicular bodies
MyD88	Myeloid differentiation primary response 88
β-NAD^+	β-nicotinamide adenine dinucleotide
NADase	β-NAD^+-glycohydrolase
NAIP	NLR family of apoptosis inhibitory proteins

NAM	Nicotinamide
NEK7	NIMA-related kinase 7
NF	Necrotizing fasciitis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide-binding domain, LRR-containing receptor
NLRC	NLR and CARD-containing receptor
NLRP	NLR and PYD-containing receptor
P2R	Purinergic receptor
P2X7R	P2X7 receptor
PAMP	Pathogen-associated molecular pattern
PI	Propidium iodide
$PI(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
POP	PYD-only protein
PRR	Pattern-recognition receptor
PYD	Pyrin domain
RIPK	Receptor-interacting serine/threonine-protein kinase
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
ROS	Reactive oxygen species
SAg	Superantigen
SLO	Streptolysin O
SLS	Streptolysin S
SNP	Single nucleotide polymorphism
Spe	Streptococcal pyrogenic exotoxin
STING	Stimulator of IFN gene
STSS	Streptococcal toxic shock syndrome
TAK1	TGF-β-activated kinase 1
TCR	T cell receptor
TIR	Toll-IL-1 receptor

TIRAP	TIR-containing adaptor protein
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF6	TNF-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor-inducing IFN-β
UBD	Ubiquitin-binding domain
wt	Wild-type

Popular scientific summary

Our immune system faces a multitude of challenges daily: it not only needs to notice differences between our own cells and those of invaders, it is also required to recognize whether a microorganism is one of our harmless symbionts or if it has the potential to cause inflammation and disease. To fight breaches in our defense we need patrolling soldiers ready to battle a wide range of enemies at a moment's notice; to resolve conflicts we need agents that are specially trained for each case. Our body has solved these issues by splitting the immune system into two parts: the innate and the adaptive. Cells of the innate immune system are trained to recognize characteristics common to a wide range of bacteria and viruses, enabling them to quickly mount a protective response and alert the adaptive immune system. The adaptive cells are specially trained to recognize specific pathogens and able to make clones of themselves when activated; the whole troop is then deployed to the site of combat.

Numerous studies on pathogenic microorganisms have shown us that we are part of a continuous arms race, but it is seldom clear who has the upper hand. This thesis has studied a small part of this arms race, where a bacterium known as Group A *Streptococcus* (GAS, Latin name *Streptococcus pyogenes*) uses two weapons, so-called virulence factors, to dampen the levels of alarm signals released by macrophages, a type of innate immune cell.

GAS gives rise to a wide range of diseases, from common and mild strep throat and impetigo to potentially life-threatening and invasive infections such as necrotizing fasciitis (known as the "flesh-eating disease") and toxic shock syndrome. With this repertoire it's not surprising to learn that GAS is high up on WHO's list of infection-related deaths worldwide. Antibiotic resistance in GAS is not (yet) a problem, but the invasive diseases often progress faster than the effect of antibiotics. There's been an increase in invasive GAS disease cases since the 1980's. Bacteria isolated from these unfortunate patients revealed that a lot of infections were caused by the same strain of GAS that had spread rapidly around the world. Genetic comparisons of this new strain and older ones showed that one of the differences between them was that the new strain produced increased levels of the two virulence factors Streptolysin O (SLO) and NADase.

Previous studies on SLO showed that it is able to kill macrophages by making large holes in them, but that macrophages on the other hand use a warning system called

the inflammasome to detect these holes and release a warning signal called IL-1β to attract other immune cells. Our studies have shown that NADase works to dampen the amount of IL-1β released, by somehow inhibiting a macrophage surface molecule called P2X7. We also discovered that SLO can cause breakdown of IL-1β inside the macrophage. That both SLO and NADase affected IL-1β was very interesting, since this signal molecule has been shown to be very important in combating GAS. This could mean that the bacterium uses SLO and NADase to prevent activation of the immune system. More research into this area would give us valuable information about the infection process, but it would also award us with increased knowledge of how our immune cells work.

Populärvetenskaplig sammanfattning

Vårt immunförsvar står inför många olika prövningar varje dag: det måste inte bara kunna göra skillnad på kroppens egna celler och inkräktare, utan behöver också kunna skilja på mikroorganismer som är våra ofarliga hyresgäster och de som har potential att orsaka sjukdomar. När våra barriärer rämnar behövs patrullerande soldater som är redo att slåss och slå larm vid första anblick av hot, men för att lösa konflikterna behövs också en trupp agenter som är specialtränade för varje fall. Vår kropp har löst detta genom att dela upp immunförsvaret i två delar: det medfödda och det adaptiva. Cellerna som tillhör det medfödda immunförsvaret har tränats för att känna igen varningssignaler gemensamma för många olika sorters bakterier och virus, vilket gör att de snabbt kan börja oskadliggöra hoten och skicka varningssignaler till det adaptiva immunförsvaret. När en adaptiv cell, specialtränad för att känna igen just denna inkräktare, aktiveras börjar den klona fler kopior av sig själv och den samlade truppen ger sig sedan ut i kroppen för att bekämpa hotet.

Om det är något vi lärt oss av många års studier av sjukdomsframkallande bakterier är det att vi befinner oss i en ständig kapprustning, där det sällan är klart vem som har överhanden. Den här avhandlingen handlar om en del av denna kapprustning – hur bakterien *Streptotoccus pyogenes* (oftast kallad Grupp A Streptokocker (GAS)) använder sig av två vapen, så kallade virulensfaktorer, för att dämpa varningssignaler från makrofager, en del av det medfödda immunförsvaret.

GAS ger upphov till infektioner av väldigt olika karaktär, från milda sjukdomar såsom halsfluss och svinkoppor till de potentiellt livshotande (invasiva) infektionerna nekrotiserande fasciit (mer känd som köttätande sjukdom) och toxiskt chocksyndrom. Med en sådan bred sjukdomsrepertoar är det inte konstigt att GAS finns med i toppen på WHO:s lista över infektionsrelaterade dödsorsaker världen över. Som tur är går det fortfarande att behandla de flesta GAS-infektioner med antibiotika, men när det gäller de invasiva infektionerna är sjukdomsförloppet ofta för snabbt för att det ska hinna verka. Sedan 1980-talet har det skett en ökning i antal rapporterade fall av invasiva infektioner. Bakterier tagna från dessa patienter visade att många av sjukdomsfallen orsakats av en och samma bakteriestam som snabbt spridits världen över. Genom genetiska jämförelser av denna stam och äldre stammar har man kommit fram till att det som skiljer dem åt är bland annat att den nya stammen producerar mer av de två virulensfaktorerna Streptolysin O (SLO) och NADase.

Tidigare studier har visat att SLO kan döda makrofager genom att göra stora hål i dem, men makrofagerna kan å andra sidan upptäcka dessa hål genom ett varningssystem som kallas inflammasomen och snabbt utsöndra en signalsubstans, IL-1β, som lockar till sig andra celler ur immunförsvaret. Våra studier har visat att NADas kan minska mängden IL-1β som utsöndras, och att detta sker genom att NADas på något sätt hämmar en av makrofagens ytmolekyler som kallas P2X7. Vi har också sett att SLO kan göra så att IL-1β bryts ner inne i cellen istället för att utsöndras. IL-1β har tidigare visats spela en viktig roll i att dämpa GAS-infektioner, så att det var just den signalsubstansen som både SLO och NADas påverkade var väldigt intressant, eftersom det skulle kunna innebära att bakterien använder dem för att undgå att upptäckas av immunförsvaret. Vidare forskning i exakt hur SLO och NADas hämmar IL-1β kommer ge viktig information om infektionsförloppet i stort men också göra att vi fördjupar våra kunskaper om hur våra immunceller fungerar.

Introduction to the immune system

Our first line of defense against invading pathogens consists of the physical and anatomical barriers that must be overcome in order to gain access to the host, *e.g.* our skin, the mucociliary apparatus of our respiratory tract and the low pH in our stomach. If these barriers are successfully breached, the next obstacle is our immune cells, classically divided into innate and adaptive. Innate immune cells recognize conserved signals relating to pathogens and danger molecules and are armed with antimicrobial defense systems such as phagocytosis and antimicrobial peptides (AMPs), enabling them to rapidly mount a response.[1] They are also tasked with initiating activation of adaptive immunity, leading to a highly specific immune response and the generation of persisting memory cells.[2]

The innate immune system includes professional phagocytes and antigen-presenting cells (APCs) such as macrophages, dendritic cells (DCs) and neutrophils.[1] They are aided by non-professional phagocytes, *e.g.* epithelial cells, and by the AMP-containing granules found in natural killer cells, eosinophils and basophils.[1] In addition, circulating macromolecules such as complement proteins and lipopolysaccharide (LPS)-binding protein (LBP) also participate in host defense and make up humoral innate immunity.[1,3] Recognition of pathogens is mediated by a number of germline-encoded receptors that recognize conserved bacterial and viral structures.[4] Upon activation (which will be dealt with in molecular detail in the following chapters) innate cells release cytokines and chemokines to recruit and activate circulating immune cells to the site of infection.[2]

Innate immune cells such as macrophages are divided into subsets depending on location, function and developmental stage. Macrophage precursors, monocytes, are derived from hematopoietic stem cells and can be found in the spleen or circulating in the blood, awaiting recruitment signals.[3] Upon sensing danger, they can migrate into the tissue and differentiate into mature macrophages, joining specialized tissue-resident macrophages such as alveolar macrophages in the lung and Kupffer cells in the liver.[3] Tissue-resident macrophages are derived from embryonic progenitors and their capacity for self-renewal makes sure that their numbers are constantly replenished.[5] Macrophages can also be viewed of as being on a spectrum, where on one end M1 macrophages are involved in the response against bacteria and viruses and on the other M2 macrophages are involved in wound and tissue healing and

resolving immune responses.[3] However, there is great plasticity and overlap between these subsets.

While the strength of innate immunity lies in its speed, the T and B cells of adaptive immunity instead have the potential to recognize virtually any antigen. Through a complicated process involving random rearrangements of gene segments, highly specific T cell receptors (TCR) and B cell receptors (BCR) are generated.[6] After a rigorous selection process to weed out cells expressing *e.g.* self-reacting receptors, T and B cells migrate from primary lymphoid organs (thymus and bone marrow) to secondary lymphoid organs (spleen and lymph nodes) where they await activation by APCs or soluble antigens, leading to proliferation and differentiation.[2,6]

Activated T cells migrate to effector sites and are roughly divided into cytotoxic or helper subsets. Cytotoxic T cells express center of differentiation (CD) 8 ($CD8^+$ T cells) and are involved in killing infected host cells, while helper T cells express CD4 ($CD4^+$ T cells) and aid in activating B cells, phagocytes and other T cells by releasing specific cytokines.[6] Depending on environmental cues, such as cytokines released by APCs, $CD4^+$ T cells differentiate into distinct subsets with specific functions and roles in influencing the immune response, *e.g.* by releasing a specific repertoire of cytokines.[2]

The humoral part of adaptive immunity is mediated by antibodies that can bind and neutralize pathogens and mediate immune cell activation.[7] Activated B cells can differentiate into short-lived plasma cells which produce a high number of low-affinity antibodies, or they can establish germinal centers, where long-lived plasma cells generate high-affinity antibodies of different isotypes, which differ in their ability to *e.g.* activate the complement pathway and bind to APCs.[8]

The antigen-specific T and B cells that persist after a primary infection is resolved are called memory cells and ensure efficient activation of the immune system if the pathogen is encountered again, thus creating a library of all the challenges our body has faced. Despite the longevity and specificity of the adaptive immune response it is only shared among vertebrates; other multicellular organisms rely solely on innate immunity, highlighting its fundamental role in our defense system.[1]

This thesis focuses on the interactions between macrophages and the human pathogen Group A *Streptococcus* (GAS; *Streptococcus pyogenes*), with particular interest in how production and release of the cytokine interleukin (IL) 1β (IL-1β) is regulated. As promised above, the following sections describe the events taking place when pathogens or danger signals are detected. It is followed by sections on ubiquitination and IL-1β release pathways and ends with a description of GAS before going into our present research and results.

Recognition of pathogens and danger signals

In 1989, Charles Janeway proposed a model for how innate immunity distinguishes self from non-self and activates adaptive immune responses in response only to the latter: a set of receptors expressed by all innate cells, pattern-recognition receptors (PRRs), that would be able to recognize pathogen-specific structures, pathogen-associated molecular patterns (PAMPs).[9] In the decade after this, a few seminal studies proved this to be true. The discovery that humans express a functional and genetical homologue of the *Drosophila melanogaster* protein Toll,[10] important for the fly's antifungal responses,[11] gave us the first PRR but not its PAMP. The PAMP, as well as the gene encoding that first human Toll-like receptor (TLR), was resolved a year later when it was reported that a defective *tlr4* gene prevented a response against LPS.[12] Since then a multitude of studies have identified additional PRRs and their ligands, leading to a modification of Janeway's first hypothesis: PRRs are not limited to recognizing PAMPs but also react to signs of cell damage (damage-associated molecular patterns [DAMPs]) and disruption of homeostasis (homeostasis-altering molecular processes [HAMPs]).[13] Recent efforts have been made to understand how the innate response is modulated, *e.g.* how are the commensal bacteria in our gut – which also express PAMPs – distinguished from pathogens? The presence of virulence factors that activate several types of PRRs are thought to be one clue, compartmentalization another; detection of PAMPs in the gut lumen is normal, while their presence in the lamina propria indicates that the epithelial lining has been breached and that an immune response needs to be initiated.[2]

The PRRs discovered so far can be divided into TLRs, C-type lectin receptors (CLRs), nucleotide-binding domain, leucine-rich repeat (LRR)-containing receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs) and stimulator of interferon (IFN) gene (STING) and cyclic GMP-AMP synthase (cGAS).[14] NLRs, RLRs, ALRs, STING and cGAS survey the cytoplasm while CLRs are present in the plasma membrane; TLRs mediate signaling from both plasma membrane and intracellular endosomes and are the most well-studied class of PRRs.[14,15]

Surface-associated PRRs

A common fate after a PRR recognizes its ligand is activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which can lead to expression and release of several inflammatory cytokines.[15] For CLRs such as Dectin-1 this happens in response to glucans from fungi and mycobacteria,[14] while cell-surface TLRs generally recognize microbial membrane components, as exemplified by the LPS-TLR4 and flagellin-TLR5 interactions.[16] TLR activation occurs after the formation of homo- or heterodimers; the ability of TLR2 to recognize diverse ligands from bacteria, viruses and fungi is thought to be due to its ability to form heterodimers with TLR1 and TLR6.[15] Pattern recognition depends on the horseshoe-like extracellular domain made up of LRRs which is connected to a transmembrane domain and a cytosolic Toll-IL-1 receptor (TIR) domain required for recruitment of adaptor molecules to mediate signaling.[17] Two sets of adaptor proteins mediate the downstream signaling events following TLR activation. As the events following TLR4 activation can be viewed as a prototypical TLR signaling cascade, the following section will describe this in detail.

TLR4 and LPS, recognition and signaling

As LPS is not a direct ligand for TLR4, several coreceptors and adaptor proteins are required for initiating the signaling cascade downstream of LPS sensing.[18] LBP binds LPS and transfers it to the coreceptor CD14, which in turn mediates transfer to MD-2 which is bound to TLR4, initiating receptor dimerization.[14] The dimerization mediates recruitment of the TIR-containing adaptor molecules TIR-containing adaptor protein (TIRAP) and myeloid differentiation primary response 88 (MyD88).[19,20] MyD88, used by all TLRs except TLR3, forms a complex termed the myddosome with the serine/threonine kinase IL-1 receptor-associated kinase 4 (IRAK4) and IRAK1, leading to their activation.[14] IRAK1, together with the E3 ubiquitin ligase tumor necrosis factor (TNF)-associated factor 6 (TRAF6), recruits and ubiquitinates a complex which includes transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1).[17] This allows for activation of the IκB kinase (IKK) complex, which frees NF-κB from its inhibitor and permits it to travel to the nucleus and induce gene expression of proinflammatory genes such as cytokines.[15] The TAK1 complex also activates mitogen-activated protein kinase (MAPK) family members, leading to induction of genes controlled by the transcription factor activator protein 1, which controls processes such as cell differentiation and death.[14]

TLR4 is unique among the TLR family in that it can signal both from the surface and from endosomes. After CD14 mediates endosomal uptake of TLR4,[21] the adaptor proteins TIR domain-containing adaptor-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) are recruited.[15,19,22] TRIF interacts with TRAF6,

which recruits receptor-interacting serine/threonine-protein kinase 1 (RIPK1), leading to activation of the TAK1 kinase and the transcription factors NF-κB and IFN regulatory factor 3 (IRF3), the former inducing expression of proinflammatory genes such as pro-IL-1β, important for antibacterial responses, and the latter resulting in production of type I IFNs, important in *e.g.* antiviral responses.[4,15]

Intracellular PRRs

PRRs located in endosomes or the cytosol generally detect the presence of intracellular pathogens. For example, TLR3, TLR7 and TLR9 respond to endosomal DNA and single- or double-stranded RNA, *e.g.* released by pathogens residing in endosomes, or liberated after phagocytic uptake and destruction.[14] TLR9 and TLR7 induce a MyD88-dependent pathway that, apart from activating NF-κB, also induces release of type I IFN *via* the transcription factor IRF7.[4,17] TLR3 instead induces type I IFNs through a pathway shared with endosomal TLR4.

RLRs induce NF-κB and IRFs in response to cytosolic viral RNA, while STING and cGAS recognize cytosolic DNA and trigger production of both type I IFNs and proinflammatory cytokines.[14,23] The NLRs NOD1 and NOD2 induce proinflammatory genes upon detection of bacterial cell wall degradation products,[15] while activation of other NLRs, and some ALRs, leads to formation of inflammasomes and production of IL-1β,[24] which will be described in detail below.

Inflammasomes

The term inflammasome was coined in 2002 to describe high-molecular weight complexes that mediate activation of inflammatory caspases.[25] What seemed like a simple system – a sensor, an adaptor and caspase-1 – has blossomed into a diverse field of sensors, activation signals and regulatory mechanisms. Inflammasome formation occurs when the activated sensor protein oligomerizes, allowing recruitment of the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD [caspase activation and recruitment domain]) *via* their mutual pyrin domains (PYD).[26] This induces ASC polymerization and the formation of a filamentous structure termed the ASC speck or pyroptosome, which serves to amplify downstream signals.[27] The ASC speck can also be released extracellularly to increase inflammation.[28] Caspase-1 is recruited to ASC *via* its CARD, leading to proximity-induced autoprocessing of caspase-1 and downstream responses: cleavage and activation of pro-IL-1β, pro-IL-18 and the pore-forming protein Gasdermin D (GSDMD), induction of an inflammatory form of cell death called

pyroptosis and release of signaling molecules called eicosanoids from intestinal epithelial cells.[24,29]

Five different sensor proteins have been described to form inflammasomes: the NLR proteins NLR and PYD containing receptor 1B (NLRP1B), NLRP3 and NLR and CARD containing receptor 4 (NLRC4), the ALR AIM2, and pyrin.[30] Proteins such as NLRP6, NLRP12 and IFNγ-inducible protein 16 have also been suggested to form inflammasomes, but the details surrounding them have not been elucidated.[31,32]

Although not the first described, the AIM2 inflammasome best fits the classical view of PRR-PAMP activation in that AIM2 directly binds its ligand, dsDNA of host or microbial origin, and recruits ASC and caspase-1.[33,34] In contrast, activation of NLRP1B, the first inflammasome sensor identified,[25] is more complicated. In response to *e.g.* lethal factor from *Bacillus anthracis*, N-terminal cleavage and partial proteasomal degradation of NLRP1B releases a C-terminal part responsible for caspase-1 activation.[35,36] The NLRC4 inflammasome relies on NLR family of apoptosis inhibitory proteins (NAIPs) to recognize intracellular flagellin and the rod and needle subunits of type III secretion systems from Gram-negative bacteria, leading to NAIP-NLRC4 colocalization and inflammasome formation.[37–39] Pyrin, the newest addition to the inflammasome family, recognizes inactivation of the GTPase RHOA caused by bacteria such as *Clostridium difficile* and can thus be classified as a HAMP sensor.[13,40] Despite being the most well-studied, several questions still surround NLRP3 inflammasome activation and regulation, presented in detail below.

The NLRP3 inflammasome

NLRP3 contains an N-terminal PYD, a central oligomerization domain and a C-terminal LRR involved in regulation of activation.[26,41] NLRP3 inflammasome activation has been suggested to augment Alzheimer's and Parkinson's disease and inflammatory diseases such as atherosclerosis and type 2 diabetes.[30] In addition, mutations in *nlrp3* give rise to cryopyrin-associated periodic syndromes, a group of hereditary autoinflammatory diseases presenting with symptoms such as recurring fever and skin rashes.[30]

What separates it from other inflammasomes is that NLRP3 activation is a two-step process. An initial priming signal is required to license NLRP3 for activation, involving induction of post-translational modifications and an increase in production of NLRP3 and pro-IL-1β.[41] The activation signal can come from a wide range of stimuli, *e.g.* DAMPs such as adenosine triphosphate (ATP)[42] and uric acid[43] and PAMPs such as pore-forming toxins[44] and nucleic acids.[45] Lastly, NLRP3 can be involved in non-canonical[46–48] and alternative[49] inflammasome formation.

Priming and posttranslational modifications of NLRP3 inflammasome components

Transcriptional upregulation of *nlrp3* is controlled by NF-κB *e.g.* after TLR activation and is dependent on MyD88, TRIF, Fas-associated protein with a death domain (FADD) and caspase-8.[50,51] To complement this process, which can take several hours,[51] TLR activation also induces non-transcriptional, or transient, priming independently of protein synthesis, but dependent on proteasome function.[52] Transient priming has been shown to involve IRAK1 associating with NLRP3-ASC or a pathway dependent on TRIF, IRAK1, FADD, caspase-8 and RIPK1.[50,53–55] Reactive oxygen species (ROS) production also serves as a signal for non-transcriptional priming.[56,57]

In addition, the NLRP3 inflammasome is regulated by posttranslational modifications. While it has been well established that NLRP3 deubiquitination promotes priming and activation,[56,58–63] a recent report shows the opposite,[64] indicating differential roles depending on cell type or residue modified. NLRP3 phosphorylation has likewise been shown to be both beneficial[65] and inhibitory[66,67] for inflammasome activation. Similarly, ASC speck formation can be both enhanced[68,69] and decreased[70,71] by ubiquitination, while its phosphorylation[72] is beneficial for inflammasome activation. Lastly, caspase-1 ubiquitination has been shown to increase inflammasome activation.[73]

Activation and termination

Over the years, several stimuli have been suggested to induce NLRP3 inflammasome formation: NLRP3 relocation to mitochondrial membranes, mitochondrial damage, cathepsin B in the cytosol and potassium (K^+) efflux.[41] Attempts to unify these pathways have not yet resulted in a conclusive model, but indicate that a drop in intracellular K^+ might be the common denominator and that NLRP3 thus reacts to reacts to cellular stress.[43] A suggested mechanism for this is a conformational change in NLRP3 upon K^+ efflux that promotes activation.[74] However, the exact mechanism has not been identified and the million-dollar question of how NLRP3 (or an unknown adaptor protein) senses K^+ efflux is still considered to be open. Recently, NIMA-related kinase 7 (NEK7) was discovered to be essential for NLRP3 activation downstream of K^+ efflux.[75–77] The mechanism is largely unknown but involves NEK7 binding to the LRR and oligomerization domains of NLRP3, independently of NEK7 kinase activity.[75,78] Figure 1 attempts to illustrate the events surrounding canonical NLRP3 inflammasome activation.

Several mechanisms are in place to regulate inflammasomes. As mentioned above, posttranslational modification of inflammasome components can prevent or decrease activation. Another way is to employ PYD-only proteins (POPs) or CARD-only proteins – both present in humans but not mice – which bind ASC or caspase-1, respectively, to inhibit their recruitment.[79] IL-10 released after prolonged TLR activation leads to a decrease in NLRP3 expression, which is thought to

prevent aberrant activation upon chronic stimulation.[80] In addition, prolonged caspase-1 activation leads to self-cleavage and destabilization, thus terminating its own activity.[81] Finally, inflammasomes can be terminated by induction of autophagy and subsequent degradation of inflammasome components.[70]

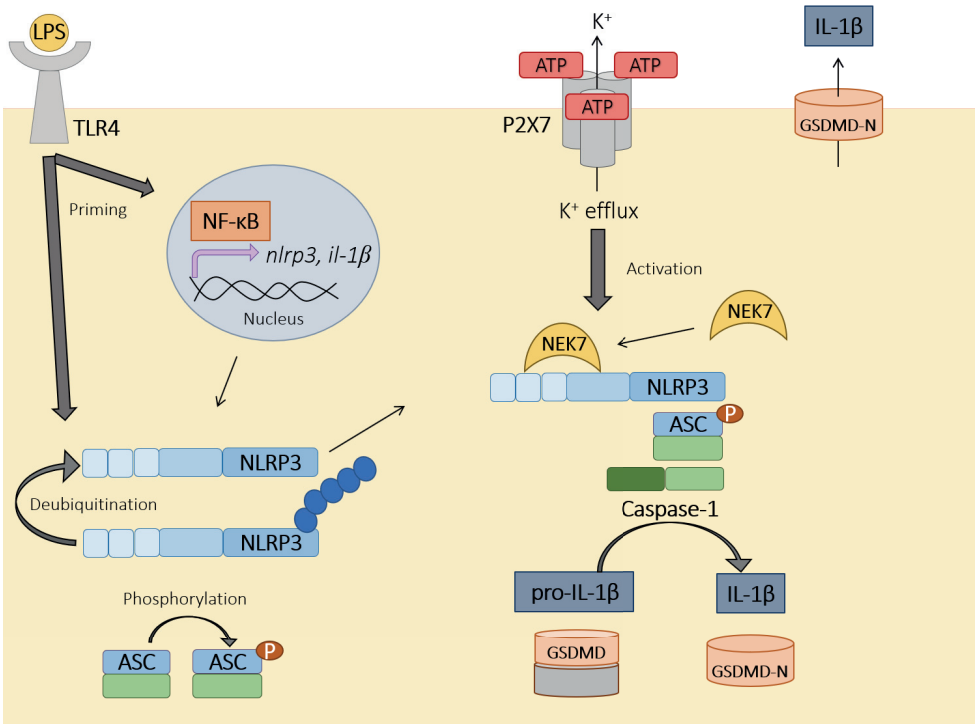

Figure 1. Canonical activation of the NLRP3 inflammasome. Activation of the NLRP3 inflammasome is a two-step process. The first step, priming, induces transcription of *nlrp3* and *il-1β* after NF-κB activation and also induces posttranslational modifications of NLRP3 and ASC. The second signal, K^+ efflux, promotes assembly and oligomerization of NEK7, NLRP3 and ASC and recruitment of caspase-1, which cleaves and activates pro-IL-1β and GSDMD. GSDMD-N forms a pore in the membrane, mediating IL-1β release.

Non-canonical and alternative inflammasomes and macrophage hyperactivation

The non-canonical inflammasome pathway is initiated when caspase-11 in mice or caspase-4 and -5 in humans recognize and directly bind cytosolic LPS *via* their CARD domain, thus enabling recognition of bacteria that have escaped vacuoles and phagosomes.[47,48,82] Non-canonical inflammasome activation can result in both pyroptosis and IL-1β release, although the latter is not mediated directly by caspase-11.[46] Instead, activated caspase-11 can induce pyroptosis and K^+ efflux after cleaving GSDMD, leading to NLRP3 inflammasome activation and IL-1β release.[83,84] Pyroptosis and downstream NLRP3 activation can also be induced by

caspase-11-dependent cleavage and activation of the pore-forming channel Pannexin-1, leading to K^+ efflux, ATP release and activation of the P2X7 receptor.[85]

Alternative inflammasome activation refers to the involvement of additional proteins other than NLRP3, ASC and caspase-1 or alternative activation stimuli or pathways. For example, stimulating human monocytes with LPS simultaneously primes and activates the NLRP3 inflammasome through a pathway involving TLR4, TRIF, RIPK1, FADD and caspase-8 in addition to NLRP3, ASC and caspase-1.[49] Caspase-8 affects inflammasome activation in additional ways: by mediating priming together with FADD,[50] functioning as a scaffold for NLRP3 activation in response to dsRNA[55] or enhancing caspase-1 activation.[50]

Cells that secrete IL-1β with retained viability, measured by absence of lactate dehydrogenase (LDH) release, are termed hyperactivated.[86,87] Hyperactivation can be seen after canonical and non-canonical inflammasome activation in response to oxidized lipids commonly released from dying cells and certain bacterial peptidoglycans and requires GSDMD for IL-1β release.[87,88]

Downstream effects of inflammasome activation

Cleavage and maturation of IL-1β and IL-18 is important in mounting inflammatory responses, *e.g.* recruiting and activating other immune cells in response to pathogens.[31] Both cytokines lack a sequence targeting them to the conventional secretion pathway and must thus rely on unconventional release,[89] which has been suggested to occur through several pathways and which will be discussed in later sections. Recently, it was shown that *Salmonella* activates the NLRC4 inflammasome in intestinal epithelial cells, leading to cell expulsion and death and release of eicosanoids and IL-18; protective against infection but also causing diarrhea and fluid loss.[29]

GSDMD cleavage by caspase-1 or caspase-11 induces an inflammatory form of cell death termed pyroptosis, characterized by the formation of a pyroptosome and cell lysis after plasma membrane permeabilization; commonly measured by release of LDH and staining with the membrane impermeable dye propidium iodide (PI).[83,88,90] GSDMD cleavage frees an N-terminal domain which oligomerizes on the plasma membrane and forms 10-15nm pores causing cell lysis,[91,92] but which have also been suggested to mediate IL-1β release.[83,90] To avoid harming neighboring cells upon extracellular release, GSDMD-N only binds the inner leaflet of the plasma membrane.[91] Pyroptosis is important in the defense against certain pathogens: intracellular bacteria are left without a niche and released DAMPs can attract and activate other immune cells.[24]

The downstream effects of inflammasome activation were previously thought to be coupled, leading to a hypothesis that cytokine release is a consequence of membrane

permeabilization and subsequent cell death.[93,94] However, several studies challenge this idea and propose that pyroptosis and cytokine release are mechanistically separate events. For example, inflammasome activation in neutrophils[95–97] as well as the alternative inflammasome described above[49] result in IL-1β and IL-18 release without pyroptosis. NLRC4 activation is another example: direct recruitment of caspase-1 to NLRC4 results in pyroptosis, while cytokine release depends on the presence of ASC.[27,98] In addition, in hyperactivated cells IL-1β release is mediated through GSDMD pores, which cause membrane permeabilization as evidenced by PI influx.[88] However, this does not lead to cell lysis, suggesting that membrane permeabilization and lysis are separable events. The mechanisms underlying separation of GSDMD-dependent IL-1β release and lysis have not been elucidated but are thought to involve a reduction in GSDMD pore size or numbers in hyperactivated cells,[88] allowing for a newly described membrane repair system to prevent lysis.[99] However, DiPeso *et al.* suggest that cell lysis and cell death can be separated: cell death is characterized by cell swelling and loss of cell movement and mitochondrial activity after GSDMD-dependent membrane permeabilization, while LDH release indicates cell lysis.[100] Further, this study suggests that glycine can block cell lysis but not cell death, indicating that previous studies might reflect dead cells with retained membrane integrity.[100]

Microbial inflammasome modulation strategies

Although there are examples of increased microbial survival and spread after inflammasome activation,[101] the numerous strategies used to inhibit inflammasomes indicate their importance in mounting a protective immune response.[102] Some pathogens affect inflammasome components directly: enteropathogenic *Escherichia coli* (*E. coli*) prevent NLRP3 deubiquitination,[103] human papilloma virus induces pro-IL-1β degradation,[104] YopM from *Yersinia pestis* inhibits caspase-1[105] and a viral POP inhibits ASC recruitment.[106] *Yersinia* YopE and YopT[107] and *Pseudomonas aeruginosa* ExoS[108] instead interfere indirectly with caspase-1 activity through unexplored pathways involving Rho GTPases and actin polymerization. Another strategy is to avoid activation entirely: *Salmonella enterica* downregulates NLRC4 expression,[109] Gram-negative bacteria can produce modified LPS[47] and *Staphylococcus aureus* modifies its cell wall to prevent lysosomal degradation and NLRP3 detection.[110] Lastly, the recent increase in research on our commensal microflora has revealed anti-inflammatory effects of microbial metabolites[102] and a role for inflammasomes in exacerbating inflammatory bowel disease.[31] Surely, continued research into the inflammasome field is likely to reveal additional strategies by which pathogenic microbes avoid and exploit this important machinery.

Ubiquitination

At any time, the inside of a cell is teeming with activity. Diverse processes such as DNA repair, endocytosis, protein degradation, signal transduction and cell-cycle regulation can and will happen simultaneously, illustrating the importance of systems to regulate this and prevent mayhem. The small protein ubiquitin – 76 amino acids long and aptly named after its abundance in eukaryotic cells – is one tool used to convey many of these signals; its addition or removal from a target protein makes up a code that is only beginning to be deciphered.

Ubiquitin and its linkage types

Covalent attachment of ubiquitin to its target is mediated by a C-terminal glycine residue which forms a bond with a lysine (K) on the target protein.[111] One or several target protein residues can be modified, creating monoubiquitination or multi-monoubiquitination, respectively.[111] Ubiquitin itself contains seven lysines that can be targeted for ubiquitin addition, thus creating chains that can be linked *via* the same lysine, homotypic, or *via* different residues, heterotypic.[112] In addition, the N-terminal methionine (Met) can also be ubiquitinated, creating what is called a linear chain.[112] Ubiquitination of two residues on one ubiquitin protein generates branched chains and adds further complexity to the code, as does the realization that ubiquitin can be modified at several sites by *e.g.* phosphorylation and acetylation, creating a staggering number of the potential combinations.[113] While the functions of branched and modified chains remain largely unexplored, more is known about K48-, K63- and Met1-linked chains. K48 chains are the most abundant and commonly signal for transport to and degradation by the proteasome; K63 chains can be a signal for autophagic degradation but may also lead to protein transport and kinase activation; Met1 chains have been found to be involved in regulating NF-κB signaling.[112,113]

Ubiquitin addition, removal and recognition

Ubiquitination is a three-step process mediated by as many enzymes. First, ubiquitin-activating or E1 enzymes use ATP to form ubiquitin adenylate, thus activating the ubiquitin.[114] Activated ubiquitin is then transferred to a ubiquitin-conjugating or E2 enzyme; lastly, interactions with a ubiquitin ligase or E3 enzyme

facilitates transfer of ubiquitin to its target protein.[111] The job of activating ubiquitin is performed by two known E1 enzymes, while about 40 different E2 and over 600 E3 enzymes work together to confer specificity to the reaction: E2 enzymes determine the chain length and linkage type and E3 enzymes recognize and bind target proteins.[111] As mentioned above, ubiquitin removal is part of the code. Deubiquitinase enzymes (DUBs) are tasked with removing ubiquitin: cleaving off whole chains, partial chains or single ubiquitin proteins.[115] One important role for DUBs is to maintain ubiquitin homeostasis, *e.g.* by removing ubiquitin molecules on proteins destined for degradation.[116]

Ubiquitin recognition, by DUBs as well as other proteins mediating *e.g.* degradation and transport, is mediated by ubiquitin-binding domains (UBDs), of which there are currently more than 150 described.[115] Ubiquitin-binding proteins can create specificity by differentially spacing their UBDs, as the distance between ubiquitin molecules differs depending on chain type: K48 linkage creates a compact chain while K63 results in an elongated version.[115]

Regulation of inflammasome activation by ubiquitin

As mentioned previously, NLRP3 deubiquitination is thought to mostly be a positive signal for inflammasome activation. The K48- and K63-linked chains are added by several E2 and E3 enzymes; similarly, multiple DUBs have been implicated in their removal,[59,60,62,63] illustrating the importance of regulating inflammasome activation. Ubiquitin-dependent degradation of pro-IL-1β is mediated by K48 chains[117] while its aberrant activation is mediated by K63-linked chains.[118,119] The E2 enzyme UBE2L3 was recently shown to add K48-linked chains to pro-IL-1β,[117] and the DUBs A20[119] and POH1[118] remove K63-linked chains. Caspase-1 is also activated by K63 ubiquitination,[73] but the E2 and E3 ligases involved are currently unknown. ASC ubiquitination can be K63- or Met1-linked; both types can increase speck formation,[68,69] but K63 chains can also lead to degradation[70,71] which can be counteracted by the DUB USP50.[71] In summary, ubiquitination plays an important role in regulation cell processes in general and inflammasomes in particular. Each published study increases our appreciation of (and perhaps frustration with) the complex interactions between the ubiquitin code, the proteins interpreting it and the downstream effects it sets in motion.

The P2X7 receptor

Extracellular ATP is an important DAMP sensed by purinergic receptors (P2Rs), divided into P2X and P2Y subfamilies.[120] While ATP and derivatives of it are the only identified ligands for the ion channel P2X receptors, the G-protein coupled P2Y receptors recognize other nucleotides as well.[120] P2X receptors are formed as homo- or heterotrimers on the plasma membrane, where ATP binding leads to calcium (Ca^{2+}) and sodium influx and K^+ efflux.[121] The seven P2X proteins identified so far (P2X1-7) are expressed by several immune cells, in many cases with unknown functions.[121,122] P2X7 is not such a case. Its effects have mostly been studied in macrophages, DCs and microglia, but it is also expressed by and affects maturation and activation of T and B cells.[123]

Receptor structure, genetics and activation

Like its family members, P2X7 consists of intracellular N- and C-termini, two transmembrane domains and a bulky extracellular domain.[124] The crystal structure of P2X4 – which shows 41% sequence homology to P2X7 – revealed that a subunit can be likened to a dolphin rising out of the membrane, with three ATP binding sites located in pockets formed by the "head" of one subunit and the "dorsal fin" of another (Figure 2).[125] Filling the pockets with ATP leads to a conformational change which opens up the receptor complex like a pore.[125]

Unlike its family members, the trimeric P2X7 receptor (P2X7R) has low affinity for ATP and requires millimolar levels for activation, which led many to doubt its physiological relevance as an ATP receptor.[126,127] However, recent studies have illustrated a dramatic increase in ATP levels at inflammatory sites, indicating that the low sensitivity of P2X7R prevents its aberrant activation.[128] Another unique aspect of each P2X7 subunit is its elongated cytoplasmic tail which shows sequence homology to the TNF receptor and LBP, although the implications of this has not been investigated further.[127,129] The cytoplasmic tail is important for receptor oligomerization and plasma membrane localization[130] and has been suggested to be involved in permeabilization of the plasma membrane, so-called macropore formation, upon P2X7R activation.[124]

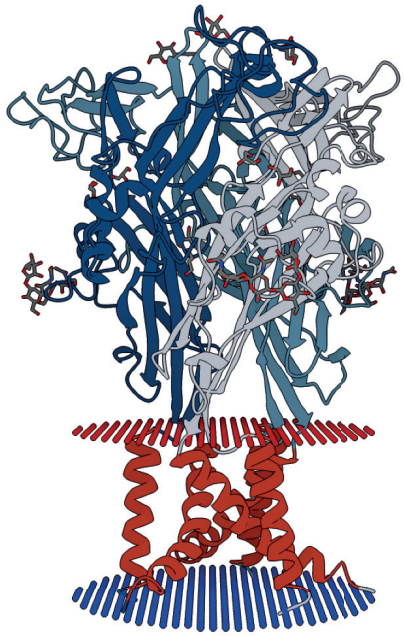

Figure 2. Crystal structure of the zebrafish trimeric P2X4 receptor. A trimeric P2X4 receptor without the intracellular C- and N-termini was crystallized with ATP bound. By Hattori *et al*,[125] Protein Data Bank ID 4DW0.

Genetic variants

There are nine human and three mouse splice variants of P2X7, which all affect activation and receptor formation differently.[131] For both human and mouse, the original, full-length variant is denoted P2X7A.[131] The human C-terminal truncation variant P2X7B forms ion channels but not macropores,[132] similar to the murine variants P2X713B and 13C, which in addition show decreased surface expression.[133] In contrast, the murine P2X7K variant, with a different exon 1 as compared to P2X7A, shows increased sensitivity to activation and macropore formation.[134] P2X7K can also be activated in response to extracellular NAD^+, while sensing of this ligand instead only lowers the activation threshold for the P2X7A variant.[135,136] Membrane-bound adenosine di-phosphate (ADP)-ribosyltransferases mediate the transfer of ADP-ribose (ADP-r) from NAD^+ to P2X7, thus inducing activation or sensitization.[135,137] T cells have been found to preferentially express P2X7K while macrophages express P2X7A,[136] explaining why NAD^+ stimulation leads to P2X7R activation and cell death in T cells but not macrophages.[135,138]

The discovery of murine P2X7 splice variants led to the realization that the two available and commonly used P2X7-deficient mice, one developed by

GlaxoSmithKline (GSK)[139] and one by Pfizer,[140] are not full receptor knock-outs. Neither strain expresses the P2X7A variant of the receptor. However, as the GSK strain was generated by disrupting P2X7 exon 1,[139] and the P2X7K splice variant contains a different first exon, P2X7K escapes deletion in the GSK strain.[134] Interestingly, the GSK mouse even exhibits enhanced P2X7-responses in some tissues and cells.[134,141] The Pfizer strain was generated by disrupting the C-terminal exon 13A, but receptor splice variants encoding the alternative exons 13B and 13C escape deletion in this strain.[133,140] The P2X713B and C receptors do however show decreased function.[133] This may offer an explanation to previously reported phenotypical differences between P2X7-deficient mice.[131]

To add another level of complexity, single nucleotide polymorphisms (SNPs) also affect P2X7R activity. In humans, gain-of-function mutations may offer protection against some infections but increase the risk of inflammatory disorders, while the reverse is suggested for loss-of-function mutations.[131] Although several SNPs have been reported in mice, only one has been fully characterized.[131] BALB/c mice are more sensitive to ATP compared to C57Bl/6 mice, which is due to a single amino acid difference in the C-terminal tail: in BALB/c mice residue 451 is a proline, in C57Bl/6 it is a leucine.[142] Further studies have shown that the 451L variant, which is found in several mouse strains, confers decreased membrane permeabilization, ion flux and cell death.[131,142,143]

Macropore formation

In addition to opening up the P2XR ion channels, ATP stimulation of macrophages also causes membrane permeabilization that mediates uptake of molecules up to 900 Da.[144] As these openings allowed the passage of molecules considerably larger than did the P2XR ion channels, they were referred to as macropores.[122] When P2X7R was discovered it was shown that this receptor could mediate the macropore functions,[127] and subsequent studies have shown that macropores may also form upon *e.g.* P2X4 and P2X2 activation.[124] The molecular identity of the P2X7 macropore is a contested subject.[124] Currently there are two opposing main hypotheses: 1. the macropore really is only a dilated P2X7R pore[145] or 2. additional pore-forming accessory proteins, such as pannexin-1, are recruited and make up the macropore.[146] However, it has been shown that pannexin-1 knock-out cells still show macropore-mediated functions after ATP stimulation,[147] and no other candidate has been proposed. For many years, it has been commonly accepted in the field that macropore formation happens upon prolonged stimulation of P2X7R, a view that has recently been challenged. Indeed, new data indicate that P2X7R activation induces an instant and stable opening of the channel, allowing the passage of large molecules. Contrary to previous beliefs, no dilation of the channel was observed, and it was suggested that previous conclusions about dilation may have

been a result of the interpretation of whole cell-population kinetics rather than kinetics of the pore itself.[148,149] The C-terminal tail of P2X7 was thought to be required for uptake of large molecules.[132,142,150,151] Recently however, Karasawa *et al.* showed macropore formation by C-terminal deletion mutants expressed in liposomes.[152] These macropores did not form in cholesterol-containing liposomes,[152] in agreement with a previously reported inhibitory effect of cholesterol on P2X7R-mediated uptake of large molecules.[153] Complementing the deletion mutants with C-terminal residues allowing palmitoylation[154] counteracted the effect of cholesterol,[152] suggesting that the C-terminal tail may not be required for pore formation *per se* but may have a regulatory effect depending on the lipid composition of the membrane.

Modulation of activation

In addition to NAD^+, P2X7R can be activated by the AMP LL-37[155] and amyloid-β,[156] although it is not known whether these substances induce cellular release of ATP[147] which then activates P2X7R, or whether these molecules are actual P2X7R ligands. The antibiotic polymyxin B,[157] LPS,[85] the antihistamine clemastine,[158] and P2X4R[159,160] all lower the P2X7R activation threshold by unknown mechanisms. P2X4R can be coimmunoprecipitated with P2X7R and has been shown to interact with the P2X7 C-terminus,[161] but reports contradict on whether they interact as homo- or heterotrimers and how this interaction modulates activation.[162,163]

In addition to lipids, P2X7R activation is regulated by a feedback loop mediated by matrix metalloproteinase 2 (MMP-2). After its P2X7-dependent release, MMP-2 cleaves P2X7 to terminate its activity, thus preventing detrimental effects of sustained receptor activation.[164] P2X7R activation is also negatively modulated by divalent cations such as magnesium (Mg^{2+}) and zinc, through chelation of ATP or through poorly understood allosteric inhibition of the receptor itself.[165] Several P2X7R inhibitors have been developed; although structurally different, some of them bind to the same site where they allosterically prevent pore opening.[166] However, due to the polymorphic nature of P2X7, the inhibitory concentrations of these inhibitors vary between both species and individuals, complicating their therapeutic potential.[131]

Downstream effects

The most well-known downstream effect of P2X7R activation is formation of the NLRP3 inflammasome, thought to be due to P2X7-dependent K^+ efflux.[42,167] However, a recent study demonstrated that the NLRP3-activating K^+ efflux in fact

is mediated by TWIK2 (two-pore domain weak inwardly rectifying K^+ channel 2) after cation influx by P2X7R activation.[168] P2X7 has been reported to directly associate with NLRP3,[169] but the importance of this observation on inflammasome activation has not been investigated.

P2X7R activation also leads to nonclassical release of e.g. IL-1β,[170] transglutaminase-2,[171] cathepsins[172,173] and MMP-9[174] through its effects on membrane blebbing[175–177] and release of exosomes[178,179] and secretory lysosomes.[180] In addition, P2X7R can influence cell migration through induction of cytokines and chemokines,[181] promote shedding of surface molecules and induce plasma membrane phosphatidylserine exposure, apoptosis and phagocytosis.[131,182,183] Interestingly, Janks *et al.* recently showed that several of these downstream events depend on P2X7R-dependent activation of a chloride channel,[184] in contrast to a previous study reporting inhibitory effects of chloride on P2X7R activation and NLRP3 activation.[185]

These diverse downstream effects reflect the many-faceted role of P2X7R during infection and inflammation. On one hand its activation can exacerbate the inflammatory response during sepsis and in response to some viruses, but on the other its inhibition during certain bacterial, fungal or protozoan infections leads to deleterious effects.[123] The same is true for cancer, where both pro- and anti-tumor effects are seen when manipulating P2X7R activation.[121] In contrast, inflammatory diseases such as diabetes and neurodegenerative diseases such as multiple sclerosis are alleviated by P2X7 inhibition, making it an attractive therapeutic target.[186]

IL-1β and its release pathways

IL-1β and its 10 IL-1 family members possess highly conserved gene structures and are mostly clustered on human chromosome 2, suggesting that the genes arose from duplication of a common ancestor gene.[187] IL-1β and IL-1α were the first cytokines to be identified; initially thought to be the same protein, two studies published in 1984 demonstrated different sequences of IL-1 cDNA.[188,189] Prior to this, Dinarello *et al.* were first in demonstrating the presence of the fever-mediating protein leukocytic pyrogen in humans, a protein that later was shown to be IL-1β.[190,191] Since then, a host of studies have attributed additional functions to these inflammatory cytokines and demonstrated their importance in the inflammatory response. In short, IL-1β induces production of cytokines and chemokines, promotes influx of immune cells by increasing expression of adhesion molecules on endothelial cells and promotes differentiation and expansion of T helper cells.[192] Its downstream effects are mediated by IL-1 receptors (IL-1R), of which there are 10 family members.[193] IL-1β binds primarily to IL-1R1, leading to *e.g.* NF-κB activation through binding of MyD88 to its TIR domain, similar to TLR signaling.[193]

Dysregulated IL-1β release can wreak havoc in our bodies, illustrated by its association with several autoinflammatory diseases and the success in treating them with anakinra, a recombinant form of the naturally occurring IL-1 receptor antagonist (IL-1Ra), which binds IL-1R1 with high affinity and prevents its activation.[191] IL-1Ra is not the only endogenous mechanism that keeps IL-1β at bay: high levels of IL-10 in the gut keeps macrophages virtually unresponsive to inflammasome activating stimuli,[194] and type I IFNs reduce the amount of pro-IL-1β via IL-10 production and/or direct inhibition of inflammasome activation.[195] Indeed, for certain patients with multiple sclerosis, IFN-β is an efficient treatment, possibly due to its role in quenching IL-1β generation.[191]

Further underlining the importance of keeping the production of IL-1β under tight control, its release is regulated by several checkpoints. First, baseline IL-1β expression is low and can be increased after NF-κB activation, *e.g.* after TLR or IL-1R activation.[51] Second, gene expression results in production of the inactive precursor pro-IL-1β that requires proteolytic cleavage in order to induce IL-1R signaling.[196] A few years after the cloning of the IL-1β gene, the enzyme mediating the activating cleavage of the pro-IL-1β protein was identified to be caspase-1.[197] However, the presence of active IL-1β in caspase-$1^{-/-}$ mice[198] demonstrated the

presence of additional proteases: host-derived MMP-9, granzyme A and neutrophil proteinase-3[192,199] and microbe-derived proteases from GAS[200] and C. albicans[201] are examples of these. Third, pro-IL-1β can be targeted for autophagosomal[202] or proteosomal degradation, the latter after its ubiquitination.[203] Last, IL-1β lack the N-terminal leader sequence that allows for release of proteins through the so-called conventional secretion pathway. Proteins that do contain this leader sequence are, upon translation, targeted to the ER lumen from which they are transported to the Golgi, packaged into secretory vesicles and finally released through vesicle fusion with the plasma membrane.[204] The only member of the IL-1 family of proteins to be secreted through this pathway is IL-1Ra.[192] Several nonconventional pathways have been suggested to govern IL-1β release, but externalization of its other family members is a largely unexplored area.

Rabouille *et al.* recently proposed a classification system for unconventional release of leaderless proteins: type I release involves pore-mediated translocation across plasma membranes, type II is mediated by ATP-binding cassette (ABC) transporter proteins in yeast and type III involves membrane-bound organelles.[205] These organelles can release their contents either by fusing with the plasma membrane, similarly to the classical release pathway, or by vesicle lysis after their release into the extracellular space.[206] Several type I and III pathways have been put forth as alternatives for IL-1β release. Figure 3 summarizes the proposed unconventional IL-1β release pathways.

Non-vesicular release (Unconventional type I release)

Non-vesicular, or type I,[205] release of IL-1β suggests that pro-IL-1β cleavage occurs in the cytosol and that the active cytokine then is transported over the membrane by a transporter protein or diffused through a pore. Recent research has focused on the pore-forming protein GSDMD, but other mechanisms have also been proposed.

GSDMD pores and membrane permeabilization

GSDMD pore characterization revealed that its diameter, 10-15nm,[92] is large enough to allow passage of IL-1β with a diameter of 4.5nm[207] as well as other cytosolic proteins of similar size, such as IL-18, suggesting a passive process.[208] In caspase^{mlt} mice, which lack caspase-1 protease activity, caspase-8 drives GSDMD-independent LDH-, IL-1α- and IL-1β release, possibly also reflecting a passive process.[209] In contrast, Monteleone *et al.* showed that IL-1β cleavage is sufficient for release, which can be rapid and GSDMD-dependent or slow and GSDMD-independent.[210] In this study it was found that pro-IL-1β cleavage led to exposure of

a specific motif that enabled relocation to and electrostatic interaction with plasma membrane ruffles enriched with phosphatidylinositol 4,5-bisphosphate (PI[4,5]P_2), suggesting an active process regardless of the presence of GSDMD.[210] On a similar note, lanthanides, a group of chemical elements, prevent Nigericin-mediated PI influx and pyroptosis, but do not affect GSDMD-dependent IL-1β release, also suggesting an active process.[211]

Several other proteins or substrates that cause membrane permeabilization may also mediate IL-1β release, such as MLKL (necroptosis-associated mixed lineage kinase domain like pseudokinase),[212] the shellfish toxin maitotoxin[213] and the bee venom melittin.[214] The organic compound punicalagin has been found to block membrane permeabilization/pore formation but not NLRP3 activation, as illustrated by the intracellular accumulation of mature IL-1β and its subsequent release after punicalagin washout.[215] The target(s) of punicalagin remain unclear.

Non-vesicular release by other proteins

Prior to the discovery of GSDMD, other pore-forming proteins were suggested to mediate IL-1β release. An early suggestion was ABC transporters, which facilitate translocation of numerous compounds,[216–218] but the results of several of these studies were complicated by the realization that the inhibitors used commonly have off-target effects.[219,220] Similarly, based on studies using inhibitors, pannexin-1 was suggested as a route across the membrane during IL-1β release.[221,222] However, subsequent studies using pannexin-1 knock-out mice have shown that this pore-forming protein is dispensable for IL-1β release.[147,223] As discussed earlier, P2X7R activation can induce several of the type III pathways discussed above, but P2X7-dependent unconventional release has also been suggested to occur through the P2X7R macropore.[204] However, IL-1β is larger[207] than the reported diameter of the macropore,[145] making it unlikely that this is the direct conduit for release.

Fibroblast growth factor 2 (FBF-2) is externalized by an intriguing mechanism. Similar to IL-1β, PI(4,5)P_2 recruits FGF-2 to the plasma membrane[224] where it oligomerizes and inserts itself, thus creating its own release pore.[225] The mechanism for disassembly and release is not fully elucidated but involves capture by cell surface heparan sulfates.[226] Although IL-1β itself so far has not been shown to possess pore-forming abilities, it is possible that PI(4,5)P_2 could mediate its release through other proteins.

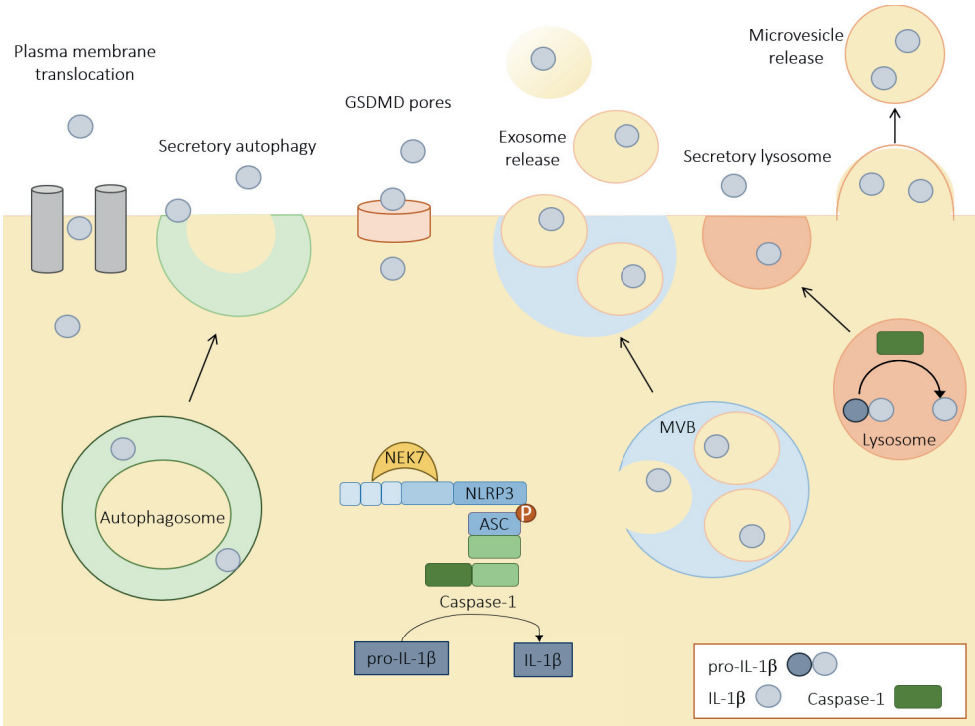

Figure 3. Unconventional IL-1β release pathways. IL-1β release has been suggested to occur through plasma membrane translocation by accessory proteins, after uptake into secretory autophagosomes or through the plasma membrane pores formed by GSDMD after inflammasome activation. In addition, IL-1β has been suggested to be taken up into exosomes and secretory lysosomes or to be captured in microvesicles.

Vesicular release (Unconventional type III release)

Secretory lysosomes

Secretory lysosomes are lysosomal organelles which have evolved to not only degrade proteins but to also mediate their release by fusing with the plasma membrane.[227] Used by *e.g.* $CD8^+$ T cells to deliver granzymes and neutrophils to release chemoattractants, secretory lysosomes depend on Ca^{2+} influx to induce microtubule-dependent migration to the plasma membrane.[206,228] IL-1β has been shown to colocalize with the lysosomal markers cathepsin D and LAMP-1, and to show similar secretion kinetics as the former, suggesting a role for a secretory lysosomal release pathway[179,180] Further studies showed that lysosomal release was blocked by inhibiting the enzymes phosphilipase A2 and C, which are activated by Ca^{2+} influx after P2X7R activation by ATP.[180] This early model proposed that pro-IL-1β processing by caspase-1 may take place in the lysosome[229] and that caspase-

1 and IL-1β may be protected from lysosomal degradation by interacting with the vesicular membrane and/or by the decrease in lysosomal pH that occurs upon migration of lysosomes to the plasma membrane.[230] However, recent studies demonstrate that caspase-1 activation and pro-IL-1β processing occurs at the cytosolic ASC speck,[231] and that mice with faulty secretory lysosomes still release IL-1β,[94] indicating that early results might have been misinterpreted by *e.g.* contamination of ASC specks during subcellular fractionation and purification of lysosome-containing fractions.[232]

Microvesicles and exosomes

Activation of P2X7R leads to formation of 200nm-1μm microvesicles derived from the plasma membrane through a process called blebbing, which has been suggested to trap IL-1β and mediate its release.[175,233] Microvesicles can in theory contain any cytosolic protein in the vicinity to the bleb, and their formation and release has been suggested to be dependent on Ca^{2+} influx, calpains and Rho kinases after P2X7R activation.[176,177] In addition to IL-1β, microvesicle cytosols have been suggested to contain caspase-1, cathepsin D and high-mobility group protein 1 and to express P2X7R and major histocompatibility complex II (MHC-II) on their surface.[234,235] As for the proposition of the involvement of secretory lysosomes, the microvesicular release theory has received some critique: there seem to be differential requirements for microvesicle and IL-1β release after P2X7R activation,[178] and IL-1β release is independent of Ca^{2+} influx.[236]

Smaller than microvesicles, exosomes also contain cytosolic proteins and can be formed by inward budding of endosomes; they are then contained in so-called multivesicular bodies (MVB) in the cytosol and are released when the MVBs fuse with the plasma membrane.[237] Qu *et al.* demonstrated that ATP stimulation leads to P2X7R- and inflammasome-dependent release of exosomes containing IL-1β and cathepsin D and expressing MHC-II.[178,238] However, in a later study the same group found that exosome release was independent of caspase-1 activity,[238] raising concerns about its importance for release of mature IL-1β.

While intact microvesicles and exosomes can deliver their cargo to neighboring cells by fusion with the plasma membrane or endocytosis, they can also bind to surface receptors such as integrins and induce downstream signaling pathways or release their content extracellularly by lysing.[237] It is not clear whether lysis of IL-1β-containing vesicles occurs actively or not; the presence of P2X7R on their membranes has led to speculation that receptor activation by extracellular ATP could induce vesicle lysis.[239]

Secretory autophagy

Like lysosomes, autophagosomes can be repurposed to mediate secretion instead of degradation, a well-known pathway in yeast that only recently has been explored in eukaryotic cells.[240] However, whether autophagy inhibits[202] or promotes[241,242] IL-1β release is debated and has been proposed to depend on type and strength of stimulus. A mechanism of IL-1β release through secretory autophagy has been described: HSP90-dependent translocation of IL-1β into the space between the two membranes of an autophagophore, which then fuses directly with the plasma membrane or with a secretory lysosome, forming a so-called amphisome.[242] Translocation was suggested to involve IL-1β unfolding and was dependent on a sequence motif previously implicated in chaperone-mediated autophagy, a pathway used to target specific proteins for degradation.[242]

IL-1β release as a spectrum reflecting signal strength

The release pathways described for IL-1β have been studied in different cell types from different species, with differing experimental settings and using different methods of analysis, making it difficult to compare the suggested pathways and their physiological relevance. In an attempt to unify results, these pathways have been proposed to reflect the intensity of the activating signal.[204,206] Initial signals would lead to secretory lysosomal or exosomal release, which is more tightly controlled but less efficient. Packaging of IL-1β into vesicles would also allow for degradation if the signal is short-lived, *e.g.* through autophagy.[202] A persistent threat would call for activation of non-vesicular release pathways, which are effective but potentially threaten cell viability. Related to this, the rate of IL-1β release can also vary depending on redox state; cells with upregulated antioxidant systems display a reduced rate of IL-1β release due to buffering of ROS produced upon activation stimuli.[243] Thus, each cell is able to activate several pathways in order to properly regulate the magnitude of the inflammatory response and to prevent the harmful effects of aberrant IL-1β release.

Group A *Streptococcus*

Streptococcus pyogenes, commonly known as Group A *Streptococcus* (GAS), is a pathogen capable of causing a wide range of diseases in its only host: humans.[244] Since up to 20% of school-age children and 25% of adults in contact with school-age children carry GAS asymptomatically, it is considered a facultative rather than obligate pathogen.[244,245] Relatively mild diseases such as throat infection (pharyngitis, "Strep throat") or skin infection (impetigo) are found at one end of the spectrum and represent the majority of cases; over 600 million pharyngitis cases are estimated yearly.[246] At the other end lies invasive but rare infections such as necrotizing fasciitis (NF, "flesh-eating disease") and Streptococcal toxic shock syndrome (STSS), complicated to treat and associated with mortality rates of 15-25%.[247] About 517 000 deaths are caused by GAS each year, placing it among the top 10 infectious causes of human mortality.[246] In a majority of cases, GAS-related death is due to post-infection sequelae such as rheumatic heart disease and acute glomerulonephritis, characterized by autoimmune damage to heart and kidney, respectively.[248] A relatively unexplored aspect of GAS infections are their effect on our nervous system: they are linked with rapid onset of obsessive-compulsive and tic disorders in children (pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections)[249] and directly activate pain-sensing neurons during NF, leading to a decreased immune response.[250] Although the number of GAS infections have decreased globally due to increased living conditions and hygiene, there has been a resurgence in invasive infections since the 1980's, due to the emergence and subsequent world-wide dissemination of strains with increased fitness and/or virulence.[251] The incidence of GAS infection follows a seasonal pattern, with rates peaking during winter and spring,[252] and a recent study suggests an association between invasive GAS infections and influenza A infection.[253]

GAS is Gram-positive and characterized from other species in *e.g.* a throat culture by complete lysis of red blood cells (β-hemolysis) on blood agar plates and presence of a group A carbohydrate (GAC) on its surface, a classification system developed by Rebecca Lancefield based on differing immunological responses to streptococcal cell wall carbohydrates.[248,254] As it is the only member of the *Streptococcus* genus expressing a GAC, the term GAS has become synonymous with the species *Streptococcus pyogenes*. Further serotyping is based on genetic differences in the

surface-expressed M protein, a major virulence factor that currently exists in over 200 variants.[255] Other cell-surface proteins such as T and R proteins can also be used to distinguish strains from each other.[248] Once identified, a GAS infection is treated with antibiotics; although increased resistance to certain antibiotic types is being reported, GAS is amazingly still sensitive to penicillin.[255] Notably, disease progression in invasive infections can be too overwhelming for antibiotic treatment to be effective: 8-23% of patients with invasive disease die within 7 days of infection even when antibiotics are administered, and the number is even higher if STSS is developed.[255] There is currently no vaccine against GAS, despite many years of research and several clinical trials. Diversity among strains and their geographic distribution is one complicating factor, incomplete understanding of adaptive immune responses and their role in inducing post-infection sequelae another.[256] As an example, molecular mimicry between the M protein and myosin allows antibodies to the former to cross-react with the latter, which may contribute to disease progression in rheumatic heart disease.[257]

Pathogenesis

GAS expresses an impressive arsenal of virulence factors: proteins, enzymes and carbohydrates that are used during an infection to ensure optimal adherence, survival and spread. Recent research has revealed that virulence factors may play different roles depending on GAS location, leading to a realization that the interplay between virulence factors and the cues dictating their regulation may be more complicated than previously thought.

Adherence and colonization

The most crucial step in GAS pathogenesis is adhesion to surfaces such as our skin and nasopharyngeal mucosa, which constantly work to remove foreign objects by exfoliation or mucus and saliva flow.[248] Adhesion is thought to occur in two steps: weak, hydrophobic interactions between bacterial lipoteichoic acid and host cell surfaces,[258] followed by specific adhesion mediated by bacterial surface molecules, such as the M protein and the hyaluronic acid capsule which may bind CD46 and CD44 on keratinocytes, respectively.[259,260] The extracellular matrix is also an important adhesion site illustrated by the presence of several fibronectin-binding proteins.[258] GAS competes with the local microflora but can also cooperate with it to form mixed-species biofilms, adherent bacterial aggregates associated with chronicity in other types of bacterial infections.[261] GAS biofilms and their role in pathogenesis are poorly studied, but presence of biofilm in biopsies from NF

patients was associated with increased bacterial load and tissue destruction, indicating a role in disease severity.[262]

While GAS as a species is versatile in its ability to infect different tissues, specific strains often show a preference to infect either skin or throat. Genetic studies have shown that the sequences of the M protein-encoding gene *emm* and other *emm*-like genes can be grouped depending on tissue tropism: throat specialists, skin specialists and generalists without preference.[244]

Invasion and evasion

GAS is typically considered an extracellular pathogen, but it has become clear that it can invade and reside in epithelial cells[263] by *e.g.* escaping autophagy.[264] Detection of intracellular GAS in removed tonsils[265] and skin infection biopsies[266] suggests that recurrent and persistent infections are due to this reservoir, which is not targeted by antibiotics. Intracellular survival and replication[267] can also occur in macrophages, by prevention of phagolysosomal fusion[268] or acidification.[269]

While opsonophagocytosis might not be an immediate death threat, it is still a crucial part of the host defense system and GAS has evolved several ways of inhibiting it. M protein[270] and the hyaluronic capsule[271] inhibit or prevent opsonization by complement proteins, IgG-degrading enzymes prevent antibody opsonization,[272] the cell wall proteinase SpyCEP inhibits neutrophil recruitment by cleaving chemoattractants such as IL-8[273,274] and cytolysins such as streptolysin O (SLO) and S (SLS) induce cell damage and death by forming large pores.[275–277] The additional immune-modulatory effects of SLO are discussed later on.

The systemic and detrimental inflammatory response during STSS is thought to be due to streptococcal pyrogenic exotoxins (Spe). These superantigens (SAg) act as a bridge between the β-chain of TCRs and MHC-II molecules, causing antigen-independent activation and proliferation of a large number of T cells.[248] Kasper *et al.* suggest that the biological role of SAgs is to establish nasopharyngeal infection, as the presence of SAgs led to a dramatic increase in colonization of mice expressing human MHC-II, arguing that nonspecific T cell activation might be an unfortunate side effect of SAg expression after colonization.[278] In accordance with this, immunization against SpeA protects against nasopharyngeal infection in a T cell-dependent manner.[279]

The GAS genome contains multiple regulatory systems that control the expression of virulence factors and thus the transition from colonization to infection, depending on *e.g.* bacterial density and environmental cues such as extracellular Mg^{2+} and LL-37 for the control of virulence regulatory system (CovRS).[280,281] This two-component system regulates 10-15% of the genome, for example it negatively regulates the expression of capsule and SLO and positively regulates SpeB, a

secreted cysteine protease which cleaves both host and microbial proteins.[282] Invasive and hypervirulent GAS isolates commonly exhibit inactivating mutations in CovRS, allowing for increased expression of virulence factors and resistance to defense mechanisms.[283,284] These mutations are almost always *de novo* mutations that occur in the host, as strains with inactive CovRS are impaired in establishing infection due to hyperencapsulation and thus also impaired in spreading to other hosts.[285,286] Invasive infections are therefore thought of as evolutionary dead-ends, arising due to colonization of and local adaptation to sites that normally are sterile.[287]

M1T1 GAS

A widespread M1T1 strain that has been circulating since the 1980's has been under much scrutiny. An epidemiologic study of invasive GAS cases in USA showed that while several M types cause invasive infections, M1 strains dominated,[288] and analysis of invasive M1 isolates from Canadian patients showed that they were all M1T1 clones.[289] However, an Australian study suggests that M1T1 strains are not inherently more invasive than other strains and that the apparent prevalence in invasive disease instead is due to the overall abundance of this strain in the population.[290] This is in line with what was discussed above regarding hypervirulence; however, as the majority of studies on M1T1 strains have focused on why they cause invasive infections, their (eventual) fitness advantage remains unexplored.

The evolution of epidemic M1T1 GAS

By sequencing the entire genome of 3615 M1T1 isolates found worldwide, and by comparing their sequences with a pre-epidemic reference strain, Nasser *et al.* repeated and solidified the findings of a previous study showing that emergence of the epidemic strain was preluded by three gene transfer events.[291,292] The first two were *via* bacteriophages: first the extracellular virulence factor streptococcal DNase D2 was acquired, followed by SpeA1, a SAg.[291] Replacing a single nucleotide in SpeA1 gave rise to SpeA2, which *in vitro* shows increased binding to MHC-II.[293] Finally, a M12 strain is thought to be the donor during a horizontal gene transfer (HGT) event leading to the acquisition of a 36 kb region which increases production of the virulence factors SLO and β-nicotinamide adenine dinucleotide (β-NAD^+, NAD^+)-glycohydrolase (NADase).[291,292] This region differs from pre-epidemic strains by a few SNPs, two of which are located in the promotor region for the *nga* gene encoding NADase; these two plus a SNP in the coding region of *nga* are sufficient and necessary for increased expression of SLO and NADase.[294] A similar HGT event is thought to precede the recent increase in M89 prevalence, as some of

these emerging M89 strains express the same promotor variant as the epidemic M1T1 strains.[294,295] M1 and M89 strains expressing increased levels of SLO and NADase have been shown to resist phagocytic killing and cause increased disease in several mouse models,[294,296–298] indicating that the two virulence factors are important in both bacterial survival and disease progression.

NADase expression in M1 strains compared to pre-epidemic strains has been noted in clinical isolates in different parts of the world.[299,300] In a portion of these isolates, mutations conferring little or no NADase activity have become established, indicating that NADase lacking enzymatic activity is under positive selection.[300–302] The role of inactive NADase in bacterial fitness or disease progression has not been fully elucidated, although Riddle *et al.* suggest that NADase-inactive strains show tissue tropism while NADase-active strains are generalists.[301]

SLO, β-NAD^+-glycohydrolase and cytolysin-mediated translocation

The relationship between SLO and NADase begins with their transcription. The *nga* gene is located upstream of *slo* and the two genes are transcribed as a single operon,[303–305] although *slo* has also been reported to have its own promoter.[306] The next part of their close association involves stability. NADase binds SLO and stabilizes both toxins upon secretion, preventing their breakdown and enhancing SLO-mediated cytotoxicity.[307,308] As this is independent of NADase activity, it is thought to be one reason for the positive selection of NADase.[307] The final part of cooperation and co-dependence between the two virulence factors is in regard to their effects on host cells.

SLO belongs to the cholesterol-dependent cytolysin (CDC) family, which includes pore-forming toxins expressed by several types of bacteria. CDC pores can be up to 30 nm in diameter and are made up of about 50 monomers which oligomerize into a pre-pore complex before insertion, a process that requires membrane cholesterol.[309] However, SLO membrane binding and pre-pore formation can occur in the absence of cholesterol; a carbohydrate-binding motif allows SLO to bind galactose-containing residues on red blood cells[310,311] and epithelial cells.[312] Additionally, NADase mediates SLO binding to epithelial cells in a manner independent of both cholesterol and carbohydrates.[312,313] Pore formation induces apoptosis in keratinocytes[314] and macrophages,[315] but can also cause an inflammatory form of cell death termed oncosis, characterized by loss of membrane integrity, organelle swelling and leakage of intracellular contents.[316] The reason why infected mice often fare better in its absence[315–317] are not limited to reduced cell death: SLO can reduce cytokine release,[318,319] protect from phagocytosis[320] and impair neutrophil degradation.[321]

In a seminal paper from 2001, Madden *et al.* described a new function for SLO: translocation of NADase into the host cell cytosol.[304] The process was termed cytolysin-mediated translocation (CMT) and was the first injection system described for Gram-positive bacteria. CMT occurs only when GAS adheres to host cells and requires secretion of SLO and NADase from the same bacterium.[304] NADase then binds an unknown receptor *via* its N-terminal domain,[312] which requires the presence of SLO.[312] NADase binding promotes translocation, as it mediates SLO binding even in the absence of cholesterol or carbohydrates.[313] Other CDCs can become CMT competent by fusing them to an N-terminal domain unique to SLO and by adding residues from the SLO membrane-binding domain; both are required but not sufficient by themselves.[313,322] Similarly, fusing the N-terminal domain and last C-terminal residues from NADase to other proteins enables their translocation.[323] Translocation was first thought to occur through the SLO pore, but as subsequent studies showed that pore formation in fact is dispensable[322,324] it is currently unknown how NADase travels across the membrane.

NADase was discovered in 1957, when it was shown that fractions of SLO purified from bacterial supernatant hydrolyzed the important coenzyme NAD^+ into nicotinamide (NAM) and ADP-ribose (ADP-r),[325] a process that can lead to energy store depletion and cell death for both pro- and eukaryotic cells.[308] This toxicity thwarted early attempts to express NADase in, and purify it from, *E. coli* until immunity factor for *Streptococcus pyogenes* NADase (IFS) was discovered; a small cytoplasmic protein which blocks the active site pocket in NADase,[305,326,327] thus protecting the bacterium from the effects of its toxin. Transcribed in the same operon as *nga* and *slo*, *ifs* is often truncated and nonfunctional in NADase-inactive mutants.[305] As the ADP-r-cyclase and -transferase abilities previously ascribed to NADase purified from bacterial supernatant[299,328] were absent when studying a cloned version, NADase is now thought to be a strict glycohydrolase.[329] Both NAD^+ cleavage products affect eukaryotic cells: NAM inhibits NF-κB activation and thus prevents the production of pro-inflammatory cytokines[330,331] and ADP-r induces Ca^{2+} signaling, which has multiple downstream effects.[332,333]

Enzymatically active NADase enhances SLO-mediated toxicity by NAD^+ and ATP depletion,[304,308,334,335] while inactive NADase induces necrosis dependent on activation of c-Jun N-terminal kinase (JNK), a MAPK.[336] However, intracellular delivery of enzymatically NADase alone also induces toxicity.[337] NADase affects cytokine release by modulating the activity of the stress-related enzyme PARP-1,[338] activated by SLO pore formation: active NADase induces release of the TLR4-binding DAMP high mobility group box 1[339] (HMGB1), inactive NADase induces release of IL-8 and TNF-α.[335] Together, SLO and NADase promote intracellular survival by inhibiting phagolysosomal acidification[269] and autophagic killing,[340] but NADase has also been shown to promote intracellular survival independently of

SLO.[337] Lastly, SLO and NADase are involved in regulating IL-1β release after NLRP3 inflammasome activation, discussed below and in Paper I and II.

The host response to GAS

Whether it is adhering to barriers such as epithelial cells or spreading into deeper tissue, GAS does not go unnoticed by its host. The continued arms race between host and pathogen has led to development of several strategies to ensure proper removal of the pathogen, among them opsonization by complement proteins and subsequent phagocytosis. Additional host responses are discussed below.

Cytokines and PRRs

Despite not being competent for phagocytosis, non-immune cells such as keratinocytes are not defenseless against invading GAS: K^+ efflux induced by pore-forming toxins triggers p38 MAPK activation and IL-8 release from epithelial cells,[341,342] recruiting *e.g.* neutrophils which limit GAS spread through production of AMPs and ROS.[343] Professional phagocytes such as macrophages and DCs are also important in limiting GAS dissemination, evidenced by increased bacterial numbers and decreased survival when either cell type is depleted.[344,345] Disease control is mediated by production of IL-6, TNF-α and IL-12 through induction of Th17 cells,[346] recruitment of macrophages[347] and protection during skin infection,[348] respectively and in addition to activation of innate cells. Lastly, IFN-β production is crucial for preventing hyperinflammation by controlling neutrophil influx.[349,350]

MyD88 plays a central role in launching defense mechanisms in response to GAS. Its deletion leads to a decrease in cytokine levels[349,351–353] and in upregulation of DC activation markers[353]; although phagocytosis is unaffected, MyD88$^{-/-}$ mice exhibit increased susceptibility, pathology and bacterial numbers.[352] MyD88 deficiency in humans leads to predisposition to invasive pyogenic infection at early age.[354] Despite this, the upstream receptors and their ligands have not been fully elucidated. TLR4 is one proposed candidate involved, as SLO has been suggested to activate TLR4[355], TLR4$^{-/-}$ mice display decreased mucosal antibody responses to GAS infection[356] and human *tlr4* polymorphisms are linked with predisposition to GAS infections.[357] On the other hand, TLR2/4/9 single or triple knockout cells from mice only exhibit a slight reduction in cytokine production *in vitro*.[351,353] Similarly, TLR2$^{-/-}$ mice have been reported to be both susceptible to[358] or unaffected by[347] *in vivo* GAS infection compared to wild-type (wt) mice. GAS nucleic acids have been suggested as TLR13[358,359] or STING[349] ligands, but a recent study was unable to

detect cytosolic GAS DNA during infection and instead suggest that the M protein induces STING-dependent IFN-β induction via an unidentified receptor.[360]

Adaptive immune responses

The adaptive response to GAS is less characterized than the innate. On one hand, mice deficient in T- and B-cells exhibit similar clearance and survival rates as wt mice,[361,362] but on the other anti-M protein antibodies provide protection against infection in mice and humans,[363] suggesting that humoral adaptive immunity is important in preventing infections. Additionally, as children have higher infection rates than adults[364] and MyD88-deficient individuals are only susceptible to infection when young, adaptive immunity developed during life seems to be able to complement innate-mediated protection.[354] In a study comparing immune responses children and adults, it was found that adults displayed higher levels of IFN-γ and IgG3 (the IgG subclass that most efficiently mediates complement activation and opsonophagocytosis) during GAS infection.[365] Thus, it is possible that differential immune responses may partly explain why adults and children present with very different diseases after GAS infection.[354]

The T cell response against GAS is dominated by Th1 and Th17 cells, with low numbers of Th2 cells, in both mice and humans.[365,366] Mouse studies have also revealed that the T helper cell response against GAS is affected by route of infection. Intravenous or subcutaneous infection results in formation of antigen-specific Th1 cells, while Th17 cells dominate after intranasal infection.[346] It has been shown that Th17 numbers are reduced in $TLR2^{-/-}$ mice in response to GAS[356] and that Th17 cell expansion upon GAS infection is dependent on production of IL-6 and TGF-β.[346,367,368] In addition, transfer of $CD4^{+}$ T cells from mice immunized with GAS into naïve hosts can promote bacterial clearance from the nasal cavity, but only if the transferred T cells can produce IL-17.[367] This indicates a protective role for Th17 responses during mucosal GAS infection, but antigen-specific Th17 cells have also been shown to promote detrimental central nervous system infiltration and blood-brain barrier leakage during GAS infection.[369]

Other host factors influencing infection outcome

In mice, sex affects susceptibility to GAS infection: males generally exhibit exacerbated symptoms and increased mortality compared to females.[362,370] Another host factor that can influence the outcome of infection is MHC-II; some haplotypes are associated with increased susceptibility to sepsis while others confer protection, in humans[371–374] and mice[361] alike. Those with protective haplotypes are better at controlling bacterial and immune cell proliferation and cytokine production, preventing the extensive tissue destruction and bacteremia seen in susceptible

haplotypes.[361,362,371–375] Susceptibility in mice has been mapped to four additional loci, and analysis of differentially expressed genes in these loci identified IL-1β as a key regulator of disease outcome.[376] The implications of this are discussed below.

The role of IL-1β during GAS infection

After identifying IL-1β as a key susceptibility regulator in mice infected with GAS, Chella Krishnan *et al.* compared *il-1β* expression in several different mouse strains and found that decreased survival was associated with high *il-1β* expression.[376] A link between high IL-1β levels and detrimental effects during GAS infection has been reported previously: type I IFN receptor$^{-/-}$ mice produce increased levels of IL-1β, which leads to increased neutrophil influx and cytokine levels and results in systemic hyperinflammation and decreased survival.[350] A similar phenotype is seen in mice deficient in NF-κB signaling; here hyperinflammation is rescued by deletion of IL-1R.[377] The presence of a neutralizing anti-IL-1β antibody also reduces wound size in a GAS skin infection model.[378] However, IL-1β is not only detrimental to an infected host: IL-1R$^{-/-}$ and IL-1β$^{-/-}$ mice or mice infected in the presence of anakinra also show decreased survival and increased lesion size and bacterial numbers,[200,350,377,379] illustrating that a balanced IL-1β response is beneficial for disease outcome. This is also reflected in humans, as patients receiving anakinra have a 300-fold higher risk of contracting invasive GAS infections, while infections by other pathogens are unchanged.[200] Despite the clear implications of removing IL-1β or its receptor during GAS infection, results are less clear when it comes to the role of specific inflammasome components *in vivo*. Harder *et al.* found no difference in susceptibility between NLRP3$^{-/-}$ mice and wt during intraperitoneal infection, despite lower serum levels of IL-1β.[44] In contrast, caspase-1/11$^{-/-}$ mice were more susceptible than wt mice in a subcutaneous infection model,[350] suggesting that there might be differences in the response depending on infection route.

Several GAS proteins have been described to induce IL-1β release. Most recent is SLS, suggested to play a partial role in IL-1β release during GAS infection,[378] but the mechanism and inflammasome components involved were not expanded on. Another GAS protein shown to activate NLRP3 is the ADP-ribosylating enzyme SpyA, leading to decreased bacterial survival in host cells.[379] In addition, recombinant M1 protein induces NLRP3-dependent IL-1β release after endocytosis, and infecting macrophages with GAS strains lacking M1 results in a partial reduction in IL-1β levels compared to wt GAS.[380] Last in this list but the first GAS inflammasome activator to be described, SLO-dependent activation of NLRP3[44] is dependent on K^+ efflux[381] and requires SLO pore formation.[382] Increased IL-1β levels in response to a partially active SLO mutant are thought to be due to decreased cytotoxicity, allowing for plasma membrane repair and a sustained response.[382] We have recently shown that GAS can regulate IL-1β release through NADase-

dependent inhibition[383] (Paper I) of a release pathway involving P2X7 and membrane permeabilization (Paper II).[384] In contrast to previously reported functions of NADase, inhibition of P2X7-dependent IL-1β release is mediated by the extracellularly located fraction of this toxin (Paper I).[383] In addition to its inflammasome-activating properties, SLO also seems to be able to regulate IL-1β levels, by inducing ubiquitination and degradation of pro-IL-1β which could be rescued by the broad autophagy inhibitor 3-methyladenine (3-MA) (Paper III).[381] This suggests that GAS has evolved several mechanisms of regulating IL-1β release (Paper I-III).

Present investigation

Original papers and manuscripts

Paper I: Inhibition of Inflammasome-Dependent Interleukin 1β Production by *Streptococcal* NAD^+-Glycohydrolase: Evidence for Extracellular Activity

Dóra Hancz, Elsa Westerlund, Benedicte Bastiat-Sempe, Onkar Sharma, Christine Valfridsson, Lena Meyer, John F. Love, Maghnus O'Seaghdha, Michael R. Wessels, Jenny J. Persson.

mBio. 2017 Jul 18;8(4). pii: e00756-17. doi: 10.1128/mBio.00756-17

Paper II: The Secreted Virulence Factor NADase of Group A *Streptococcus* Inhibits P2X7 receptor-mediated Release of IL-1β

Elsa Westerlund, Christine Valfridsson, Daisy X Yi, Jenny J. Persson.

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Paper III: Streptolysin O Induces Ubiquitination and Degradation of pro-IL-1β

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Paper IV: *Streptococcal* β -NAD^+-glycohydrolase binds to and induces cytokine release from macrophages in the absence of Streptolysin O

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Manuscript

Summary and discussion of papers

Paper I: Inhibition of Inflammasome-Dependent Interleukin 1β Production by Streptococcal NAD^+-Glycohydrolase: Evidence for Extracellular Activity

Background and aim

The two virulence factors SLO and NADase are genetically and functionally linked: SLO protein stability requires co-expression of NADase[307] and SLO can mediate translocation of NADase into host cell cytosols.[304] Their combined effects include cell death and phagolysosomal escape,[269] and strains expressing them show increased disease symptoms and are overrepresented in invasive infections.[289,299]

The inflammatory cytokine IL-1β is important during GAS infection – without it, bacterial numbers increase and disease symptoms worsen.[200,350,379] The NLRP3 inflammasome can be activated by at least three different GAS proteins,[44,379,380] one of which is SLO and the pores it forms.[44,382] However, the role of NADase during SLO-dependent inflammasome activation inflammasome has not been studied previously. We set out to investigate this using a macrophage infection model and a set of isogenic bacterial mutants.

Key findings

- NADase inhibits IL-1β release after SLO-dependent NLRP3 activation.
- NLRP3 activation and inhibition of IL-1β release are executed by extracellularly located bacteria.
- Differences in IL-1β release are not due to differential induction of cell death or priming.
- Caspase-1 activation levels are similar in absence or presence of NADase, suggesting an inhibitory effect of NADase downstream of inflammasome activation.
- Inhibition of IL-1β release is performed by non-translocated NADase, revealing a novel role and functional niche for the enzyme.

Discussion

After the discovery that cell adherent GAS may perform CMT, SLO-mediated translocation of NADase into the cell cytosol,[304] much effort has been invested in elucidating its mechanism and understanding the effects of intracellularly located

NADase. During an infection however, it is likely the case that NADase is both translocated and present extracellularly. By taking advantage of the fact that translocation occurs only upon secretion from the same bacterium[304] and by using a strain not competent for translocation,[269] our study is the first to show that extracellular NADase has a specific function during macrophage infection: inhibition of IL-1β release. We also found that NADase cannot affect IL-1β release induced by SLO when only pure SLO and NADase proteins are used (unpublished data), implying that an infection context and possibly other host-pathogen interactions are required for this event to occur. It remains to be investigated whether this immune regulatory property is common to all NAD^+-hydrolase enzymes expressed by other bacterial species, such as the newly described NAD^+ and $NADP^+$ glycohydrolase expressed by *Mycobacterium tuberculosis*.[385,386]

Patients receiving the IL-1R antagonist anakinra have a significantly increased risk of specifically contracting invasive GAS diseases, illustrating the importance of IL-1 signaling for infection control. In contrast, in a study of susceptibility to GAS among mouse strains, high levels of IL-1β correlated with decreased survival,[376] and yet another indicated that uncontrolled IL-1β levels cause lethal hyperinflammation and tissue damage.[350] This indicates that IL-1β responses need to be finely balanced for optimal host survival and bacterial clearance. Our findings suggest that GAS have evolved a way to limit IL-1β levels during infection, possibly to skew the immune response in its favor. However, the contribution of NADase-dependent inhibition of IL-1β during GAS infection still requires further investigation before we can fully understand its effects on disease progression.

While several pathogens have the ability to interfere with the generation of IL-1β, *e.g.* by preventing inflammasome formation and activity,[103,105] our study indicates that NADase inhibits IL-1β release *per se*. Several non-classical IL-1β release pathways have been described, among them release *via* exosomes, secretory lysosomes or through GSDMD pores.[206] It is possible that NADase targets one or several of these pathways to prevent IL-1β release. In summary, our results describe a previously unknown role for NADase and functionality of the toxin in a novel location. Inhibition of IL-1β release may mediate regulation of the immune response, potentially leading to increased bacterial spread in the host.

Paper II: The Secreted Virulence Factor NADase of Group A *Streptococcus* Inhibits P2X7 receptor-mediated Release of IL-1β

Background and aim

The inflammatory cytokine IL-1β lacks a leader sequence directing it to the classical ER-Golgi secretion pathway and thus has to rely on unconventional protein release pathways.[206] Several pathways have been suggested, including packaging into

secretory lysosomes or exosomes, active membrane transporters or GSDMD pores.[206] Activation of the purinergic receptor P2X7 leads to Ca^{2+} and K^+ flux, which has been implicated both in NLRP3 inflammasome activation and unconventional protein release.[170] Innate immune cells such as macrophages activate the NLRP3 inflammasome and release IL-1β in response to K^+ efflux induced by GAS and its pore-forming toxin SLO.[44] We have previously shown that extracellularly located NADase can counteract this response by inhibiting IL-1β release through an unknown mechanism (Paper I).[383] The focus of Paper II was to investigate this pathway further.

Key findings

- In absence of functional NADase, GAS induces a P2X7-dependent IL-1β release pathway.
- P2X7-dependent IL-1β release occurs independently of extracellular ATP and is not affected by NAD^+ cleavage products.
- Protein degradation or vesicular release does not differ in absence or presence of functional NADase.
- P2X7-dependent IL-1β release is dependent on permeabilization of the plasma membrane.
- GSDMD is not involved in P2X7-dependent or -independent IL-1β release in response to GAS.

Discussion

As the presence of NADase or PEGs only partially inhibits IL-1β release, our results suggest that this cytokine is externalized through two pathways in response to GAS, one of which involved the P2X7 receptor and membrane permeabilization. This partial inhibition could represent a way for the host to create redundancy – by releasing IL-1β through several non-classical pathways, immune system activation in response to GAS is ensured. Release through several pathways has also been suggested to reflect signal strength; in response to a weak signal, one pathway is activated, while another might be switched on as the signal intensity increases.[204] In addition, maintaining IL-1β at appropriate levels could also be beneficial for the bacterium. Invasive infections caused by complete lack of IL-1 signaling are detrimental for the host, but GAS which in these cases are located in normally sterile sites may also be less likely to spread to new individuals.[287] Host mortality likely also limits the spread of GAS in a population suggesting that lethal GAS infections may be an evolutionary dead end for the bacterium.[287]

The P2X7 receptor is well-known for its importance in ATP-mediated NLRP3 activation, but has also been implicated in the regulation of unconventional protein

release pathways.[170] Our data suggest P2X7-mediated IL-1β release is not vesicular or autophagic, but indicates a requirement for plasma membrane permeabilization. In light of the recent discovery that GSDMD pores mediate IL-1β release in response to several inflammasome-activating stimuli,[206] we were thus surprised to find that GAS-induced IL-1β release is completely independent on GSDMD. Activation of the P2X7 receptor has been reported to lead to opening of a large membrane pore, the so-called P2X7 macropore. This structure has been suggested to mediate unconventional release of proteins,[387] but whether the macropore is formed by the trimeric P2X7 receptor itself or by accessory proteins is debated.[124] However, as the estimated size of the pore formed by P2X7 is smaller than IL-1β,[145,207] direct release through P2X7 itself is questionable and accessory proteins are likely involved.[124] Our data supports this hypothesis, although we were not able to specifically identify additional proteins involved. In addition, our data does not allow us to predict the sequence of events surrounding P2X7-dependent IL-1β release, *i.e.* whether P2X7 and eventual accessory proteins are involved in an upstream regulatory process or if they are involved in the actual release. The C-terminal tail of P2X7 harbors predicted binding motifs for multiple proteins and lipids[129] and is important for membrane localization[130] and permeabilization.[388] It is thus attractive to speculate that P2X7-dependent functions can be dictated or modified by different binding partners to these domains, including the release of IL-1β in response to GAS infection.

In summary, our results indicate that NADase acts to inhibit an IL-1β release pathway dependent on the P2X7 receptor and membrane permeabilization. While the exact mechanism is still unknown, our study sheds light on IL-1β release in response to a setting where several stimuli are present and pinpoints P2X7 as a future research interest in relation to GAS infections.

Paper III: Streptolysin O Induces Ubiquitination and Degradation of pro-IL-1β

Background and aim

Addition or removal of the small protein ubiquitin can modify the activation and function of a target protein as well as mark it for degradation or secretion.[112] The presence of one or several ubiquitin moieties, and whether the chains they form are linear or branched, determines which of the diverse fates a target protein meets.[112] An example of how ubiquitination affects cellular processes relates to NLRP3 inflammasome activation and subsequent IL-1β release. Caspase-1 ubiquitination enhances its activity[73] while NLRP3 activity instead requires deubiquitination, preventing its proteasomal degradation.[60,62] ASC speck formation can be both enhanced[68] and inhibited[71] by ubiquitination, as can pro-IL-1β levels; either leading

to aberrant activation[118,119] or degradation.[117,203] During our investigations into NADase-dependent inhibition of IL-1β,[383,384] we found that pro-IL-1β was ubiquitinated in macrophages in response to GAS infection and that this was dependent on SLO, thus prompting us to further investigate this phenomenon.

Key findings

- SLO-expressing GAS induce ubiquitination of pro-IL-1β.
- SLO pore formation is required but not sufficient for ubiquitination of pro-IL-1β.
- SLO-dependent ubiquitination occurs independently of inflammasome activation or inflammasome proteins.
- Pro-IL-1β ubiquitination is of mixed linkage specificity.
- Pro-IL-1β is degraded upon infection with SLO-expressing GAS.

Discussion

SLO activates the NLRP3 inflammasome,[44] leading to release of IL-1β which plays an important role in the host response against GAS.[200] In this study, we describe a novel role for SLO in regulating the levels of this cytokine by ubiquitination and degradation of pro-IL-1β.

Pro-IL-1β ubiquitination has previously been shown to be of the K63 or K48 linkages, leading to increased release[118,119] or degradation,[117] respectively. We found that the ubiquitination pattern induced in response to GAS contained a mixture of these linkages, but we have not determined the exact composition of the ubiquitin pattern, *i.e.* whether the two types of linkages are part of the same chain or stem from separate ubiquitination sites. In addition, we have not investigated whether the basal pro-IL-1β ubiquitination seen after LPS priming is responsible for one linkage type and GAS infection the other, or whether LPS-induced ubiquitination is required for the SLO-induced process. Although the ubiquitination pattern is heterogeneous, the overall signal seems to be related to degradation, which can be blocked by the autophagy inhibitor 3-MA. However, whether pro-IL-1β degradation actually proceeds through the autophagy pathway is uncertain at this point; 3-MA is a broad PI3K inhibitor that can interfere witht several processes in addition to autophagy, and Bafilomycin, an inhibitor that prevents the formation of the autophagolysosome, did not prevent pro-IL-1β degradation.

A lysine at position 133 in pro-IL-1β has been identified as a site for K63-linked ubiquitination that supports increased release of mature IL-1β,[119] but whether this ubiquitination site is of relevance for our observations remains to be determined. Future studies will be aimed at defining the residues involved in ubiquitination in response to GAS, and specifically mutating these residues would aid in the

investigation of the role and importance of pro-IL-1β ubiquitination during infection. It would also be of interest to determine whether the mature, released cytokine is ubiquitinated and whether this could affect binding to IL-1R and subsequent signaling.

Presence of the inflammasome components NLRP3, ASC or caspase-1 do not seem to be required for pro-IL-1β ubiquitination induced by GAS, nor does inflammasome activation. In contrast, ubiquitination is increased in macrophages lacking some of the inflammasome components, indicating that they may play a role in regulating the ubiquitination processes. Indeed, it has been suggested that inflammasome proteins may be involved in non-inflammasome events, such as regulation of metabolic pathways.[389]

Several pathogens exploit the ubiquitin system in general, including its role in inflammasome activation; *E. coli* prevents NLRP3 deubiquitination[103] and a ubiquitin ligase from human papilloma virus induces IL-1β degradation.[104] Whether SLO-mediated ubiquitination belongs to this category or if pro-IL-1β degradation is a regulatory mechanism induced by the host is a question for further studies.

Another question that remains to be answered is the identity of the ubiquitin ligase responsible for pro-IL-1β ubiquitination, and whether it comes from GAS or the host. The requirement of an infection setting, *i.e.* that no ubiquitination occurs when recombinant SLO alone is sensed by the cell, suggests that additional bacterial factors are involved, possibly including a bacterial E3-ligase.

Another involved factor might be translocated NADase, and the striking similarities between the requirements for ubiquitination and CMT provides some support for this speculation. However, studying this possibility is complicated by the fact that NADase stabilizes SLO and that full SLO stability requires that NADase expresses a domain needed for its translocation.[307] To investigate the involvement of NADase in SLO-mediated ubiquitination, a translocation mutant with retained SLO stability would need to be constructed.

Paper IV: *Streptococcal* β-NAD^+-glycohydrolase binds to and induces cytokine release from macrophages in the absence of Streptolysin O

Background and aim

Much effort has gone into elucidating the mechanism behind CMT, the SLO-dependent translocation of NADase into host cell cytosols. For CMT to occur, the co-association of SLO and NADase at the cell surface requires that NADase binds to a yet unknown receptor; a binding event that also requires the presence of SLO.[312] During our previous studies, we unexpectedly found NADase activity associated

with the membrane even in the absence of SLO, which prompted our further investigation.

Key findings

- NADase associates to the plasma membrane in absence of SLO during GAS infection of macrophages.
- Recombinant NADase binds to a cell surface protein independently of other bacterial proteins.
- Recombinant NADase induces MyD88- and TRIF-dependent cytokine release from macrophages expressing TLR4 and CD14.
- TLR4 and CD14 are not required for NADase binding to macrophages.
- Inactive recombinant NADase harbouring a G330D mutation exhibit decreased binding to macrophages and does not induce cytokine release.

Discussion

SLO has previously been suggested to induce TLR4 activation, leading to production of inflammatory cytokines.[355] We also propose involvement of this receptor in GAS pathogenesis in the downstream signaling pathways induced upon NADase binding to macrophages. However, as NADase does not seem to bind directly to TLR4 or CD14 it is currently unclear how the receptor is activated: does NADase induce release of an endogenous TLR4 agonist or does NADase binding to one or several of the surface proteins involved in TLR4 signaling? Cross-linking of NADase when present on the macrophage surface and subsequent immunoprecipitation using an anti-NADase antibody might let us identify the different components involved in NADase binding and cytokine induction.

NADase binding to epithelial cells involves an N-terminal region with a putative carbohydrate-binding motif.[312] Although binding to macrophages seems to depend on a cell-surface protein, it is still possible that the carbohydrate-binding motif is important for the interaction to occur. Recombinant NADase carrying a mutation in the carbohydrate-binding motif would thus be of interest to our continued studies. A screen of surface molecules expressed on macrophages but not epithelial cells could narrow down the list of possible candidates for NADase binding. In addition, future studies should investigate whether the interaction between NADase and macrophages is unique or whether it extends to *e.g.* other innate immune cells such as DCs and neutrophils and, importantly, to cells of human origin.

Interestingly, in a subpopulation of M1T1 isolates, specific mutations rendering NADase enzymatically inactive have been fixed, suggesting that the presence of an inactive NADase is under positive evolutionary pressure.[300–302] Strains expressing inactive NADase have been suggested to belong to the so-called specialist strains,

i.e. strains showing a preference for causing infections at either throat or skin, while strains expressing active NADase are generalists, equally likely to infect both tissues.[301] The pressure to express enzymatically inactive NADase is thought to partly be due to its importance in the stability and function of SLO,[307] but might also be due to unknown roles in bacterial fitness and/or virulence. Our study suggests that active NADase induces cytokine release, while the inactive form of the protein does not, and it remains to be determined whether host or bacterium benefits from the induction of pro-inflammatory cytokines. Elevated cytokine release could be important in restricting bacterial dissemination through influx and activation of immune cells, but could also increase the permeability of tissues and vasculature, which may facilitate bacterial spread into deeper tissues and improve bacterial establishment and disease progression. Conversely, decreased cytokine release might similarly also benefit both bacterium and host. To further elucidate this newly described function of NADase and its possible role in immune regulation by GAS, studies comparing NADase-active and -inactive strains and the immune responses generated against them would be an interesting place to start.

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