Adjuvant immunotherapies as a novel approach to bacterial infections

The rapid emergence of multidrug-resistant pathogens, especially Gram-negative bacteria and mycobacteria, represents one of the major medical challenges of the 21st century. The gradual loss of effective classical antibiotics for many bacterial pathogens, combined with an increasing population density and mobility, urgently calls for the development of novel treatments. Here, we discuss the potential of adjuvant immunotherapies to selectively stimulate protective immune responses as a treatment option for bacterial infections. In order to elicit appropriate immune responses and to avoid unwanted inflammatory tissue damage, it is essential to identify ligands and receptor pathways that specifically control protective responses at the site of infection. We summarize existing data and discuss suitable candidate targets for future immunotherapies of infectious diseases.

KEYWORDS: adjuvant immunotherapy ▪ bacterial infections ▪ immune checkpoints ▪ infectious threat ▪ innate immunity ▪ multidrug resistance ▪ vita-PAMPs

Elisa T Helbig[1], Bastian Opitz[1] & Leif E Sander*[1]

[1]Department of Infectious Diseases & Pulmonary Medicine, Charité University Hospital, Augustenburger Platz 1, 13353 Berlin, Germany
**Author for correspondence: leif-erik.sander@charite.de*

In light of the sweeping success of antibiotics and vaccines in the mid-20th century, there was the widespread fallacious notion in the 1960s and 1970s that infectious diseases had been defeated once and for all. One of the world's leaders in infectious diseases at that time, Petersdorf, predicted a millennium in which clinical infectious disease fellows would have to "culture themselves" [1,2]. Unfortunately, these and other anecdotal prophecies were false. Today, infectious diseases continue to represent one of the largest medical problems and socio-economic challenges worldwide [3]. In low-income countries, infectious diseases represent the leading cause of death. While in middle- and high-income countries most people die from chronic conditions such as cardiovascular diseases and cancer, mortality rates of infectious diseases such as pneumonia remain stubbornly high despite easy access to healthcare and antimicrobials [201]. Why did the optimistic prophecies of the mid-last century fail? The reasons are probably simple: as environments and hosts change, so do the microorganisms and their vectors, creating new host–microbe interactions and new diseases. Simply speaking, modern medicine was unable to outrun microbial evolution. In the past century alone, several hundred documented infectious diseases have emerged or re-emerged [4]. The most prominent examples are viral diseases such as HIV/AIDS, SARS or Ebola; however, strikingly, bacterial infections represent the majority of all emerging infectious diseases [4].

Introduction of antibiotic therapy in the 1930s was undoubtedly one of the greatest medical breakthroughs of all time, which saved millions of lives because infectious disease (e.g., pneumonia)-related mortality was dramatically reduced [5]. Today, many standard procedures such as abdominal surgery are hard to imagine without the ability to prevent and treat bacterial infections with broad-spectrum antibiotics. However, the easy availability and widespread (mis-)use of antibiotics has come at the price of a sharply increasing bacterial drug resistance due to Darwinian selection. Unfortunately, at the same time, the antibiotic pipeline has successively dried up in the past few decades [6].

Antibiotic resistance is a rapidly growing global problem; however, with specific geographic distribution patterns [202]. For instance, eastern Europe and Asia are experiencing alarmingly high rates of multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis* infections [7,8], which, in combination with an increased population mobility, is likely to lead to a renaissance of tuberculosis in western countries. Antibiotic-resistant Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* or vancomycin-resistant *Enterococci* have drawn significant public attention [9]. Fortunately, infection rates are slowly decreasing in recent years [10] and most isolates are still sensitive to second- and third-line antibiotics such as linezolid. By contrast, the drastic increase of multidrug resistance among Gram-negative bacteria, especially *Enterobacteriaceae* such as

Future Medicine part of fsg

Escherichia coli, *Klebsiella* spp. and nonfermenters such as *Pseudomonas* and *Acinetobacter* spp., is a cause for major concern [11–13]. As extended-spectrum β-lactamase (ESBL) rates are soaring in parts of Asia, Latin America and southern Europe, with frequencies of ESBL for *E. coli* and *Klebsiella* spp. reaching >80% of clinical isolates in India [11], classical antibiotics such as third-generation cephalosporins are falling by the wayside. Rising cephalosporin resistance has in turn greatly increased carbapenem consumption. Until recently, carbapenems were typical reserve antibiotics with near-universal activity against all *Enterobacteriaceae,* most other Gram-negative and several Gram-positive bacterial species. Lately, several different carbapenemases have been isolated. Carbapenemase-producing strains are on the rise and they are usually resistant to most antibiotics with the (partial) exception of tigecycline and some old and sub-optimal agents such as colistin or fosfomycin [14,15]. However, tigecycline has little or no activity against *Pseudomonas* and tigecycline-resistant *Enterobacteriaceae* are also emerging [16]. Furthermore, tigecycline has shown limited clinical effectiveness in treating severe infections, possibly due to its pharmacokinetic/pharmacodynamic profile and its bacteriostatic rather than bacteriolytic activity [17]. Moreover, even completely colistin-resistant carbapenemase-producing *Klebsiella pneumoniae* strains have already emerged [18], leaving no more arrows in the antibiotic quiver. These scenarios have led WHO and leading infectious disease professionals to warn that modern medicine is on the verge of a postantibiotic era [203], an ironic contrast to the euphoric predictions of the 1960s.

Antibiotic resistance is often encoded on promiscuous plasmids, which can be easily transferred among strains and species, thereby facilitating the fast spread of drug resistance. Gut resident microflora [19] or contaminated food and water [20,21] have been shown to serve as a reservoir and source of spread for antibiotic resistance such as ESBL. Misuse of antibiotics creates selective pressure which favors the development of drug resistance. Some authors have estimated up to 50% of all antibiotic prescriptions worldwide to be considered inappropriate [22]. Such misuse includes antibiotic treatment of viral infections, prescription of broad-spectrum antibiotics for banal infections, too low dosing or too short intake periods, all of which create ideal conditions for Darwinian selection of antibiotic-resistant mutants. Another cause for concern is the massive consumption of broad-spectrum antibiotics in animal farming. In addition to, for example, high ESBL carriage rates in poultry, published evidence suggests that soil resident bacteria are becoming increasingly antibiotic-resistant as a result of uncontrolled antibiotic use in meat production and plant agriculture [23]. In an excellent study, Forsberg *et al.* recently demonstrated that soil bacteria may serve as an important environmental source of resistance genes [24]. The authors found perfect sequence identity in several resistance genes and flanking mobile elements of soil bacteria and human pathogens as a direct indicator of horizontal gene transfer of resistance genes [24].

The combination of rapidly emerging multi-drug resistance and increasing global population density urgently calls for novel, broadly effective and cost-efficient therapies for infectious diseases. While the development of new antibiotics is sorely needed, the pipeline may still fail to keep pace with the emergence of resistance [6]. It will therefore be important to design strategies to limit our dependence on antibiotics as the sole therapeutic option for bacterial infections. Instead of the traditional pathogen-centered therapies, we discuss the prospects of host-focused approaches to selectively stimulate protective antimicrobial immune responses. Such treatments are referred to as adjuvant immunotherapies and could be used as an alternative or a potent addition to conventional antibiotics [25]. Intentional induction of antimicrobial immunity is an old and very efficient prophylactic concept, first introduced by Edward Jenner in 1798 and widely known as vaccination [26]. Specific vaccines against problematic pathogens known to frequently carry antibiotic resistance could critically limit their spread. However, besides technical obstacles to develop efficient targeted vaccines, there is growing vaccination reluctance in the population due to safety concerns [27]. In addition, acute bacterial infections demand quick-acting therapies. Classical vaccines confer protection by generating adaptive immune responses, which normally require several days or even weeks to fully unfold. By contrast, the innate immune system possesses a large arsenal of antimicrobial effectors, which can be activated within minutes or hours. Furthermore, innate immune responses are antigen-independent, allowing a broader application of adjuvant immunotherapies, at least against the same class of pathogens (e.g., Gram-negative extracellular bacteria). It has also been suggested that immunotherapies may be favorable for managing chronic infections such as *M. tuberculosis* infections [28]. Here, we

discuss novel strategies for targeted activation of innate immunity and review previously published studies on innate immune stimulants as adjuvant immunotherapeutic agents. We will particularly focus on novel insights into the immunological decision-making mechanisms that govern antimicrobial responses and means to manipulate them therapeutically.

Innate immune detection of microbial infections

The innate immune system comprises a complex network of cellular and molecular components that collectively detect microbial invaders and orchestrate protective immune responses. Targeted manipulation of this sophisticated machinery may therefore represent a promising strategy against bacterial infections without the risk of further aggravating antibiotic resistance through increased selective pressure.

As predicted by Charles Janeway Jr in 1989, the innate immune system senses microorganisms through pattern recognition receptors (PRRs), a large group of germline-encoded immune receptors (**Box 1**). PRRs recognize highly conserved pathogen-associated molecular patterns (PAMPs) [29], which are defined as integral parts of all microorganisms and mark them as foreign to the immune system [30]. PRR ligation activates intracellular signaling cascades, often converging

Box 1. Pattern recognition receptors.

TLRs

- TLRs were the first group of PRRs to be discovered. In humans, ten functional TLRs have been found, which detect a broad range of PAMPs including lipids, lipoproteins, lipopolysaccharide, proteins and nucleic acids derived from bacteria, fungi, viruses and parasites, as well as some synthetic compounds. According to their subcellular localization, TLRs can be divided into surface (TLR-1–6 and -10) and endosomal (TLR-3 and -7–9) TLRs
- TLRs are transmembrane proteins and the extracellular LRR domain is thought to bind microbial ligands, which results in the formation of receptor homo- or hetero-dimers. Dimerization of cytosolic TIR domains is required for the recruitment of adaptor molecules. All TLRs, except TLR-3, signal through MyD88. TLR-3 recruits TRIF, which can also be recruited to TLR-4 upon receptor internalization. Through a series of downstream signaling partners, MyD88 activates NF-κB and MAPKs, whereas TRIF primarily activates IRF3 and NF-κB. TLRs can also detect endogenous molecules modified by microbes, such as hemozin, which is a produced by *Plasmodium falciparum* through digestion of hemoglobin

NLRs

- NLRs form a large family of at least 20 proteins in humans, characterized by a LRR, NOD domain and variable effector domain. NLR activation by PAMPs, as well as DAMPs, induces different responses. NLRs can be functionally divided into three groups:
  - NLRs that initiate proinflammatory responses through RIP2 kinase and downstream NF-κB and MAPK activation (e.g., NOD1 and NOD2)
  - NLRs that form inflammasomes leading to the activation of caspase-1 (e.g., NLRP1, NLRP3 and NLRC4/NAIP5)
  - NLRs that regulate other cellular responses such as type-I IFN production or MHC molecule expression (e.g., NLRC5 and NLRP12)
- Many NLR functions and ligands remain unknown. Owing to their cytosolic localization, NLRs are considered gatekeepers of the host cell cytosol that detect invading pathogens

RLRs

- RLRs contain an N-terminal CARD (not present in LGP2) and a DExD/H-box RNA helicase domain. RIG-I and MDA5 detect viral dsRNA, whereas LGP2 might function as a negative regulator or cofactor of these receptors. Upon detection of viral RNA, RIG-I and MDA5 signal through IPS-1, also called MAVS or VISA, and induce type-I IFN production

CLRs

- CLRs form a large family of proteins that all share the common feature of a CTLD. Several CLRs are considered PRRs, involved in the sensing of mycobacterial, fungal and viral carbohydrate, as well as noncarbohydrate PAMPs. Downstream-signaling via SYK and CARD9 leads to the production of proinflammatory cytokines, inflammasome activation and ROS generation. CLEC9A, also known as DNGR1, is a CLR expressed primarily on cross-presenting DCs. It detects F-actin as a DAMP exposed by necrotic cells and promotes cellular immunity

Other cytosolic PRRs

- AIM2 was recently identified as a sensor for cytosolic DNA, leading to ASC recruitment, inflammasome formation and caspase-1 activation. AIM2 is a member of the PHYIN protein family, which comprises four human and at least 11 mouse proteins. Another PYHIN family member, IFI16, and its mouse ortholog, p204, were recently proposed as sensors of cytosolic DNA inducing type-I IFN production
- DHX9 and DHX36 were recently found to bind cytosolic CpG DNA and activate MyD88 in human plasmacytoid DCs. A complex consisting of DHX36, DDX1 and DDX21 was also found to sense cytosolic dsRNA and to induce TRIF-dependent IFN-β production. STING plays a key role in the cellular response to cytosolic DNA and it was recently shown to detect bacterial-derived c-di-GMP and c-di-AMP, and induce type-I IFNs

CARD: Caspase activation and recruitment domain; CLR: C-type lectin receptor; CTLD: C-type lectin-like domain; DAMP: Danger-associated molecular pattern; DC: Dendritic cell; LRR: Leucin-rich repeat; NOD: Nucleotide oligomerization domain; NLR: Nucleotide oligomerization domain-like receptor; PAMP: Pathogen associated molecular pattern; PRR: Pattern recognition receptor; RLR: RIG-I-like receptor; ROS: Reactive oxygen species; TIR: Toll-IL-1 receptor; TLR: Toll-like receptor.

on NF-κB, MAPKs and/or interferon regulatory factors [31], leading to cytokine expression, enhanced phagocytosis and the generation of reactive oxygen species or production of antimicrobial peptides [32]. Upon activation, some members of the nucleotide oligomerization domain (NOD)-like receptor (NLR) family of PRRs can assemble into large cytosolic protein complexes, termed inflammasomes, which serve as platforms for autocatalytic activation of caspase-1 and subsequent maturation and release of IL-1β and IL-18 [33]. In APCs, PRR activation promotes antigen processing and presentation, upregulation of co-stimulatory molecules and cytokine production. Thus, PRR ligation links innate and adaptive immunity [34]; a fact that is frequently exploited since many known vaccine adjuvants act as PRR ligands [35,36].

The pattern recognition dilemma: how to discriminate dangerous from harmless microbial contacts

Inflammatory immune responses must be tightly controlled in order to avoid unnecessary or excessive tissue damage, while ensuring efficient microbial clearance. Thus, antimicrobial immune responses are scaled to the level of infectious threat posed by a given microbial encounter [37]. Indeed, pathogenic and invasive bacteria generally elicit more robust immune responses compared with nonpathogenic or commensal microbes [38]. Given that all microorganisms per definition contain PAMPs, finer immunological distinctions, for example, between pathogens and commensals, require additional layers of regulation. We have recently proposed a concept of five immune checkpoints that collectively allow the immune system to integrate microbial signals along with (micro-) environmental cues to accurately measure the infectious threat [37]. We proposed that the infectious threat level is measured through a series of checkpoints that discriminate between soluble and particulate (checkpoint 1), live and dead (checkpoint 2), pathogenic and nonpathogenic (checkpoint 3) and invasive and colonizing (checkpoint 4) microbial stimuli. In addition, tissue (mucosal and parenchymal) and compartment (local, systemic, sterile and nonsterile)-specific cues are integrated to shape the ensuing immune response (checkpoint 5) [37]. While the molecular mechanisms underlying many of these delicate distinctions still remain to be fully elucidated, we believe that the targeted manipulation of individual or several of these immune checkpoints may represent a powerful strategy to elicit protective antimicrobial immunity [39]. Based on our recent findings, we particularly favor the activation of signaling pathways that are centrally involved in the detection of microbial viability, bacterial pathogenicity and tissue damage [39,40].

Viability-associated PAMPs are signatures of microbial life & indicators of increased infectious threat

Live microorganisms are historically known to induce more vigorous immune responses than their killed counterparts, illustrated by the often-observed superiority of live vaccines [27,41,42]. Given that microbial viability is the fundamental basis of infectivity, we hypothesized that the innate immune system actively discriminates between live and dead microbes. Indeed, we found that only viable but not killed avirulent *E. coli* activate the NLRP3 inflammasome, leading to caspase-1 activation and subsequent cleavage and release of IL-1β, and pyroptosis. In addition, stimulation of murine macrophages or dendritic cells with live avirulent bacteria significantly enhanced the production of type-I IFN. These responses were not restricted to *E. coli* and were also observed with other nonpathogenic or severely attenuated bacteria. This study demonstrated an inherent ability of innate immune cells to detect bacterial viability *per se*, independently of virulence factors [40]. Previously, superior immune responses to viable microorganisms (e.g., in vaccines) compared with inanimate stimuli had been attributed to an allegedly higher virulence and persistence of live microbes [27]. When we searched for unique molecular characteristics of viable bacteria, we identified bacterial mRNA as a labile component, which was rapidly lost upon bacterial killing. Addition of purified bacterial mRNA to heat-killed *E. coli* completely restored their ability to induce inflammasome activation and IFN-β production, indicating that prokaryotic mRNA is a molecular signature of microbial life, the detection of which triggers robust immune responses not warranted for dead microbes. We consequently defined prokaryotic mRNA as the first member of a novel class of PAMPs, called viability-associated PAMPs (vita-PAMPs) [40]. Vita-PAMPs indicate microbial viability and thus an elevated infectious threat to the immune system. Cellular responses to vita-PAMPs require NLRP3, ASC and caspase-1 for IL-1β production and pyroptosis in addition

to the transcription factor IRF3 for IFN-β expression. Importantly, the Toll-like receptor (TLR) adaptor protein TRIF is required for both inflammasome activation and IFN-β production, indicating its central role in the detection of microbial viability [40]. Recently, Rathinam *et al.* found that TRIF induces caspase-11 expression via auto/parakrine type-I IFN signaling, which leads to a noncanonical activation of the NLRP3 inflammasome in response to live Gram-negative bacteria [43]. Moreover, TRIF-dependent responses are crucial for host defense against systemic *E. coli* infections in mice [40], which is further underscored by studies demonstrating protective effects of TRIF signaling during respiratory and intestinal Gram-negative bacterial infections [44–46].

Besides the robust innate immune responses triggered by viable bacteria and vita-PAMPs, we found that bacterial RNA is a potent stimulator of adaptive immunity. Vaccination of mice with a combination of heat-killed *E. coli* and purified bacterial RNA elicited class-switched *E. coli*-specific IgG serum levels comparable with or even higher than immunization with viable bacteria [40]. Besides bacterial mRNA, other vita-PAMPs likely exist, such as soluble mediators indicative of bacterial metabolism. Cyclic dinucleotides are bacterial second messenger molecules, which were recently shown to activate innate immune responses. Most notably, the bacterially-produced cyclic dinucleotides c-di-AMP and c-di-GMP stimulate robust production of type-I IFN, which is also a hallmark response to viable bacteria [40,47]. Two recent studies identified STING and the cytoplasmic helicase DDX41 as host cell receptors for c-di-AMP and c-di-GMP [48,49]. Besides these and other intracellular signaling molecules, bacteria utilize a sophisticated system, named quorum-sensing (QS), for intercellular communication. QS serves to coordinate population behavior, for example, growth, motility or biofilm formation [50]. This communication requires the synthesis of small, diffusible molecules, termed QS molecules (QSMs). Gram-negative bacteria use autoinducer (AI)-1, which is a *N*-acyl homoserine lactone; or AI-2, a cyclic furanosyl borate diester, and AI-3, as well as a host of other compounds, many of which have not yet been identified [50]. Gram-positive bacteria use post-translationally modified autoinducing peptides [50]. Once a threshold concentration is reached within a population, the QS molecule–receptor complex regulates the expression of quorum sensing-dependent genes [50]. Interestingly, QSMs can also affect mammalian cells. For instance, *N*-(3-oxo-dodecanoyl) homoserine lactone (C12) from *Pseudomonas aeruginosa* inhibits TLR-induced NF-κB signaling through a hitherto undefined pathway [51]. Reduced viability and IL-12 production, along with lower expression of TLR-2 and TLR-4 in human monocytes in the presence of C12 has also been observed [52]. By contrast, a previous study reported increased chemotaxis and phagocytic activity of neutrophils in response to C12 secreted by *P. aeruginosa* in the early phase of biofilm formation [53]. Given that the production of signaling molecules such as cyclic dinucleotides and QSMs depends on active bacterial metabolism and thus viability, they could be valuable indicators for the innate immune system by relaying information on the viability status and population density of infecting bacteria.

Inflammasome activation as a signature response to microbial virulence & tissue damage

Inflammasome-forming NLRs are located in the cytosol and are therefore prime sensors of invasive pathogens. Inflammasome formation is strongly stimulated by many bacterial virulence factors [38]. For example, bacterial pore-forming toxins including listeriolysin of *Listeria monocytogenes* [54], pneumolysin of *Streptococcus pneumoniae* [55] or α-hemolysin of *S. aureus* [56] stimulate the NLRP3 inflammasome, components of bacterial type III secretion systems and flagellin are recognized by the NAIP5/NLRC4 inflammasome [57], and lethal factor of *Bacillus anthracis* is sensed by the NLRP1 inflammasome [58]. Moreover, inflammasomes have been shown to react to endogenous host molecules such as ATP or uric acid released after tissue damage caused by sterile insults [59]. It is also likely that inflammasomes are activated by these danger signals at later stages of infections associated with high degrees of tissue damage. Given that inflammasome activation is a critical response to most virulent bacteria, it seems reasonable to consider inflammasome activators as candidate therapeutic agents for infections with low-virulent or opportunistic bacteria in immunocompromised patients.

We will next discuss the prospects of using immune stimulants that simulate an elevated infectious threat level to the immune system as a means of amplifying antimicrobial host immune responses to bacterial infections.

Stimulation of innate immunity as an adjuvant anti-infective therapy

As conventional antibiotics are becoming an increasingly blunt sword due to rapidly emerging multidrug resistance, alternative concepts, for instance, targeted activation of host immune responses, are urgently needed. Such adjuvant therapies would ideally mobilize protective responses without causing inappropriate collateral tissue damage.

We will summarize previous efforts in the development of immune adjuvants to treat infectious diseases and further discuss ways to use vita-PAMPs and other viability-associated molecules, as well as inflammasome activators, as therapeutics to enhance protective immunity during infections.

Broad innate immune stimulation: targeting TLRs & NODs, among other PRRs

Their central role in the detection of microorganisms and ability to rapidly induce innate immune responses have made PPR ligands obvious candidates for vaccine adjuvants and therapeutics for diseases ranging from cancer to viral infections [60–65]. Here, we will summarize some previous studies with a focus on bacterial infections. An overview of PRR ligands that have been evaluated in preclinical studies and clinical trials is given in Table 1.

Toll-like receptors

Ligation of TLRs elicits proinflammatory responses and although most TLRs share substantial similarities with regards to their intracellular signaling, individual TLRs or certain combinations of TLRs have distinct effects on ensuing immune responses.

Pretreatment with soluble TLR agonists such as Pam3-CSK4 or MALP2 (TLR-2), polyinosinic:polycytidinic acid (polyI:C, TLR-3), lipopolysaccharide (LPS, TLR-4) or CpG DNA (TLR-9) has been shown to enhance host defense through increased phagocytosis and bacterial killing in mice [66–68]. Since LPS is not suitable for clinical applications due to its high toxicity, which is mainly associated with the lipid A portion of the molecule, other ligands such as monophosphoryl lipid A (MPLA) and aminoalkyl glucosaminide phosphates (AGPs) have been tested. MPLA and AGP are less toxic, TRIF-biased TLR-4 agonists, and MPLA is already used as adjuvant in several vaccine formulations [69]. Synthetic TLR-4 agonists can also enhance host defense. Prophylactic administration of the AGP CRX-524 or a combination of CRX-524 and CRX-527 increases resistance to *L. monocytogenes* and *Yersinia pestis* infections in mice [70,71]. MPLA can promote clearance of *Haemophilus influenzae* and *Moraxella catarrhalis* from the nasal mucosa of mice [72]. Another interesting ligand is flagellin, the main component of bacterial flagella. It binds TLR-5 and elicits MyD88-dependent responses, but it is also recognized by NAIP5, which forms an inflammasome with NLRC4 and induces the release of IL-1β and IL-18 [57]. Mucosal administration of flagellin promotes pulmonary immunity and protects mice from bacterial pneumonia with *P. aeruginosa* and *S. pneumoniae* [73,74]. Despite these promising preclinical results, there are only limited clinical data available regarding the use of TLR agonists in the treatment of bacterial infections in patients. The few examples include heat-killed or lysed bacteria, or bacterial mixtures such as CADI-05 [75] or Luivac [76,77], all of which have shown only limited clinical success so far. Nonetheless, TLRs remain attractive targets, especially for infections with poorly TLR-stimulatory bacteria such as *Francisella tularensis*, which expresses a modified form of LPS [78]. Some pathogens, on the other hand, exploit TLR-controlled cellular responses. *Salmonella enterica* serovar Typhimurium requires TLR signaling in order to switch on virulence genes encoded by SPI2, which allows the establishment of the salmonella-containing vacuole and bacterial replication [79], indicating that broad TLR stimulation may not be universally efficient.

Despite the limited success of TLR ligands for the treatment of bacterial infections, these are routinely used to treat viral infections and certain types of skin cancers [80]. Endosomal TLRs (TLR-3, TLR-7, TLR-8 and TLR-9) detect nucleic acids such as viral RNA or DNA. Imiquimod, a synthetic TLR-7 agonist, is approved as a first-line therapy for HPV-associated genital warts [81] and has also been successfully used to treat HSV infections [82]. Resiquimod (R-848), a dual TLR-7 and TLR-8 agonist, and ANA773, a prodrug of a small-molecule TLR-7 agonist, were shown to (transiently) lower HCV RNA serum levels when administered to chronically HCV-infected patients [83,84]. Interestingly, it was recently demonstrated that exogenous TLR stimulation can restore repressed innate immune responses during chronic fungal infections [85].

In addition to stimulating individual TLRs, efforts have also been undertaken to target

Table 1. Overview of tested immunostimulants.

Adjuvant	Function	Experimental and preclinical data	Clinical trials†	Ref.
Poly(I:C)	Synthetic ligand of TLR-3, RIG-I, MDA5 and DDX1, DDX21 and DHX36	Increased phagocytosis and intracellular killing of *Escherichia coli* Transiently increased cytokine production and pulmonary neutrophil recruitment, and prolonged survival in *Francisella tularensis*-infected mice Increased cytokine production and bacterial killing by human macrophages Pretreatment causes increased *Streptococcus pneumoniae*-induced lethality	Trials evaluating use in cancer vaccines and cancer treatment NCT01437605 NCT01008527 NCT00694551 NCT00374049 NCT01720836 NCT00986609	[67,102, 106,144]
MPLA	Synthetic TRIF-biased TLR-4 agonist	Reduction of bacterial burden in the nasopharynx and recruitment of neutrophils in *Haemophilus influenzae*- and *Moraxella catarrhalis*-infected mice	Approved in FENDrix® (GlaxoSmithKline Biologicals, Rixensart, Belgium; HBV vaccine) and Cervarix® (GlaxoSmithKline Biologicals; HPV vaccine)	[145,146]
CRX-524, CRX-527, RC-529	Synthetic TRIF-biased TLR-4 agonist	Increased survival of *Listeria monocytogenes*-infected mice Reduction of bacterial burden in lung and increased IFN-γ and IL-12p70 production in *Yersinia pestis*-infected mice Enhanced survival in combination with gentamicin Increased cytokine production *in vitro* and *in vivo*, and increased survival and reduction of bacterial burden in lung, liver and spleen in *F. tularensis*-infected mice	RC-529 approved in Supervax® (Dynavax, CA, USA; HBV vaccine)	[70,71,78]
Flagellin	Bacterial protein, ligand for TLR-5 and NAIP5/NLRC4	Increased survival, reduced bacterial burden and enhanced pulmonary cytokine production and neutrophil recruitment in *S. pneumoniae*-infected mice Increased survival and bacterial clearance, decreased dissemination, and enhanced induction of antimicrobial peptides in *Pseudomonas aeruginosa*-infected mice Increased survival and protection of intestinal integrity, and decreased bacterial burden in antibiotic-pretreated mice infected with *Clostridium difficile* Increased survival and delayed onset of *Salmonella typhimurium* infection Reduced colonization and enhanced expression of RegIIIγ in antibiotic-treated VRE-infected mice	Several trials explore flagellin as vaccine adjuvant for influenza NCT01172054 NCT00966238 NCT00730457 NCT0096623 NCT0921973 NCT00921947 NCT00603811 *Campylobacter* NCT00124865 *Y. pestis* NCT0138744	[73,74, 147–151]
mesoDAP containing PGN	NOD1 agonist	Increased killing of *S. pneumoniae* and enhanced neutrophil functions	–	[95]
Nigericin	NLRP3 agonist	Augmented IL-1β production and decreased morbidity and mortality in elderly mice infected with influenza	–	[136]
CNF1	*E. coli*-derived effector, modifies host Rho GTPases	Increased survival and transcription of antimicrobial peptides in CNF1-expressing *Drosophila* infected with highly virulent *P. aeruginosa*	–	[152]
c-di-GMP	Bacterial second messenger, STING/DDX41 ligand, induces type-I IFN	Increased leukocyte recruitment and reduced bacterial burden in a *Staphylococcus aureus* mouse mastitis model Maturation of murine and human DCs *in vitro* Increased survival and reduced bacterial burden in lung and blood in lethal *S. pneumoniae* infection in mice Increased survival and enhanced chemokine/cytokine production, and reduced bacterial burden in lung and blood in *Klebsiella pneumoniae*-infected mice Increased pulmonary neutrophil recruitment and reduced bacterial burden in lung and spleen in *Acinetobacter baumannii*-infected mice	–	[49, 113–116, 153]

†*For more information on the clinical trials, please see ClinicalTrials.gov* [204].
AHL: N-*acyl* L-*homoserine lactone; COPD: Chronic obstructive pulmonary disease; DC: Dendritic cell; Poly(I:C): Polyinosinic:polycytidylic acid; mesoDAP: Meso-diaminopimelic acid; MPLA: Monophosphoryl lipid A; NOD: Nucelotide oligomerization domain; PGN: Peptidoglycan; PRR: Pattern recognition receptor; QSM: Quorum-sensing molecule; TLR: Toll-like receptor; VRE: Vancomycin-resistant* Enterococcus.

Table 1. Overview of tested immunostimulants (cont.).

Adjuvant	Function	Experimental and preclinical data	Clinical trials†	Ref.
AHLs	Bacterial QSMs	Increased survival, enhanced neutrophil recruitment and reduced bacterial burden in spleen, lung and blood in a lethal *Aeromonas hydrophila* infection in mice Enhanced phagocytosis by murine macrophages	–	[126]
Whole bacteria and bacterial lysates	Agonists of multiple PRRs	Lysed, aerosolized *H. influenzae* increases survival in *S. pneumoniae-, P. aeruginosa-, K. pneumoniae-, S. aureus-, Bacillus anthracis-, Y. pestis- or F. tularensis*-infected mice Local protection and production of AMPs in *S. pneumoniae*-infected mice Reduced frequency of recurrent respiratory tract infections in children Improved symptoms in chronic bronchitis and COPD, unclear if Luivac can prevent exacerbations	Multiple trials CADI-05 Luivac	[76,77,93,94]

†*For more information on the clinical trials, please see ClinicalTrials.gov* [204].
AHL: N-*acyl* L-*homoserine lactone; COPD: Chronic obstructive pulmonary disease; DC: Dendritic cell; Poly(I:C): Polyinosinic:polycytidylic acid; mesoDAP: Meso-diaminopimelic acid; MPLA: Monophosphoryl lipid A; NOD: Nucelotide oligomerization domain; PGN: Peptidoglycan; PRR: Pattern recognition receptor; QSM: Quorum-sensing molecule; TLR: Toll-like receptor; VRE: Vancomycin-resistant* Enterococcus.

the major signaling adaptors of TLRs, namely MyD88 and TRIF. Their important function in humans was demonstrated in individuals with genetic deficiency of central TLR signaling molecules. These patients suffer from severe invasive bacterial and viral infections [86,87]. However, no selective activators of these molecules are currently available for clinical use, and most data regarding the protective potential of MyD88 and TRIF during bacterial infections are derived from mouse studies [45,46,88].

To date, TLR-targeting strategies for the treatment of bacterial infections are still at an experimental stage.

Nucleotide oligomerization domains

NLRs constitute a large group of cytosolic proteins generally characterized by a three-domain structure, consisting of a leucine rich repeat domain, a NOD and a variable effector domain [89]. Their cytosolic location makes NLRs ideal sensors for invasive pathogens or translocated microbial products. They can be functionally subdivided into three main groups:

- NOD proteins that activate inflammatory signaling through RIP2 and downstream NF-κB and MAPK signaling (e.g., NOD1 and NOD2) [90];
- Inflammasome-forming NLRs (e.g., NLRP1, NLRP3 and NLRC4/NAIP5) [33];
- NLRs that regulate other cellular responses such as type-I IFN production or MHC molecule expression (e.g., NLRC5, CIITA and NLRP12) [91], in addition to a number of NLRs with hitherto unknown functions.

The potential use of NLR ligands to treat acute infections has been predominantly evaluated for NOD1 and NOD2 ligands in several murine infection models. The recognition of peptidoglycan from *H. influenzae* through NOD1 enhanced opsonophagocytic killing of *S. pneumoniae* by neutrophils, an effect that could be mimicked by a synthetic muropeptide (FK-156) [92]. Besides its possible therapeutic impact, this study also elegantly demonstrates how microbes exploit the host's immune system to compete with one another. These results were later also confirmed with aerosolized *H. influenzae* lysates [93,94].

Recently, it was shown than intestinal microbiota-derived NOD1 ligands translocate into the circulation and enhance protective neutrophil functions in the periphery [95]. This effect is lost upon antibiotic treatment, which depletes the resident microflora, but it can be restored by exogenous administration of M-TriDAP, a NOD1 ligand derived from Gram-negative bacteria. Thus, artificial NOD1 stimulation may represent a promising strategy to enhance innate immunity against bacterial pathogens, especially during broad-spectrum antibiotic treatment that depletes or severely alters the endogenous microflora.

NOD2 agonists also augment the phagocytic activity of peripheral blood leukocytes and peritoneal, liver and lung macrophages [96]. Treatment of human alveolar macrophages with the NOD2 ligand muramyl dipeptide induces autophagy and expression of antimicrobial peptide LL37, leading to improved mycobacterial killing [97], whereas mice lacking NOD2 are more susceptible to *M. tuberculosis* infections [98].

These studies collectively indicate the potential value of NOD stimulation as an addition to tuberculostatic therapies.

Other PRRs

Synthetic dsRNA mimetic poly(I:C) is probably one of the best studied immunostimulants [99]. It activates several PRRs and had been known as a potent inducer of type-I IFNs for decades before PRRs were discovered [99]. Poly(I:C) and derivates thereof have shown promising effects as vaccine adjuvants in various mouse models [100]. However, its therapeutic potential during acute infections needs further investigation, although the poly(I:C) derivate poly(ICLC) protected mice against lethal inhalational anthrax [101] and prolonged survival after intranasal *F. tularensis* challenge [102]. The latter study is also a good example of the potential combination of immunostimulatory and classical antimicrobial therapies, as it demonstrated that poly(I:C) treatment provided a time window to initiate antibiotic therapy. The dual function of poly(I:C) to induce type-I IFNs as well as proinflammatory mediators, and the fact that both pathways are protective against most Gram-negative bacteria such as *Legionella pneumophila* [103] and *P. aeruginosa* [104] make poly(I:C) derivates good candidates for the treatment of Gram-negative infections. However, poly(I:C) application can also have harmful effects, since it was shown to cause exacerbation of *M. tuberculosis* infection [105] and increased susceptibility to pulmonary secondary bacterial infections, effects that are not observed with the TLR-7 agonist imiquimod [106].

C-type lectin receptors (CLRs) represent a relatively young group of PRRs **(Box 1)** [107] and their therapeutic potential has not been explored in great detail. Interestingly, it was recently discovered that the adjuvant activity of mycobacterial cord factor and its analog trehalose-6,6-dibehenate is mediated by the CLR MINCLE [108–110]. It is also well established that CLRs play a critical role in host defense against fungal infections, alone or in concert with TLRs [111]. The ongoing identification of CLR functions and ligands could provide new targets, and may hold therapeutic potential for difficult-to-treat mycobacterial or fungal infections.

Generally, bacterial pathogens contain multiple PAMPs and activate several signaling pathways emanating from distinct cellular compartments leading to unique innate immune responses. Hence, instead of targeting individual PRRs, a combined stimulation of different receptor modules might be the favorable approach to adjuvant immunotherapies of bacterial infections.

Simulating a highly infectious threat

The immune system carefully scales the infectious threat of microbial encounters in order to mount appropriate immune responses [37]. Detection of molecular signatures of microbial viability, microbial virulence or tissue damage alerts the immune system to an elevated level of infectious threat and elicits robust responses [37,39]. Thus, manipulation of the cellular detection machineries involved in the recognition of microbial threats might be a good strategy to generate specific, host protective immunity. We will therefore discuss ligands and receptors that might be used to mimic highly infectious threats.

Recently, we discovered an inherent capacity of the immune system to distinguish between live and dead bacteria through the recognition of a specialized class of PAMPs selectively associated with viable microbes called vita-PAMPs [40]. We found that bacterial mRNA represents such a vita-PAMP because it is only found in significant amounts in viable bacteria and it elicits typical innate and adaptive immune responses when detected in conjunction with phagocytosed bacteria. One signature response to live bacteria and vita-PAMPs is the activation of the NLRP3 inflammasome and subsequent release of IL-1β. Importantly, this pathway plays a critical role in host defense against bacterial infections such as pneumococcal pneumonia [55]. Targeted NLRP3 activation could therefore prove beneficial during bacterial infections of immunocompromised patients. Several conditions, such as chronic obstructive pulmonary disease, smoking, mechanical ventilation or viral infections can predispose patients to bacterial respiratory infections, probably due to a combination of structural mucosal alterations and local immunosuppression. Prophylactic delivery of vita-PAMPs (e.g., in the form of aerosolized particles) to stimulate local immunity against viable microbes might protect at-risk patients from acquiring bacterial pneumonia, for example, during mechanical ventilation. Direct experimental and clinical evidence for such an approach is currently missing; however, indirect evidence stems from studies in gene-knockout mice lacking key components of the signaling cascade triggered by live bacteria. Mice deficient

in TRIF or NLRP3 are highly susceptible to pneumonia caused by extracellular bacteria [46,55,88]. Activation of TRIF by viable Gram-negative bacteria plays a crucial role for the activation of the NLRP3 inflammasome, for example, via autocrine IFN-β signaling and caspase-11 [40,43,45]. However, TRIF activation alone, for example, through TLR-3 or TLR-4 activation, does not suffice to activate the inflammasome, and pretreatment of mice with poly(I:C) was shown to actually increase their susceptibility to Gram-positive pathogens [106]. Thus, vita-PAMPs are likely to confer their protective effects in concert with other bacterial ligands. To date, bacterial mRNA is the only known vita-PAMP, yet, the cellular receptor for bacterial mRNA is currently unknown [40]. Detailed knowledge about the cellular receptors and signaling pathways could allow the development of ligands capable of stimulating viability-induced immunity. Moreover, combining vita-PAMPs with other immunostimulatory molecules to target additional checkpoints of immunological risk assessment could further improve host protective immune responses [37,39].

In addition to prokaryotic mRNA, other vita-PAMPs or related molecules and activities very likely exist [37,39]. The bacterial second messenger molecules c-di-GMP and c-di-AMP are potent stimulators of type-I IFNs, and might represent a novel group of vita-PAMPs [112]. Treatment of murine and human dendritic cells with c-di-GMP promotes maturation and cytokine production, and enhances their ability to stimulate T cells, independently of TLR or NLR signaling [113]. In a mouse model, local or systemic c-di-GMP treatment protected against subsequent *K. pneumoniae* infection by enhancing innate immune responses [114]. *Ex vivo*-cultured lung macrophages from c-di-GMP-treated mice showed increased cytokine production and iNOS expression [114]. Beneficial effects of c-di-GMP administration were later confirmed in *S. pneumoniae* [115] and *Acinetobacter baumannii* infection models [116]. However, the molecular mechanisms of c-di-GMP-mediated immune activation remained unclear until recently, when two separate studies identified STING and DDX41 as mammalian sensors of c-di-AMP and c-di-GMP [48,49]. In addition to potent innate immune activation, a superior capacity of c-di-GMP and c-di-AMP to induce specific class-switched antibodies and Th1 type immunity compared with LPS, CpG DNA and alum was recently reported [117,118]. Both c-di-AMP and c-di-GMP have potent adjuvant activity when intranasally administered [117,119], which is interesting given the constitutive PAMP exposure of the nasal mucosa due to microbial colonization. The additional stimulatory effect of intranasal cyclic dinucleotide administration indicates that these second messenger molecules may convey increased infectious threat levels to the mucosal immune system [37,120]. Despite these promising results, cyclic dinucleotides have not been evaluated as immune adjuvants in clinical trials so far.

Until recently, cyclic dinucleotides were considered to be produced exclusively in bacteria. However, in two groundbreaking studies, Wu *et al.* and Sun *et al.* discovered the synthesis of cyclic GMP-AMP (cGAMP) molecules in mammalian cells [121,122]. Production of cGAMP is triggered upon recognition of cytosolic dsDNA by the enzyme cGAMP synthase (cGAS). Subsequently, cGAMP binds to STING and induces IFN-β production. These studies demonstrate for the first time the generation of cyclic dinucleotides in eukaryotic cells and provide an exciting new mechanism for innate immune responses to cytosolic DNA [121]. Importantly, similar responses could be evoked by synthetic cGAMP, indicating the immunostimulatory potential of cGAMP or related molecules, comparable with c-di-GMP or c-di-AMP.

QSMs represent another class of molecules that could serve to inform the immune system about the infectious threat level, since their production closely correlates with bacterial population density and pathogenicity [50]. Bacteria use QS to regulate growth and virulence, for example, by controlling the expression of genes required for biofilm formation and invasiveness. Interestingly, several bacterial QSMs modulate responses in mammalian immune cells however, with different outcomes. While some studies have shown that certain QSMs disrupt NF-κB signaling and induce apoptosis in macrophages [51,123], other groups have observed immunostimulatory effects such as enhanced neutrophil chemoattraction [53,124]. Moreover, the mammalian receptors responsible for the cellular effects of QSMs are still largely elusive, with few exceptions. In an elegant study Lee *et al.* identified the bitter taste receptor T2R38 expressed in the mucosa of the upper respiratory tract as a sensor of *P. aeruginosa*-derived QSM *N*-acyl homoserine

lactone [125]. They revealed a critical impact of T2R38 signaling on mucosal host defense and discovered that *T2R38* gene polymorphisms are associated with an increased susceptibility to upper respiratory tract infections with Gram-negative bacteria [125]. A previous study in mice had shown that administration of exogenous *N*-acyl homoserine lactone confers protection against *Aeromonas hydrophila* infection [126]. At this point it is too early to predict a therapeutic potential of QSMs as immunostimulatory agents, especially since the mechanisms of interaction between QSMs and the mammalian immune system still remain largely unclear. However, in contrast to the little-known cross-kingdom interactions, the role of QSMs in the regulation of bacterial virulence and infection is well documented [127]. Thus, intercepting with QS to suppress bacterial virulence and growth, a concept that has been termed quorum quenching, is viewed as a novel antimicrobial strategy [128]. Several approaches to quorum quenching, also called signal interference, have been experimentally tested. Small-molecule inhibitors of QS systems have been successfully used to inhibit *P. aeruginosa* and *A. baumannii* biofilm production [129–131]. Moreover, pretreatment with QS inhibitors renders Gram-positive and -negative bacterial biofilms more susceptible to available antibiotics in clinically relevant concentrations, and enhances bacterial clearance in mice [132,133]. Biofilms constitute a notorious obstacle for successful antibiotic therapy, thus suppressing biofilm formation would represent a major therapeutic advance and powerful adjuvant strategy. As an alternative to competitive QSM inhibition, it has also been proposed to interfere with QSM synthesis and induce its degradation [134,135]. Therapeutic manipulation of QS is in its infancy and further studies are required to determine its clinical value, possibly in combination with other immunotherapeutic and antibiotic strategies.

Inflammasome activation represents a response to elevated infectious threat [37,38] and the subsequent production of inflammasome-dependent mediators including IL-1β and IL-18 are required for the protective early innate immune response to possibly most pathogenic bacteria. Moreover, inflammasome-dependent cytokines also critically shape the ensuing adaptive immune response to bacteria. However, infections with less virulent or opportunistic bacteria often do not lead to strong inflammasome activation, especially when they are in a persistent, nonreplicating form of their life cycle. These bacteria can nonetheless pose a great threat for immunocompromised patients. In addition, some pathogenic bacteria have evolved mechanisms to evade recognition by inflammasomes. For example, we have recently shown that certain invasive types of pneumococci express a pneumolysin variant that is no longer recognized by the NLRP3 inflammasome [55]. We therefore propose that artificial inflammasome activation by synthetic agonists might be a suitable approach to strengthen protective immunity. Experimental evidence for this assumption comes from viral infection models. It has been shown that elderly mice expressed reduced levels of NLRP3, which resulted in impaired inflammasome function and enhanced susceptibility to influenza virus infection [136]. Treatment of infected elderly mice with the NLRP3 agonist nigericin augmented IL-1β production and decreased morbidity and mortality. Interestingly, alum that has been in clinical use for decades as a vaccine adjuvant is also a strong inflammasome activator [35]. Although there is some controversy, it is likely that alum's effect on the inflammasome contributes to its adjuvancy [35]. In addition to these protective therapeutic effects during acute viral infections in mice, alum might be a good candidate as an adjuvant therapeutic for bacterial infections. Furthermore, flagellin or other synthetic or natural inflammasome activators might be considered as suitable candidates. Flagellin-induced NLRC4 activation and subsequent IL-18 release was shown to elicit host protective $CD8^+$ T-cell responses in a mouse model of Salmonellosis [137]. Overall, inflammasome agonists have shown protective effects in different infection models in mice and should be further examined as adjuvant therapeutics in severe bacterial infections, especially those in elderly and immunocompromised patients caused by multidrug-resistant bacteria.

Disinhibition as an alternative strategy to immune stimulation

One obvious disadvantage of adjuvant immune stimulation is its inherent risk of inducing unwanted inflammation and collateral tissue damage. Rather than actively stimulating immune responses, it could therefore be a valid alternative to release natural breaks on inflammatory pathways in order to selectively trigger immune activation at the site of infection.

Such strategies are currently being explored for anti-viral or -tumor immunotherapies. For instance, expression of inhibitory receptors by virus-specific T cells predicts HCV persistence and blockade of inhibitory receptors PD-1 or Tim-3 can improve viral clearance [138]. PD-1 blockade also enhances antiviral immunity in SIV-infected nonhuman primates [139]. Moreover, anti-PD-1 antibody treatment shows promising results for refractory melanoma and small-cell lung cancer [140]. In contrast to adaptive immune responses, far less is known about the therapeutic potential of targeting inhibitory molecules in innate immune signaling cascades [141]. For instance, SOCS proteins inhibit cellular signaling through cytokine and PRRs, and it has been shown that certain bacteria and viruses exploit SOCS proteins for immune evasion [142,143]. Thus, in similarity to anti-tumor and -viral immunotherapies, disinhibiting inflammatory signal cascades through the blockade of suppressor molecules could help to selectively activate immune responses in infected tissues.

Conclusion & future perspective

Immunotherapies hold great promise for future treatments of autoimmunity, cancer and infectious diseases. Classical vaccination is the oldest and probably most successful immunotherapy; it is also the most efficient public health measure against infectious diseases. Rapidly emerging antibiotic resistance, especially of Gram-negative bacteria, represents an enormous medical and socioeconomic challenge of the near future. The gradual loss of effective antibiotics is a serious threat to the very existence of modern medicine as we know it today [203]. Thus, alternative strategies are sorely needed. We should therefore start to utilize our expanding knowledge of the molecular mechanisms of innate immune responses to design novel antimicrobial immunotherapies. The obvious risks of immunotherapies, including autoimmunity, autoinflammation and consecutive organ damage, have to be taken very seriously and remain the major obstacles on the road to clinical applications. Thus, despite the urgency of the situation, it is absolutely essential to identify specific ligands and responding signaling pathways that can mediate host protection while sparing healthy tissues. In order to maintain immunological homeostasis, antimicrobial immune responses are tightly scaled to the level of infectious threat [37]. Identification of molecular switches to manipulate the immunological risk assessment machinery may be the key to successful adjuvant immunotherapies of infectious diseases.

Financial & competing interests disclosure

ET Helbig is supported by the Jürgen Manchot foundation, B Opitz is supported by the German Research Council (SFB-TR84/A1 and OP86/7-2) and LE Sander is supported by the German Research foundation (DFG SA-1940/2-1, SFB TR84/EN), the Else Kröner-Fresenius Foundation and the European Society for Clinical Microbiology and Infectious Diseases. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Infectious diseases represent a major medical challenge and socioeconomic burden worldwide.
- Newly emerging infectious diseases and sharply increasing multidrug resistance rates, especially among Gram-negative bacteria, call for immediate action and the development of novel therapies.
- Adjuvant immunotherapies should be considered as an addition and alternative to classical antibiotics.
- Targeted stimulation of innate immunity promotes pathogen clearance.
- The immune system scales its responses to the level of infectious threat.
- Manipulating immunological threat evaluation could represent a valid strategy to elicit protective immune responses.
- The immune system can detect bacterial viability through the recognition of viability-associated pathogen-associated molecular patterns.
- The immune system discriminates between virulent and less virulent bacteria through the detection of virulence factors by, for example, inflammasomes.
- Viability-associated pathogen-associated molecular patterns, inflammasome activators or other molecules indicative of high infectious threat levels may serve as potent immunostimulants.
- It will be critical to identify ligands and responding pathways that selectively elicit protective immune responses but minimize unwanted inflammation and tissue damage.
- Risk patients may profit from prophylactic immune stimulation to prevent hospital-acquired infections.

References

Papers of special note have been highlighted as:
▪ of interest
▪▪ of considerable interest

1 Petersdorf RG. The doctors' dilemma. *N. Engl. J. Med.* 299(12), 628–634 (1978).

2 Petersdorf RG. Whither infectious diseases? Memories, manpower, and money. *J. Infect. Dis.* 153(2), 189–195 (1986).

3 Morens DM, Folkers GK, Fauci AS. The challenge of emerging and re-emerging infectious diseases. *Nature* 430(6996), 242–249 (2004).

4 Jones KE, Patel NG, Levy MA *et al.* Global trends in emerging infectious diseases. *Nature* 451(7181), 990–993 (2008).

▪▪ **Impressive documentation of emerging infectious diseases between 1940 and 2005, most of which are bacterial zoonoses and show a specific geographical distribution pattern.**

5 Finland M. Chemotherapy of pneumonia – with special reference to the present status of sulfadiazine. *N. Engl. J. Med.* 225, 187–195 (1941).

6 Coates AR, Halls G, Hu Y. Novel classes of antibiotics or more of the same? *Br. J. Pharmacol.* 163(1), 184–194 (2011).

7 Jean SS, Hsueh PR. High burden of antimicrobial resistance in Asia. *Int. J. Antimicrob. Agents* 37(4), 291–295 (2011).

8 Dalton T, Cegielski P, Akksilp S *et al.* Prevalence of and risk factors for resistance to second-line drugs in people with multidrug-resistant tuberculosis in eight countries: a prospective cohort study. *Lancet* 380(9851), 1406–1417 (2012).

9 Calfee DP. Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, and other Gram-positives in healthcare. *Curr. Opin. Infect. Dis.* 25(4), 385–394 (2012).

10 Johnson AP. Methicillin-resistant *Staphylococcus aureus*: the European landscape. *J. Antimicrob. Chemother.* 66(Suppl. 4), iv43–iv48 (2011).

11 Livermore DM. Current epidemiology and growing resistance of Gram-negative pathogens. *Korean J. Intern. Med.* 27(2), 128–142 (2012).

12 Peleg AY, Hooper DC. Hospital-acquired infections due to Gram-negative bacteria. *N. Engl. J. Med.* 362(19), 1804–1813 (2010).

13 Arias CA, Murray BE. Antibiotic-resistant bugs in the 21st century – a clinical super-challenge. *N. Engl. J. Med.* 360(5), 439–443 (2009).

14 Lascols C, Hackel M, Marshall SH *et al.* Increasing prevalence and dissemination of NDM-1 metallo-beta-lactamase in India: data from the SMART study (2009). *J. Antimicrob. Chemother.* 66(9), 1992–1997 (2011).

15 Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiol. Rev.* 35(5), 736–755 (2011).

16 Spanu T, De Angelis G, Cipriani M *et al.* *In vivo* emergence of tigecycline resistance in multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli*. *Antimicrob. Agents Chemother.* 56(8), 4516–4518 (2012).

17 Yahav D, Lador A, Paul M, Leibovici L. Efficacy and safety of tigecycline: a systematic review and meta-analysis. *J. Antimicrob. Chemother.* 66(9), 1963–1971 (2011).

18 Kontopoulou K, Protonotariou E, Vasilakos K *et al.* Hospital outbreak caused by *Klebsiella pneumoniae* producing KPC-2 beta-lactamase resistant to colistin. *J. Hosp. Infect.* 76(1), 70–73 (2010).

19 Perry JD, Naqvi SH, Mirza IA *et al.* Prevalence of faecal carriage of Enterobacteriaceae with NDM-1 carbapenemase at military hospitals in Pakistan, and evaluation of two chromogenic media. *J. Antimicrob. Chemother.* 66(10), 2288–2294 (2011).

20 Calbo E, Freixas N, Xercavins M *et al.* Foodborne nosocomial outbreak of SHV1 and CTX-M-15-producing *Klebsiella pneumoniae*: epidemiology and control. *Clin. Infect. Dis.* 52(6), 743–749 (2011).

21 Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect. Dis.* 11(5), 355–362 (2011).

22 Dellit TH, Owens RC, McGowan JE Jr *et al.* Infectious diseases Society of America and the Society for Healthcare Epidemiology of America guidelines for developing an institutional program to enhance antimicrobial stewardship. *Clin. Infect. Dis.* 44(2), 159–177 (2007).

23 Heuer H, Schmitt H, Smalla K. Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.* 14(3), 236–243 (2011).

24 Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO, Dantas G. The shared antibiotic resistome of soil bacteria and human pathogens. *Science* 337(6098), 1107–1111 (2012).

▪ **Formal demonstration that soil bacteria are a source of antibiotic resistance genes for human pathogens.**

25 Kak V, Sundareshan V, Modi J, Khardori NM. Immunotherapies in infectious diseases. *Med. Clin. N. Am.* 96(3), 455–474, ix (2012).

26 Jenner E. The three original publications on vaccination against smallpox. *Harvard Classics* 38(4), 1–14 (1909).

27 Detmer A, Glenting J. Live bacterial vaccines – a review and identification of potential hazards. *Microb. Cell Fact.* 5, 23 (2006).

28 Grange JM, Brunet LR, Rieder HL. Immune protection against tuberculosis – when is immunotherapy preferable to vaccination? *Tuberculosis (Edinb.)* 91(2), 179–185 (2011).

29 Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* 54(Pt 1), 1–13 (1989).

30 Medzhitov R, Janeway CA Jr. Decoding the patterns of self and nonself by the innate immune system. *Science* 296(5566), 298–300 (2002).

31 Kawai T, Akira S. TLR signaling. *Semin. Immunol.* 19(1), 24–32 (2007).

32 Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature* 449(7164), 819–826 (2007).

33 Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu. Rev. Immunol.* 27, 229–265 (2009).

34 Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. *Science* 327(5963), 291–295 (2010).

35 Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453(7198), 1122–1126 (2008).

36 Seubert A, Calabro S, Santini L *et al.* Adjuvanticity of the oil-in-water emulsion MF59 is independent of Nlrp3 inflammasome but requires the adaptor protein MyD88. *Proc. Natl Acad. Sci. USA* 108(27), 11169–11174 (2011).

37 Blander JM, Sander LE. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. *Nat. Rev. Immunol.* 12(3), 215–225 (2012).

38 Vance RE, Isberg RR, Portnoy DA. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microb.* 6(1), 10–21 (2009).

39 Sander LE. Improved vaccines through targeted manipulation of the body's immunological risk-assessment? *Bioessays* 34(10), 876–884 (2012).

40 Sander LE, Davis MJ, Boekschoten MV *et al.* Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature* 474(7351), 385–389 (2011).

▪▪ **Discovery of an inherent capacity of the innate immune system to discriminate live from dead bacteria through vita-pathogen-associated molecular patterns, which may explain the high efficacy of live vaccines.**

41 Lauvau G, Vijh S, Kong P *et al.* Priming of memory but not effector CD8 T cells by a killed bacterial vaccine. *Science* 294(5547), 1735–1739 (2001).

42 Kawamura I, Yang J, Takaesu Y, Fujita M, Nomoto K, Mitsuyama M. Antigen provoking gamma interferon production in response to *Mycobacterium bovis* BCG and functional difference in T-cell responses to this antigen between viable and killed BCG-immunized mice. *Infect. Immun.* 62(10), 4396–4403 (1994).

43 Rathinam VA, Vanaja SK, Waggoner L *et al.* TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by Gram-negative bacteria. *Cell* 150(3), 606–619 (2012).

44 Gurung P, Malireddi RK, Anand PK *et al.* Toll or interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-beta (TRIF)-mediated caspase-11 protease production integrates Toll-like receptor 4 (TLR4) protein- and Nlrp3 inflammasome-mediated host defense against enteropathogens. *J. Biol. Chem.* 287(41), 34474–34483 (2012).

45 Sotolongo J, Espana C, Echeverry A *et al.* Host innate recognition of an intestinal bacterial pathogen induces TRIF-dependent protective immunity. *J. Exp. Med.* 208(13), 2705–2716 (2011).

46 Jeyaseelan S, Young SK, Fessler MB *et al.* Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF)-mediated signaling contributes to innate immune responses in the lung during *Escherichia coli* pneumonia. *J. Immunol.* 178(5), 3153–3160 (2007).

47 Woodward JJ, Iavarone AT, Portnoy DA. c-di-AMP secreted by intracellular *Listeria* monocytogenes activates a host type I interferon response. *Science* 328(5986), 1703–1705 (2010).

48 Burdette DL, Monroe KM, Sotelo-Troha K *et al.* STING is a direct innate immune sensor of cyclic di-GMP. *Nature* 478(7370), 515–518 (2011).

49 Parvatiyar K, Zhang Z, Teles RM *et al.* The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. *Nat. Immunol.* 13(12), 1155–1161 (2012).

50 Williams P, Winzer K, Chan WC, Camara M. Look who's talking: communication and quorum sensing in the bacterial world. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 362(1483), 1119–1134 (2007).

51 Kravchenko VV, Kaufmann GF, Mathison JC *et al.* Modulation of gene expression via disruption of NF–kappaB signaling by a bacterial small molecule. *Science* 321(5886), 259–263 (2008).

▪ **Important demonstration of interkingdom communication between bacteria and mammalian immune cells through quorum-sensing molecules.**

52 Lu Q, Lin Y, Yang X *et al.* Regulation on expression of Toll-like receptors on monocytes after stimulation with the 3-*o*-C12-HSL molecule from *Pseudomonas aeruginosa*. *Curr. Microbiol.* 65(4), 384–389 (2012).

53 Wagner C, Zimmermann S, Brenner-Weiss G *et al.* The quorum-sensing molecule *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL) enhances the host defence by activating human polymorphonuclear neutrophils (PMN). *Anal. Bioanal. Chem.* 387(2), 481–487 (2007).

54 Meixenberger K, Pache F, Eitel J *et al. Listeria monocytogenes*-infected human peripheral blood mononuclear cells produce IL-1beta, depending on listeriolysin O and NLRP3. *J. Immunol.* 184(2), 922–930 (2010).

55 Witzenrath M, Pache F, Lorenz D *et al.* The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia. *J. Immunol.* 187(1), 434–440 (2011).

56 Munoz-Planillo R, Franchi L, Miller LS, Nunez G. A critical role for hemolysins and bacterial lipoproteins in *Staphylococcus aureus*-induced activation of the Nlrp3 inflammasome. *J. Immunol.* 183(6), 3942–3948 (2009).

57 Kofoed EM, Vance RE. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477(7366), 592–595 (2011).

58 Boyden ED, Dietrich WF. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat. Genet.* 38(2), 240–244 (2006).

59 Leemans JC, Cassel SL, Sutterwala FS. Sensing damage by the NLRP3 inflammasome. *Immunol. Rev.* 243(1), 152–162 (2011).

60 Krieg AM. CpG still rocks! Update on an accidental drug. *Nucleic Acid Ther.* 22(2), 77–89 (2012).

61 Hennessy EJ, Parker AE, O'Neill LA. Targeting Toll-like receptors: emerging therapeutics? *Nat. Rev. Drug Discov.* 9(4), 293–307 (2010).

62 Zhang X, Kraft A, Broering R, Schlaak JF, Dittmer U, Lu M. Preclinical development of TLR ligands as drugs for the treatment of chronic viral infections. *Expert Opin. Drug Discov.* 7(7), 597–611 (2012).

63 Goutagny N, Estornes Y, Hasan U, Lebecque S, Caux C. Targeting pattern recognition receptors in cancer immunotherapy. *Target Oncol.* 7(1), 29–54 (2012).

64 Hancock RE, Nijnik A, Philpott DJ. Modulating immunity as a therapy for bacterial infections. *Nat. Rev. Microbiol.* 10(4), 243–254 (2012).

65 Geddes K, Magalhaes JG, Girardin SE. Unleashing the therapeutic potential of NOD-like receptors. *Nat. Rev. Drug Discov.* 8(6), 465–479 (2009).

66 Ribes S, Adam N, Ebert S *et al.* The viral TLR3 agonist poly(I:C) stimulates phagocytosis and intracellular killing of *Escherichia coli* by microglial cells. *Neurosci. Lett.* 482(1), 17–20 (2010).

67 Ribes S, Ebert S, Czesnik D *et al.* Toll-like receptor prestimulation increases phagocytosis of *Escherichia coli* DH5alpha and *Escherichia coli* K1 strains by murine microglial cells. *Infect. Immun.* 77(1), 557–564 (2009).

68 Reppe K, Tschernig T, Luhrmann A *et al.* Immunostimulation with macrophage-activating lipopeptide-2 increased survival in murine pneumonia. *Am. J. Respir. Cell Mol. Biol.* 40(4), 474–481 (2009).

69 Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* 316(5831), 1628–1632 (2007).

70 Airhart CL, Rohde HN, Bohach GA *et al.* Induction of innate immunity by lipid A mimetics increases survival from pneumonic plague. *Microbiology* 154(Pt 7), 2131–2138 (2008).

71 Cluff CW, Baldridge JR, Stover AG *et al.* Synthetic Toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. *Infect. Immun.* 73(5), 3044–3052 (2005).

72 Hirano T, Kodama S, Kawano T, Maeda K, Suzuki M. Monophosphoryl lipid A induced innate immune responses via TLR4 to enhance clearance of nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis* from the nasopharynx in mice. *FEMS Immunol. Med. Microbiol.* 63(3), 407–417 (2011).

73 Munoz N, Van Maele L, Marques JM, Rial A, Sirard JC, Chabalgoity JA. Mucosal administration of flagellin protects mice from *Streptococcus pneumoniae* lung infection. *Infect. Immun.* 78(10), 4226–4233 (2010).

74 Yu FS, Cornicelli MD, Kovach MA *et al.* Flagellin stimulates protective lung mucosal immunity: role of cathelicidin-related antimicrobial peptide. *J. Immunol.* 185(2), 1142–1149 (2010).

75 Parkinson T. The future of Toll-like receptor therapeutics. *Curr. Opin. Mol. Ther.* 10(1), 21–31 (2008).

76 Ruah SB, Ruah C, Van Aubel A, Abel S, Elsasser U. Efficacy of a polyvalent bacterial lysate in children with recurrent respiratory tract infections. *Adv. Ther.* 18(4), 151–162 (2001).

77 Steurer-Stey C, Bachmann LM, Steurer J, Tramer MR. Oral purified bacterial extracts in chronic bronchitis and COPD: systematic review. *Chest* 126(5), 1645–1655 (2004).

78 Lembo A, Pelletier M, Iyer R *et al.* Administration of a synthetic TLR4 agonist protects mice from pneumonic tularemia. *J. Immunol.* 180(11), 7574–7581 (2008).

79 Arpaia N, Godec J, Lau L *et al.* TLR signaling is required for *Salmonella typhimurium* virulence. *Cell* 144(5), 675–688 (2011).

80 Hedayat M, Takeda K, Rezaei N. Prophylactic and therapeutic implications of Toll-like receptor ligands. *Med. Res. Rev.* 32(2), 294–325 (2010).

81 Arican O, Guneri F, Bilgic K, Karaoglu A. Topical imiquimod 5% cream in external anogenital warts: a randomized, double-blind, placebo-controlled study. *J. Dermatol.* 31(8), 627–631 (2004).

82 Lascaux AS, Caumes E, Deback C *et al.* Successful treatment of aciclovir and foscarnet resistant Herpes simplex virus lesions with topical imiquimod in patients infected with human immunodeficiency virus type 1. *J. Med. Virol.* 84(2), 194–197 (2012).

83 Pockros PJ, Guyader D, Patton H *et al.* Oral resiquimod in chronic HCV infection: safety and efficacy in 2 placebo-controlled, double-blind Phase IIa studies. *J. Hepatol.* 47(2), 174–182 (2007).

84 Bergmann JF, de Bruijne J, Hotho DM *et al.* Randomised clinical trial: anti-viral activity of ANA773, an oral inducer of endogenous interferons acting via TLR7, in chronic HCV. *Aliment. Pharmacol. Ther.* 34(4), 443–453 (2011).

85 Sousa Mda G, Reid DM, Schweighoffer E *et al.* Restoration of pattern recognition receptor costimulation to treat chromoblastomycosis, a chronic fungal infection of the skin. *Cell Host Microbe* 9(5), 436–443 (2011).

86 Sancho-Shimizu V, Perez De Diego R, Lorenzo L *et al.* Herpes simplex encephalitis in children with autosomal recessive and dominant TRIF deficiency. *J. Clin. Invest.* 121(12), 4889–4902 (2011).

87 von Bernuth H, Picard C, Jin Z *et al.* Pyogenic bacterial infections in humans with MyD88 deficiency. *Science* 321(5889), 691–696 (2008).

88 Cai S, Batra S, Shen L, Wakamatsu N, Jeyaseelan S. Both TRIF- and MyD88-dependent signaling contribute to host defense against pulmonary *Klebsiella* infection. *J. Immunol.* 183(10), 6629–6638 (2009).

89 Ye Z, Ting JP. NLR, the nucleotide-binding domain leucine-rich repeat containing gene family. *Curr. Opin. Immunol.* 20(1), 3–9 (2008).

90 Fritz JH, Ferrero RL, Philpott DJ, Girardin SE. Nod-like proteins in immunity, inflammation and disease. *Nat. Immunol.* 7(12), 1250–1257 (2006).

91 Kufer TA, Sansonetti PJ. NLR functions beyond pathogen recognition. *Nat. Immunol.* 12(2), 121–128 (2011).

92 Lysenko ES, Clarke TB, Shchepetov M *et al.* Nod1 signaling overcomes resistance of *S. pneumoniae* to opsonophagocytic killing. *PLoS Pathog.* 3(8), e118 (2007).

93 Clement CG, Evans SE, Evans CM *et al.* Stimulation of lung innate immunity protects against lethal pneumococcal pneumonia in mice. *Am. J. Respir. Crit. Care Med.* 177(12), 1322–1330 (2008).

94 Evans SE, Scott BL, Clement CG *et al.* Stimulated innate resistance of lung epithelium protects mice broadly against bacteria and fungi. *Am. J. Respir. Cell Mol. Biol.* 42(1), 40–50 (2010).

▪ **Good example of an efficient experimental immunotherapy against pneumonia in mice.**

95 Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16(2), 228–231 (2010).

▪▪ **The authors reveal an unexpected link between commensal microbiota-derived NOD-1 ligands and systemic antimicrobial immunity.**

96 Wardowska A, Dzierzbicka K, Szarynska M *et al.* Analogues of muramyl dipeptide (MDP) and tuftsin limit infection and inflammation in murine model of sepsis. *Vaccine* 27(3), 369–374 (2009).

97 Juarez E, Carranza C, Hernandez-Sanchez F *et al.* NOD2 enhances the innate response of alveolar macrophages to *Mycobacterium tuberculosis* in humans. *Eur. J. Immunol.* 42(4), 880–889 (2012).

98 Divangahi M, Mostowy S, Coulombe F *et al.* NOD2-deficient mice have impaired resistance to *Mycobacterium tuberculosis* infection through defective innate and adaptive immunity. *J. Immunol.* 181(10), 7157–7165 (2008).

99 Field AK, Tytell AA, Lampson GP, Hilleman MR. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proc. Natl Acad. Sci. USA* 58(3), 1004–1010 (1967).

100 Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. *Immunity* 33(4), 492–503 (2010).

101 Walberg K, Baron S, Poast J *et al.* Interferon protects mice against inhalation anthrax. *J. Interferon Cytokine Res.* 28(10), 597–601 (2008).

102 Pyles RB, Jezek GE, Eaves-Pyles TD. Toll-like receptor 3 agonist protection against experimental *Francisella tularensis* respiratory tract infection. *Infect. Immun.* 78(4), 1700–1710 (2010).

103 Lippmann J, Muller HC, Naujoks J *et al.* Dissection of a type I interferon pathway in controlling bacterial intracellular infection in mice. *Cell. Microbiol.* 13(11), 1668–1682 (2011).

104 Carrigan SO, Junkins R, Yang YJ *et al.* IFN regulatory factor 3 contributes to the host response during *Pseudomonas aeruginosa* lung infection in mice. *J. Immunol.* 185(6), 3602–3609 (2010).

105 Antonelli LR, Gigliotti Rothfuchs A, Goncalves R *et al.* Intranasal poly-IC treatment exacerbates tuberculosis in mice through the pulmonary recruitment of a pathogen-permissive monocyte/macrophage population. *J. Clin. Invest.* 120(5), 1674–1682 (2010).

106 Tian X, Xu F, Lung WY *et al.* Poly I:C enhances susceptibility to secondary pulmonary infections by Gram-positive bacteria. *PLoS ONE* 7(9), e41879 (2012).

107 Sancho D, Reis e Sousa C. Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annu. Rev. Immunol.* 30, 491–529 (2012).

108 Schoenen H, Bodendorfer B, Hitchens K *et al.* Cutting edge: mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. *J. Immunol.* 184(6), 2756–2760 (2010).

109 Ishikawa E, Ishikawa T, Morita YS *et al.* Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *J. Exp. Med.* 206(13), 2879–2888 (2009).

110 Lang R, Schoenen H, Desel C. Targeting Syk-Card9-activating C-type lectin receptors by vaccine adjuvants: findings, implications and open questions. *Immunobiology* 216(11), 1184–1191 (2011).

111 Hardison SE, Brown GD. C-type lectin receptors orchestrate antifungal immunity. *Nat. Immunol.* 13(9), 817–822 (2012).

112 Bowie AG. Innate sensing of bacterial cyclic dinucleotides: more than just STING. *Nat. Immunol.* 13(12), 1137–1139 (2012).

113 Karaolis DK, Means TK, Yang D *et al.* Bacterial c-di-GMP is an immunostimulatory molecule. *J. Immunol.* 178(4), 2171–2181 (2007).

▪ **First demonstration of the immunostimulatory potential of bacterial second messenger molecule c-di-GMP.**

114 Karaolis DK, Newstead MW, Zeng X *et al.* Cyclic di-GMP stimulates protective innate immunity in bacterial pneumonia. *Infect. Immun.* 75(10), 4942–4950 (2007).

115 Ogunniyi AD, Paton JC, Kirby AC *et al.* c-di-GMP is an effective immunomodulator and vaccine adjuvant against pneumococcal infection. *Vaccine* 26(36), 4676–4685 (2008).

116 Zhao L, Kuolee R, Harris G, Tram K, Yan H, Chen W. c-di-GMP protects against intranasal *Acinetobacter baumannii* infection in mice by chemokine induction and enhanced neutrophil recruitment. *Int. Immunopharmacol.* 11(9), 1378–1383 (2011).

117 Ebensen T, Libanova R, Schulze K, Yevsa T, Morr M, Guzman CA. Bis-(3′,5′)-cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant. *Vaccine* 29(32), 5210–5220 (2011).

118 Gray PM, Forrest G, Wisniewski T *et al.* Evidence for cyclic diguanylate as a vaccine adjuvant with novel immunostimulatory activities. *Cell Immunol.* 278(1–2), 113–119 (2012).

119 Madhun AS, Haaheim LR, Nostbakken JK *et al.* Intranasal c-di-GMP-adjuvanted plant-derived H5 influenza vaccine induces multifunctional Th1 $CD4^+$ cells and strong mucosal and systemic antibody responses in mice. *Vaccine* 29(31), 4973–4982 (2011).

120 Libanova R, Becker PD, Guzman CA. Cyclic di-nucleotides: new era for small molecules as adjuvants. *Microb. Biotechnol.* 5(2), 168–176 (2012).

121 Wu J, Sun L, Chen X *et al.* Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* 339(6121), 826–830 (2012).

122 Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* 339(6121), 786–791 (2012).

123 Tateda K, Ishii Y, Horikawa M *et al.* The *Pseudomonas aeruginosa* autoinducer N-3-oxododecanoyl homoserine lactone accelerates apoptosis in macrophages and neutrophils. *Infect. Immun.* 71(10), 5785–5793 (2003).

124 Karlsson T, Musse F, Magnusson KE, Vikstrom E. *N*-acylhomoserine lactones are potent neutrophil chemoattractants that act via calcium mobilization and actin remodeling. *J. Leukoc. Biol.* 91(1), 15–26 (2012).

125 Lee RJ, Xiong G, Kofonow JM *et al.* T2R38 taste receptor polymorphisms underlie susceptibility to upper respiratory infection. *J. Clin. Invest.* 122(11), 4145–4159 (2012).

▪ **Elegantly shows that bitter taste receptors of the upper respiratory tract sense *Pseudomonas aeruginosa* quorum-sensing molecules and promote host defense.**

126 Khajanchi BK, Kirtley ML, Brackman SM, Chopra AK. Immunomodulatory and protective roles of quorum-sensing signaling molecules *N*-acyl homoserine lactones during infection of mice with *Aeromonas hydrophila*. *Infect. Immun.* 79(7), 2646–2657 (2011).

127 Miller MB, Bassler BL. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55, 165–199 (2001).

128 Kalia VC, Purohit HJ. Quenching the quorum sensing system: potential antibacterial drug targets. *Crit. Rev. Microbiol.* 37(2), 121–140 (2011).

129 Hentzer M, Givskov M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J. Clin. Invest.* 112(9), 1300–1307 (2003).

130 Hentzer M, Wu H, Andersen JB *et al.* Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J.* 22(15), 3803–3815 (2003).

131 Stacy DM, Welsh MA, Rather PN, Blackwell HE. Attenuation of quorum sensing in the pathogen *Acinetobacter baumannii* using non-native *N*-acyl homoserine lactones. *ACS Chem. Biol.* 7(10), 1719–1728 (2012).

132 Brackman G, Cos P, Maes L, Nelis HJ, Coenye T. Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* 55(6), 2655–2661 (2011).

133 Wu H, Song Z, Hentzer M *et al.* Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice. *J. Antimicrob. Chemother.* 53(6), 1054–1061 (2004).

134 Storz MP, Maurer CK, Zimmer C *et al.* Validation of PqsD as an anti-biofilm target in *Pseudomonas aeruginosa* by development of small-molecule inhibitors. *J. Am. Chem. Soc.* 134(39), 16143–16146 (2012).

135 Chan KG, Atkinson S, Mathee K *et al.* Characterization of *N*-acylhomoserine lactone-degrading bacteria associated with the *Zingiber officinale* (ginger) rhizosphere: co-existence of quorum quenching and quorum sensing in Acinetobacter and Burkholderia. *BMC Microbiol.* 11, 51 (2011).

136 Stout-Delgado HW, Vaughan SE, Shirali AC, Jaramillo RJ, Harrod KS. Impaired NLRP3 inflammasome function in elderly mice during influenza infection is rescued by treatment with nigericin. *J. Immunol.* 188(6), 2815–2824 (2012).

137 Kupz A, Guarda G, Gebhardt T *et al.* NLRC4 inflammasomes in dendritic cells regulate noncognate effector function by memory CD8(+) T cells. *Nat. Immunol.* 13(2), 162–169 (2012).

138 Mcmahan RH, Golden-Mason L, Nishimura MI *et al.* Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed *in vitro* cytotoxicity. *J. Clin. Invest.* 120(12), 4546–4557 (2010).

139 Velu V, Titanji K, Zhu B *et al.* Enhancing SIV-specific immunity *in vivo* by PD-1 blockade. *Nature* 458(7235), 206–210 (2009).

140 Topalian SL, Hodi FS, Brahmer JR *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* 366(26), 2443–2454 (2012).

141 O'Neill LA. When signaling pathways collide: positive and negative regulation of Toll-like receptor signal transduction. *Immunity* 29(1), 12–20 (2008).

142 Baetz A, Zimmermann S, Dalpke AH. Microbial immune evasion employing suppressor of cytokine signaling (SOCS) proteins. *Inflamm. Allergy Drug Targets* 6(3), 160–167 (2007).

143 Pauli EK, Schmolke M, Wolff T *et al.* Influenza A virus inhibits type I IFN signaling via NF–kappaB-dependent induction of SOCS-3 expression. *PLoS Pathog.* 4(11), e1000196 (2008).

144 Butowski N, Chang SM, Junck L *et al.* A Phase II clinical trial of poly-ICLC with radiation for adult patients with newly diagnosed supratentorial glioblastoma: a North American Brain Tumor Consortium (NABTC01–05). *J. Neurooncol.* 91(2), 175–182 (2009).

145 Romero CD, Varma TK, Hobbs JB, Reyes A, Driver B, Sherwood ER. The Toll-like receptor 4 agonist monophosphoryl lipid a augments innate host resistance to systemic bacterial infection. *Infect. Immun.* 79(9), 3576–3587 (2011).

146 Steinhagen F, Kinjo T, Bode C, Klinman DM. TLR-based immune adjuvants. *Vaccine* 29(17), 3341–3355 (2011).

147 Vijay-Kumar M, Aitken JD, Sanders CJ *et al.* Flagellin treatment protects against chemicals, bacteria, viruses, and radiation. *J. Immunol.* 180(12), 8280–8285 (2008).

148 Kinnebrew MA, Ubeda C, Zenewicz LA, Smith N, Flavell RA, Pamer EG. Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant *Enterococcus* infection. *J. Infect. Dis.* 201(4), 534–543 (2010).

149 Jarchum I, Liu M, Lipuma L, Pamer EG. Toll-like receptor 5 stimulation protects mice from acute *Clostridium difficile* colitis. *Infect. Immun.* 79(4), 1498–1503 (2011).

150 Taylor DN, Treanor JJ, Sheldon EA *et al.* Development of VAX128, a recombinant hemagglutinin (HA) influenza-flagellin fusion vaccine with improved safety and immune response. *Vaccine* 30(39), 5761–5769 (2012).

151 Talbot HK, Rock MT, Johnson C *et al.* Immunopotentiation of trivalent influenza vaccine when given with VAX102, a recombinant influenza M2e vaccine fused to the TLR5 ligand flagellin. *PLoS ONE* 5(12), e14442 (2010).

152 Boyer L, Magoc L, Dejardin S *et al.* Pathogen-derived effectors trigger protective immunity via activation of the Rac2 enzyme and the IMD or Rip kinase signaling pathway. *Immunity* 35(4), 536–549 (2011).

153 Brouillette E, Hyodo M, Hayakawa Y, Karaolis DK, Malouin F. 3′,5′-cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection. *Antimicrob. Agents Chemother.* 49(8), 3109–3113 (2005).

Websites

201 WHO: the top ten causes of death. www.who.int/mediacentre/factsheets/fs310/en/index.html

202 European Antimicrobial Resistance Network ECFDPaC. http://ecdc.europa.eu/en/activities/surveillance/EARS-net/Pages/index.aspx

203 Chan M. Antimicrobial resistance in the European Union and the world. Conference on combating antimicrobial resistance: time for action. Copenhagen 2012 key note address (2012). http://www.who.int/dg/speeches/2012/amr_20120314/en/index.html

204 ClinicalTrials.gov. www.clinicaltrials.gov