Modulating immunity as a therapy for bacterial infections

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Abstract | Despite our efforts to halt the increase and spread of antimicrobial resistance, bacteria continue to become less susceptible to antimicrobial drugs over time, and rates of discovery for new antibiotics are declining. Thus, it is essential to explore new paradigms for anti-infective therapy. One promising approach involves host-directed immunomodulatory therapies, whereby natural mechanisms in the host are exploited to enhance therapeutic benefit. The objective is to initiate or enhance protective antimicrobial immunity while limiting inflammation-induced tissue injury. A range of potential immune modulators have been proposed, including innate defence regulator peptides and agonists of innate immune components such as Toll-like receptors and NOD-like receptors.

Adjunctive therapies
Treatments that are used together with a primary treatment to increase its potency. For example, an immunomodulatory treatment can be adjunctive to primary treatment with an antibiotic.

From the moment of birth and throughout our lifetime, we continuously interact with a multitude of microbial species. These include the commensal bacteria that colonize the mucosal surfaces of our bodies and the pathogenic organisms that, in most cases, are effectively targeted by our immune defences and prevented from causing an infection. Thus, the evolution of immunity and of many other aspects of our physiology has been driven by continuous interactions with commensals and the other microbial species in our environment. The dysregulation of such interactions can result in life-threatening infectious and inflammatory disorders.

Immune defence mechanisms can be broadly divided into the innate and adaptive arms, although this division is to a large extent arbitrary, as the two branches are highly interdependent and use similar effector mechanisms, and in most cases an effective immune response requires the coordinated activity of both arms (BOX 1). Pathogenic organisms actively suppress the normal mechanisms of the immune response by expressing specific virulence factors, and this contributes to a large extent to their pathogenicity and disease progression. At the same time, it is becoming increasingly recognized that the altered or inappropriate activity of the immune system also contributes to the pathology of such conditions. For example, the hyperactivation of immune responses contributes to lethality in individuals with sepsis and those infected with an influenza virus[1].

The ability to modulate immune responses, by either suppression or enhancement depending on the need, has proved to be a useful therapeutic strategy in many contexts, including the prevention and treatment of infections, the suppression of autoimmune and inflammatory responses, and the stimulation of antitumour immunity in patients with cancer[2,3]. When the currently available immunomodulatory treatments are used as anti-infectives, they typically work by correcting an acquired or congenital defect in immune system function and often use recombinant forms of the natural immunomodulators produced by the host immune system. They are largely designed as adjunctive therapies to support and extend the effectiveness of antibiotics and antivirals. For example, type I interferons (IFNs) have been used clinically to stimulate immune responses in patients with viral infections[4]. Vaccination can also be considered as a form of immunomodulatory therapy and is one of the most successful and cost-effective forms of medical intervention for the prevention of infectious diseases. It relies on the induction of immune responses to specific antigen combinations, typically delivered in formulation with an appropriate adjuvant that activates innate immunity and enhances adaptive immune responses (BOX 2). Monoclonal antibodies are another type of immunomodulator and have been in clinical use for several decades, with more than 20 antibody-based therapies approved in the United States alone to date and >150 others currently in clinical trials[5]. However, their application against infectious diseases is not common and focuses mainly on inhibiting deleterious inflammatory responses.

Immunomodulation offers certain advantages. By targeting the host rather than the pathogen, immunomodulation largely avoids selective pressure for the evolution of microbial resistance. Indeed, the stimulation of

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Box 1 | **Overview of the immune system**

Innate immunity is the first line of defence against pathogenic organisms and the interface of the interactions between the host and the microbiota[107]. At the cellular level, the innate immune response is mediated by epithelial cells in mucosal surfaces and phagocytic cells that reside in the tissues or are recruited from the blood, including neutrophils, monocytes and macrophages. At the molecular level, innate immune cells sense microorganisms through pattern recognition receptors (PRRs), which recognize molecular signatures (also known as pathogen-associated molecular patterns) from microbial cells[107,108], including proteins, lipids and nucleic acids. PRRs also recognize host signature molecules that are indicative of disease and cellular damage. Pathogen recognition through these receptors results in the activation of cellular defence mechanisms and the production of secreted pro-inflammatory cytokines, which alert other host cells to the presence of infection, drive further recruitment of immune cells from the blood to the site of infection, and induce systemic responses to the disease, such as fever. Pathogen recognition through PRRs also stimulates the microbicidal mechanisms of innate immunity, such as the production of reactive oxygen species and antimicrobial peptides, in part through the activation of phagocytic cells.

The defence mechanisms of adaptive immunity are based on the recognition of 'foreign' molecular shapes termed antigens. Adaptive immunity is activated more slowly (on the time scale of 3 days to a couple of weeks) by a combination of signals from the innate immune system and by antigens, and is largely mediated by B cells and T cells. These cells carry receptors that recognize foreign molecular patterns but have no intrinsic bias towards pathogen recognition. Adaptive immunity is therefore dependent on the innate immune system for initial pathogen recognition. Signals from the innate immune response drive the selective expansion and activation of the B cell and T cell populations with specificity for the ongoing infectious challenge. The main effector mechanisms of adaptive immunity include the production of antibodies by B cells (to act as blocking antibodies, and/or as opsonins for complement- and phagocyte-mediated killing), the killing of infected host cells by cytotoxic T cells, and various helper T cell-mediated actions. Crucially, activation of adaptive immunity results in the production of memory B cells and T cells, which can provide life-long specific protection against subsequent infections with a pathogen bearing the same antigens.

adaptive immunity through vaccination has remained resilient to microbial resistance over decades of clinical use[6]. Furthermore, the nonspecific nature of innate immune defences suggests that their modulation affords broad-spectrum protection against a range of microbial pathogens, enabling prophylactic use in high-risk groups and early treatment before the identification of causative infectious agents, for example.

However, inappropriate activation of innate immunity can result in harmful pro-inflammatory responses and tissue damage, as observed in inflammatory diseases, sepsis and some viral respiratory infections[7]. Therefore, successful application of immunomodulatory therapies requires controlled stimulation of protective immunity without an increase in systemic pro-inflammatory responses. Our continually expanding understanding of the complex mechanisms of innate immunity will aid in the development of such therapies.

This Review critically appraises the potential for the stimulation of protective antibacterial immunity by a range of innate immune modulators, including agonists of the major classes of pattern recognition receptors (PRRs), immunomodulatory host defence peptides (HDPs; also called antimicrobial peptides) and other natural bacterial ligands. The use of analogous molecules, including antimicrobial peptides, as direct microbicidal agents has been covered in depth elsewhere[8,9] and is not discussed here.

Targeting innate immune receptors

One immunomodulatory approach currently being investigated is the targeting of innate immune PRRs, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Agonists of the intracellular nucleic acid sensors RIG-I (also known as DDX58) and AIM2 also have potential as therapeutic adjuvants, but they have not been formally tested (and are therefore not discussed below).

Targeting Toll-like receptors to prevent and treat infections. The TLR family of PRRs in humans includes ten transmembrane proteins[10]. Individual members of the TLR family are specialized for the recognition of different classes of ligand and are located either on the cell surface or in the endosomal compartment. The cell surface TLRs typically sense signature components of microbial cell envelopes or flagella; for example, TLR4 and accessory proteins recognize lipopolysaccharide (LPS)[11,12], TLR2 in complex with either TLR1 or TLR6 recognizes lipoteichoic acid and various lipopeptides, and TLR5 recognizes flagellin. Endosomal TLRs primarily sense microbial nucleic acids; for example, TLR3 recognizes double-stranded RNA, TLR7 and TLR8 bind single-stranded RNA, and TLR9 recognizes microbial DNA with unmethylated CpG

Box 2 | **Adjuvants**

Adjuvants are agents that help to enhance and appropriately orient immune responses[109]. In this Review we use this term for both therapeutic adjuvants and vaccine adjuvants. Therapeutic adjuvants, also termed immunomodulators, usually enhance innate immune mechanisms that lead to the resolution of infections, and may be used alone or more often as an adjunctive therapy together with antibiotics. For example, Toll-like receptor (TLR) and NOD-like receptor (NLR) agonists stimulate innate immunity through the same basic mechanisms (that is, the same receptors, pathways and effector mechanisms) as microorganisms. One of the main challenges in the application of therapeutic adjuvants is to avoid the exacerbation of potentially harmful inflammation, which is usually triggered together with protective immunity. The fact that innate defence regulator (IDR) peptides offer protection against bacterial infections while suppressing excessive inflammation (in animal models) indicates that it is possible to separate protective innate immune mechanisms from inflammation, and encourages the belief that safe and effective immunomodulators can be developed.

By contrast, vaccine adjuvants are administered together with a vaccine and work to enhance and appropriately skew antigen-specific adaptive immune responses. There seem to be three basic mechanisms associated with different vaccine adjuvants[110]: recruitment of innate immune cells (such as antigen-presenting dendritic cells), appropriate presentation of the antigen to such cells (the 'depot effect') and activation of such cells. These mechanisms are broadly based on the stimulation of innate immunity, which in turn activates the adaptive immune response to clear the pathogen.

Pattern recognition receptors
(PRRs). Host receptors, such as Toll-like receptors (TLRs) or NOD-like receptors (NLRs), that can sense pathogen signatures (pathogen-associated molecular patterns) and endogenous damage-associated molecular patterns and then initiate signalling cascades that lead to an innate immune response. These proteins can be membrane-bound receptors (such as TLRs) or cytoplasmic receptors (such as NLRs).

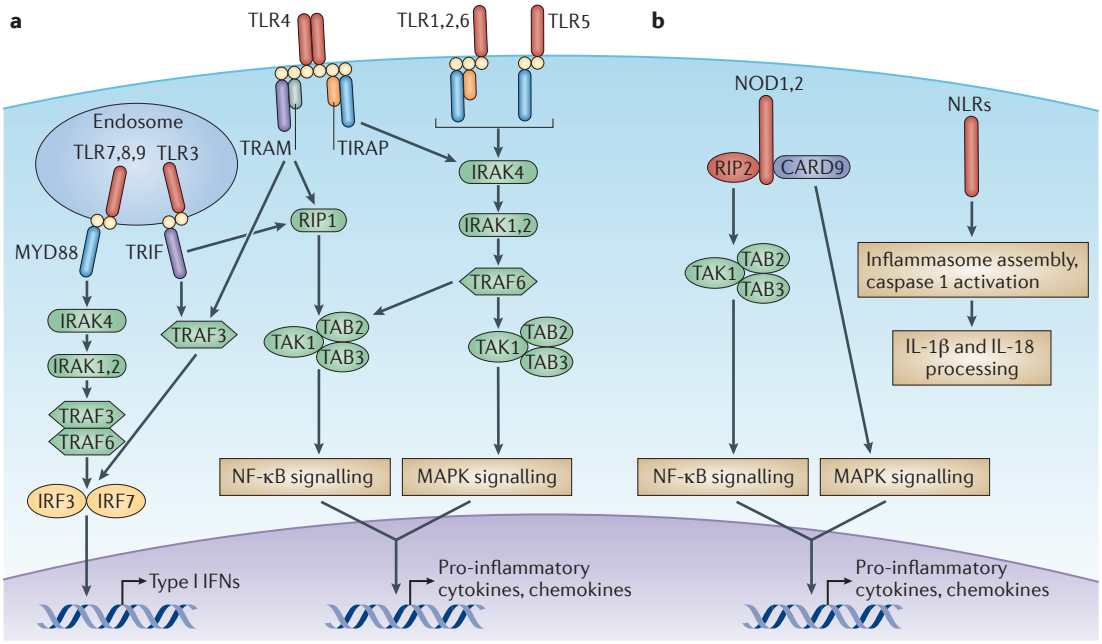

Figure 1 | **Pattern recognition receptors of the innate immune system and their signalling pathways. a** | Toll-like receptors (TLRs), which function as dimers (shown for TLR4 only), sense microbial molecular patterns (such as lipopolysaccharide and double-stranded RNA) on the cell surface and in the endosomal compartment and activate signalling pathways through TIR domain-containing adaptor proteins (myeloid differentiation 88 (MYD88), TIR domain-containing adaptor protein (TIRAP), TIR domain adaptor molecule (TRAM) and TIR domain-containing adaptor protein inducing IFNβ (TRIF)) and other downstream signalling mediators. Note that TLR4–TRIF signalling is initiated in endosomes after internalization of the receptor (not shown). TLR signalling ultimately leads to the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling (pathways not shown in detail), as well as IFN-regulatory factors (IRFs), resulting in the induction of pro-inflammatory cytokines, chemokines and type I interferons (IFNs) and, consequently, the activation of cellular antimicrobial functions (for example, phagocytosis, oxidative burst and production of antimicrobial peptides). **b** | NOD (nucleotide-binding oligomerization domain-containing) receptors sense components of peptidoglycan in the cytosol and activate receptor-interacting protein 2 (RIP2) and downstream signalling pathways, including the MAPK and NF-κB signalling pathways. This leads to the induction of pro-inflammatory cytokines and chemokines, and the activation of antimicrobial functions. NOD-like receptors (NLRs) are activated by diverse microbial signature molecules and other danger signals in the cytosol. After activation, they recruit the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), leading to inflammasome assembly, caspase 1 activation, and the processing and secretion of the cytokines interleukin-1β (IL-1β) and IL-18. CARD9, caspase recruitment domain-containing 9; IRAK, IL-1 receptor-associated kinase; TAK1, TGF-β-activated kinase 1; TRAF, TNF receptor-associated factor.

motifs. In addition to these microbial ligands, TLRs recognize endogenous ligands, often referred to as damage-associated molecular patterns (DAMPs) or alarmins, including high-mobility group protein B1 (HMGB1), S100A8–S100A9, heat shock proteins, uric acid, heparin, DNA and purine metabolites[10]. These 'molecular patterns' are, however, perhaps misnamed, as they represent very different molecules from microbial signatures and probably act through different mechanisms or binding sites. The interaction of microbial signature molecules with TLRs triggers the activation of signal transduction pathways, such as the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways, resulting in the activation of innate immune cells. This, in turn, drives the production of pro-inflammatory cytokines and promotes the induction of antimicrobial effector functions (FIG. 1a). The importance of TLR signalling in antimicrobial defence is illustrated by the fact that polymorphisms in the TLR signalling network have been associated with impaired control of bacterial infections in humans[13].

There are two possible methods of modulating TLR-driven responses, and both cases use molecules that often mimic natural ligands. Agonists have an adjuvant effect on innate immune pathways, promoting protective responses but potentially enhancing inflammation. By contrast, antagonists can suppress immune pathways and potentially harmful inflammation that is either linked to infection or the result of altered immune interactions with the bacterial microbiota (termed dysbiosis). However, antagonists might also repress protective mechanisms.

TLRs are already targeted by several approved immunomodulatory therapies and are being investigated as drug targets for many diseases through ongoing clinical trials and research programmes (TABLE 1; for a recent review on the use of TLR agonists and antagonists, see REF. 14). For example, CADI-05, which acts as an agonist for many TLRs (and probably for other PRRs) and consists of heat-killed *Mycobacterium indicus pranii*, is used as an adjunctive therapy in combination with

Table 1 | **Immunomodulatory therapies that target pattern recognition receptors to treat microbial infections**

Drug	Target and activity	Application	Phase of development	Companies
Imiquimod[121]	TLR7 agonist	Keratosis, basal cell carcinoma, HPV-associated genital warts	Clinical	Graceway Pharmaceuticals, iNova Pharmaceuticals, Mochida Pharmaceutical
MPL[122,123]	TLR4 agonist	Adjuvant in the Cervarix (GlaxoSmithKline) vaccine against HPV infections and associated cervical cancer	Clinical	GlaxoSmithKline
Luivac (lysate of a combination of bacterial species)[18,19]	Agonist for various TLRs	Recurring respiratory tract infections	Clinical	Daiichi Sankyo
CADI-05 (heat-killed *Mycobacterium indicus pranii*)[15]	Agonist for various TLRs	Leprosy (in combination with antimicrobial drugs) and tuberculosis	Clinical and Phase III	Cadila Pharmaceuticals
CpG-7909 (REF. 16)	TLR9 agonist	Improving the efficacy of the anthrax vaccine BioThrax (Coley Pharmaceutical)	Phase II	Coley Pharmaceutical
DIMS-0150 (an oligonucleotide-based drug)[17]	TLR9 agonist	Steroid-resistant ulcerative colitis	Phase III	InDex Pharmaceuticals
E-5564 (eritoran)	TLR4 antagonist	Sepsis	Phase III	Eisai Pharmaceuticals
TAK-242 (resatorvid)	TLR4 antagonist	Sepsis	Suspended in Phase III	Takeda Pharmaceutical
MF-59 (squalene and water emulsion) and MTP–PE (mifamurtide)	NOD2 agonist	HIV, influenza viruses	Phase I	Chiron/Novartis

HPV, human papillomavirus; MPL, monophosphoryl lipid A; MTP–PE, phosphatidylethanolamine-linked muramyl tripeptide; NOD2, nucleotide-binding oligomerization domain-containing 2; TLR, Toll-like receptor.

antimicrobial drugs for the treatment of leprosy[15] and is in Phase III clinical trials as a potential therapy for tuberculosis. The synthetic TLR9 agonist CpG-7909 successfully completed Phase II clinical trials for improving the efficacy of the anthrax vaccine BioThrax (Coley Pharmaceutical)[16], and the DNA-based immunomodulator DIMS-0150 (from InDex Pharmaceuticals), which also targets TLR9, is entering Phase III trials for the treatment of steroid-resistant ulcerative colitis[17]. Crude bacterial lysate preparations have also shown some efficacy against bacterial infections[18]; for example, Luivac (Daiichi Sankyo) reduces the incidence of recurring respiratory tract infections in children[19] and probably acts in part through TLR stimulation.

These clinical successes and ongoing trials are based on a large body of evidence from animal models showing that TLR agonists can act as potent and safe vaccine adjuvants, can induce the clearance of existing bacterial infections and can promote protection against future infections or colonization by pathogenic organisms[20]. This nonspecific stimulation of innate immunity as a mechanism of broad-spectrum protection against bacterial infections is particularly interesting and has been described as stimulated innate resistance. It is illustrated by recent studies in mouse models showing that LPS (a TLR4 agonist) administered at the time of infection with *Bordetella pertussis* offers partial protection against the infectious challenge[21] and that treatment with flagellin (a TLR5 agonist) is similarly protective against *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* infections when administered either concurrently with or following bacterial challenge[22,23]. Flagellin also reduces gut colonization with vancomycin-resistant *Enterococcus faecium* in animals treated with broad-spectrum antibiotics[24]. Crude microbial preparations have been found to have similar protective effects, possibly through stimulation of multiple PRRs, including TLRs. For example, protective activity against a range of bacterial pathogens is seen in mice pretreated with whole-cell lysates of *Haemophilus influenzae*[21,25] or a membrane protein fraction from an attenuated strain of *Francisella tularensis* formulated with cationic liposomes and DNA[26]. Pre-stimulation with crude *H. influenzae* lysates or with specific TLR2 and TLR4 ligands is also protective against fungal and viral pathogens, highlighting the broad protective activity of the response[21,27,28]. The use of probiotic bacteria, which may act as TLR agonists because they contain signature molecules that are recognized by TLRs, is another effective immunomodulatory strategy to prevent infections (BOX 3).

However, owing to the intrinsic complexity of host–pathogen interactions, the outcomes of innate immune stimulation may be difficult to predict. For example, disease pathology is actually exacerbated in mice that are chronically infected with *Mycobacterium tuberculosis* and then treated with a synthetic version of the TLR3 ligand polyinosinic–polycytidylic acid (polyI:C)[29], indicating that modulation of innate immune responses should be undertaken with caution and should use appropriate systems level analyses.

As TLR stimulation is a strong inducer of inflammation, it has been widely implicated in the pathologies of inflammatory disorders and infectious diseases associated with inappropriate hyperactivation of immune responses. Thus, there is an ongoing effort to develop

Box 3 | **Probiotics and therapies targeting the microbiota**

The role of the microbiota in shaping our immune system, as well as other aspects of our physiology, is becoming increasingly recognized[111], emphasizing the potential effects of medical interventions, such as antibiotics, on our homeostatic interactions with the microbiota. Antibiotic regimens can severely affect the composition of mucosal microbial communities, leading to increased risks of *Clostridium difficile*-associated diarrhoea and vaginal *Candida albicans* infections, and the exacerbation of asthma and allergic diseases[112]. Although changes in immune function as the result of immunomodulatory therapies may similarly disrupt the homeostasis of mucosal microbiota, it is also likely that appropriately tailored immunomodulators can be designed to maintain, enhance and restore mucosal homeostasis.

Probiotics typically consist of microorganisms that naturally colonize our mucosal surfaces, including *Lactobacillus* spp. and *Bifidobacterium* spp., *Streptococcus salivarius* and *Escherichia coli* str. Nissle 1917. The ease of production and administration for probiotics, and their lack of toxic side effects even in long-term treatment, make them an attractive therapeutic option. Probiotics act through several complementary mechanisms to elicit their therapeutic effects. These include modulation of local and systemic immune responses by interacting with mucosal epithelial cells and resident innate and adaptive immune system cells, and protection against colonization through direct competition with, or possibly bacteriocin-mediated action against, bacterial pathogens[111,113]. These complex mechanisms of action probably resemble the natural host interactions with the microbiota and may include induction and/or suppression of cytokines, chemokines and antimicrobial peptides[114,115], recruitment or activation of cell populations in the gut mucosa, stimulation of mucosal immunoglobulin A responses, and enhancement of epithelial repair and barrier functions[36,111,113]. Importantly, such activities are likely to include both pro- and anti-inflammatory effects, thus resulting in enhanced immune defences and promoting the maintenance of mucosal homeostasis. This combination of pro- and anti-inflammatory activity is illustrated by the fact that chemokine production and nuclear factor-κB signalling are inhibited by *S. salivarius* str. K12 (REF. 116), but Toll-like receptor signalling and the secretion of innate immune mediators are activated in response to *E. coli* str. Nissle 1917 in epithelial cells[117].

The administration of probiotics has shown promise in a range of clinical trials in patients with infectious and inflammatory diseases[17,118,119]. However, optimal treatment protocols and the identification of the patient groups that would benefit the most need to be established before probiotics can gain wider clinical use. There is also ongoing research into the applications of transgenic lactic acid bacteria as vehicles for the delivery of immunomodulatory compounds to mucosal surfaces (reviewed in REF. 120). This approach has produced highly promising results in animal models as a means for the delivery of vaccine antigens, as well as antibodies, cytokines and other immunomodulators for the treatment of inflammatory, allergic and infectious diseases[120].

TLR antagonists for the treatment of such conditions[14]. In particular, sepsis is a highly lethal complication associated with severe infection and tissue damage (triggered by uncontrolled systemic production of pro-inflammatory mediators) followed by endotoxin tolerance and multiple organ failure. However, blockade of TLR4 (or pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1β (IL-1β)) with monoclonal antibody therapies has failed to improve disease outcomes in patients with sepsis[30]. The reasons for failure are likely to be complex and include patient heterogeneity, the timing of treatment (as patients with late-stage sepsis actually suffer from suppressed inflammation), the multiple PRRs that are probably involved in sepsis, and the importance of maintaining effective immune defences against infections. Furthermore, recent clinical trials testing TLR antagonists for the treatment of sepsis have had disappointing results: the TLR4 signalling inhibitor TAK-242 (also known as resatorvid; from Takeda Pharmaceutical)[31], which was in a Phase III trial, was discontinued, and in a Phase II trial the TLR4 antagonist E-5564 (also known as eritoran; from Eisai Pharmaceuticals) had no effect in reducing mortality in patients with severe sepsis, despite previously encouraging results in animal models and endotoxin challenges in healthy volunteers[32,33].

In spite of these discouraging results, some TLR antagonist antibodies and blocking peptides are in preclinical development for treating acute and chronic inflammatory disorders, including diseases involving dysbiosis, such as inflammatory bowel disease (IBD)[14,34]. Further development of such therapies will need to take into account not only the deleterious role of immune responses in the pathology of such conditions, but also the crucial role of these responses in defence of the affected mucosal surfaces[35,36]. For example, loss of immune function in the gut mucosa can result in defects in tissue repair and can cause intestinal inflammation[37,38], whereas activation of TLRs through agonists might exacerbate pro-inflammatory responses.

Modulating NOD-like receptor signalling. NLRs are a family of cytoplasmic innate immune receptors that detect both microbial and endogenous danger signals to induce inflammation and direct antimicrobial activities[39]. Within this family, NOD1 (nucleotide-binding oligomerization domain-containing 1) and NOD2 detect peptidoglycan fragments from the bacterial cell wall to trigger NF-κB and MAPK signalling[40] (FIG. 1b). Distinct substructures of this bacterial cell wall product, termed muropeptides, are required for the activation of NOD1 and NOD2: NOD1 detects diaminopimelate (DAP)-containing muropeptides, which are mainly found in Gram-negative organisms, whereas NOD2 recognizes muramyl dipeptide (MDP), which is found in all bacteria. DAP, as well as the D-amino acids that make up the stem peptides of peptidoglycan, is present only in bacteria, making it a unique signature to alert the host about microbial infection[40]. Another member of this family, NLRP3 (NOD-, LRR- and pyrin domain-containing 3), detects both microbial and endogenous danger signals, leading to formation of the caspase 1-dependent inflammasome and the consequent cleavage of pro-IL-1β and pro-IL-18 to their active, secreted forms (FIG. 1b). The stimulation of innate immunity through NLR activation is key to priming adaptive immunity, and NLR activators such as muropeptides have the potential to be used as vaccine adjuvants. Moreover, numerous studies have explored the potential to use immunomodulation through NLRs as an anti-infective strategy.

To date, NLR agonists have been used primarily to enhance both innate and adaptive immune responses. For example, the NLRP3 agonist alum is the most highly used adjuvant for vaccines, but its mode of action, as well as whether its activity *in vivo* requires NLRP3 activation, is still under debate[41,42]. However, alum is a poor inducer of T_H1 immunity (T helper 1 immunity). Similarly, stimulation of NOD2 by MDP[43,44], a key component of Freund's complete adjuvant[45], and stimulation of other NLRs induces adaptive immune activation skewed towards T_H2 immunity[46,47]. This has led to the design of second-generation adjuvants that combine alum with other additives, usually TLR agonists, to enhance overall potency

Bacteriocin
A small ribosomally synthesized, heat-stable peptide that is produced by one bacterium and is active against another bacterium, either of the same species (narrow spectrum) or across species and even genera (broad spectrum).

Inflammasome
A molecular complex of several proteins that, following its assembly, cleaves pro-interleukin-1β (pro-IL-1β) and pro-IL-18, thereby producing active IL-1β and IL-18.

and promote balanced T_H1/T_H2 immunity[48]. Testing of the potential for using NOD1 and NOD2 agonists, in particular as human vaccine adjuvants, is currently in its infancy, and further work is required to understand how these molecules boost adaptive immune responses to co-injected antigens. For example, Chiron/Novartis is carrying out a Phase I trial of an influenza vaccine using an adjuvant called phosphatidylethanolamine-linked muramyl tripeptide (MTP–PE; also known as mifamurtide), a NOD2 agonist[48], with results still pending.

In addition to their potential use as adjuvants, many NOD agonists have been shown to have anti-infective properties. The first of these agonists to be exploited for a potential clinical application was an MDP derivative called murabutide, a NOD2 agonist with nonspecific immunomodulatory activities in individuals infected with HIV-1 (REF. 49). Further evidence of such anti-infective properties came from early studies showing that NOD1 ligands have protective effects in mice when they are given orally or parenterally before systemic infection with different bacteria[50]. Since then, many studies have shown that pretreatment of mice with NOD agonists can enhance host protection against sepsis[51], numerous bacterial infections (reviewed in REF. 52), viruses[53] and even parasites[54]. The protective effect of NOD agonists in these cases probably stems from their broad immunostimulatory properties, which enhance host protective functions at the cellular and tissue level by increasing the levels of protective factors such as nitric oxide[55] and pro-inflammatory cytokines, as well as the phagocytic capacity of immune cells (reviewed in REF. 52).

Interestingly, immunomodulatory muropeptides in combination with antibiotics offer greater resistance to infection with numerous pathogens (including bacteria, parasites and fungi) than antibiotic treatment alone (reviewed in REF. 56). Moreover, the use of muropeptides in some cases lowers the effective dose of the antibiotics used. These findings suggest that muropeptides can serve as an adjunctive therapy to conventional antibiotics. The major drawback in the use of these combined regimens is that the muropeptides must be administered prophylactically to promote anti-infective immunity.

In contrast to the concept of upregulating NLR activity to enhance immune activation, the ability to block NLR function might be advantageous in some cases. A recent study showed that viral infection upregulates the expression and activity of NOD1, NOD2 and receptor-interacting protein 2 (RIP2; also known as RIPK2; a downstream effector kinase). However, when the viral challenge is followed by a bacterial infection (specifically, a norovirus challenge followed by superinfection with *Escherichia coli* in mice), the upregulated activity of the NOD pathway triggers uncontrolled inflammation and lethality[57]. Because secondary bacterial infections can often follow viral infections in humans, the use of NOD antagonists might act to blunt pro-inflammatory responses and decrease morbidity and mortality. To date, however, there are no known antagonists of NOD1 or NOD2 and only one known family of RIP2 inhibitors, which have secondary effects on the p38 kinase pathway[58]. The use of small-molecule inhibitor screens to identify NOD antagonists and the discovery of novel and specific RIP2 inhibitors will probably open up new avenues for the modulation of these pathways that might improve treatment.

Innate defence regulator peptides

Innate defence regulator (IDR) peptides are synthetic immunomodulatory and anti-infective compounds that are broadly based on the sequences of natural HDPs. These natural peptides are produced by innate immune cells, including leukocytes and epithelial cells, in all multicellular organisms[8], and their amino acid composition is biased towards cationic and hydrophobic residues[59]. The important role of HDPs in innate immune defences, particularly at mucosal surfaces and epithelial barriers, is demonstrated by their extreme diversity and abundance in all multicellular life forms[8], by the infection susceptibility phenotypes of knockout mouse models[60] and by the increased susceptibility to infections in human conditions linked to reduced HDP production[61].

Natural HDPs, such as α-defensins, tend to have weak direct antimicrobial activities under *in vivo* conditions, but these activities may be significant at certain sites, particularly at the high concentrations found within phagosomes of innate immune cells and in intestinal crypts. The direct antimicrobial activities of HDPs are due to the action of multiple microbial molecular targets, including membrane-associated and cytoplasmic enzymes and macromolecules, as well as bacterial membranes. However, in most extracellular environments, at physiological ionic strengths and modest concentrations, the anti-infective functions of natural peptides are probably primarily mediated through their diverse immunomodulatory effects on the host. The combination of immunostimulatory effects, such as chemokine induction and stimulation of cell differentiation, and regulatory activities, such as the suppression of pro-inflammatory cellular responses to LPS and IFNγ[62], indicate that peptides act to promote a local non-inflammatory resolution of infections. This is a highly desirable mode of action, as it avoids the dangers associated with strong systemic pro-inflammatory responses.

Natural immunomodulatory HDPs provide general templates for the production of synthetic IDR peptides. Traditionally, peptide optimization efforts focused on the development of compounds that are optimized for direct killing of bacterial pathogens, although many of the drugs developed through this approach also have immunomodulatory activities and their *in vivo* functions may ultimately be mediated through a combination of these mechanisms. IDR1 was designed to have no direct antimicrobial activity but is protective in many animal models of multidrug-resistant infections, demonstrating that the protective effects of cationic peptides *in vivo* can be mediated entirely through immunomodulatory activity[63]. Indeed, immunomodulatory peptides offer many advantages, including lower potential for emergence of microbial resistance, lower toxicity to host cells and a requirement for fewer doses, addressing the issue of the

T_H1 immunity
An immune response that is characterized by a subset of T helper (T_H) cells that secrete a particular set of cytokines, including interleukin-2 and interferon-γ, the main function of which is to stimulate phagocytosis-mediated defences against intracellular pathogens.

Freund's complete adjuvant
A mixture of heat-killed mycobacteria with mineral oil. When animals are immunized with antigen mixed with Freund's complete adjuvant, a strong immune response to the antigen is induced.

T_H2 immunity
A type of immune response that is characterized by the production of interleukin-4 (IL-4), IL-5 and IL-13, and by humoral immunity mediated by B cells and immunoglobulin A (IgA) and IgE antibody classes; this response is mediated by T helper 2 (T_H2) cells.

Intestinal crypts
Tubular invaginations of the intestinal epithelium. Crypts contain intestinal stem cells that continuously divide and are the source of all intestinal epithelial cells. Paneth cells are found at the base of the crypts and produce antimicrobial proteins and peptides, including phospholipase A2 and defensins.

Table 2 | **Innate defence regulator peptides that are in clinical trials to treat bacterial infections**

Peptide	Sequence and/or structure	Activity	Potential clinical applications	Phase	Company
EA-230	4 amino acid peptide	Anti-inflammatory activity in experimental endotoxin challenge in healthy volunteers	Prevention of sepsis, and protection against renal failure following cardiac surgery	Phase I–II	Exponential Biotherapies
hLF1-11	11 amino acid cationic peptide, a fragment of lactoferricin	Direct antibacterial and antifungal activity, and stimulation of innate immunity	Prevention of infections in immunocompromised patients	Phase I	AM-Pharma
IC-31	Synthetic cationic peptide ($KLKL_5KLK$) in combination with a synthetic oligonucleotide (ODN1a)	Potent adjuvant activity in formulation with immunostimulatory oligonucleotides in animal models	Component of an adjuvant formulation in a novel vaccine against tuberculosis	Phase I	Intercell
IMX-942	5 amino acid synthetic cationic peptide loosely derived from indolicidin and IDR1	Immunomodulatory, with no antimicrobial activity; shows protective effects in mouse models of antibiotic-resistant bacterial infections	Prevention of infections in immunosuppressed patients undergoing chemotherapy for cancer	Phase II	Inimex Pharmaceuticals
MSI-78	22 amino acid cationic peptide derived from magainin	Antibacterial activity; potential immunomodulatory activity unknown	Treatment of foot ulcers in patients with diabetes	Phase III	MacroChem
MX-226 (omiganan)	12 amino acid cationic peptide derived from indolicidin	Direct antimicrobial and immunomodulatory activity	Topical antiseptic for the prevention of catheter infections	Phase III	BioWest Therapeutics
Opebacan	21 amino acid peptide derived from BPI	Antibacterial and anti-inflammatory activity	Treatment of endotoxaemia and GVHD in patients receiving bone marrow transplantations	Phase I–II (terminated)	XOMA
PMX-30063	Small-molecule structural mimetic of defensin	Antibacterial activity against MRSA; potential immunomodulatory activity unknown	Treatment of acute *Staphylococcus aureus* skin infections	Phase II	PolyMedix
XOMA-629	9 amino acid peptide derived from BPI	Direct antibacterial activity; immunomodulatory activity unknown	Treatment of impetigo skin infections caused by *Staphylococcus aureus* and *Streptococcus pyogenes*	Phase IIA	XOMA

Table is modified, with permission, from REF. 67. BPI, bactericidal permeability-increasing protein; GVHD, graft-versus-host disease; IDR1, innate defence regulator peptide 1; MRSA, methicillin-resistant *S. aureus*.

high cost of peptide drugs. However, in the long term, optimal peptides might combine antimicrobial and immunomodulatory properties or add to their immunomodulatory activity one of the other properties that are known to be present in cationic peptides, including an ability to inhibit biofilm growth[64] or promote wound healing[65,66].

Several recent clinical trials have tested the efficacy of peptide-based therapeutics[9], including those of peptides developed entirely for their immunomodulatory effects[63], those of peptides originally developed as microbicidal drugs but also known to have immunomodulatory properties and those of compounds with potent direct antimicrobial but untested immunomodulatory activities[67] (TABLE 2). The medical conditions that are targeted by the ongoing clinical trials of peptide-based drugs reflect the combined immunostimulatory and regulatory properties of this class of compound. These conditions include bacterial infections, such as infections with antibiotic-resistant pathogens, and inflammatory disorders, such as endotoxaemia and sepsis. Most clinical trials to date have focused on topical applications, such as treatment of skin infections and inflammation, although safe systemic administration, in particular of immunomodulatory peptides, has also been demonstrated.

Dendritic cells
Professional antigen-presenting cells that are found in the T cell areas of lymphoid tissues and as minor cellular components in most tissues. They have a branched, or dendritic, morphology and are the most potent stimulators of naive T cell responses.

Bacterial signalling molecules

Certain microbial signalling molecules, including *N*-acyl homoserine lactones (AHLs) and cyclic nucleotides, can also modulate host immune responses.

Cyclic nucleotides stimulate immune responses. Cyclic di-GMP (c-di-GMP) and c-di-AMP are important second-messenger molecules that are used in signal transduction in a wide range of bacteria. Mammalian cells also respond to these molecules[68]. Indeed, c-di-GMP attenuates the bacterial burden in a mouse model of *Staphylococcus aureus* infection independently of its effects on bacterial intercellular communication[69]. Furthermore, treatment of dendritic cells with c-di-GMP leads to the upregulation of numerous co-stimulatory molecules and the production of pro-inflammatory cytokines, including IL-8, IL-12 and CC-chemokine ligand 2 (CCL2)[70]. It was also found that c-di-GMP has adjuvant activity: injection of c-di-GMP along with

an antigen results in enhanced immunogenicity, and gives balanced T_H1/T_H2 immunity when given systemically and T_H1-skewed responses when administered mucosally[71]. Similar adjuvant activities were recently described for c-di-AMP[72].

Both c-di-GMP and c-di-AMP are detected in the host cytosol, and c-di-GMP was recently shown to be directly detected by stimulator of IFN genes (STING; also known as TMEM173)[73]. This engages a defence response that triggers the activation of the MAPK and STING–TBK1–IRF3 (STING–TANK-binding kinase 1–IFN-regulatory factor 3) signalling pathways. These downstream pathways, in turn, activate the expression of pro-inflammatory cytokines and type I IFNs[74,75], respectively, which have integral roles in anti-infective immunity (FIG. 2a). Further research is required to unleash the potential of these microbial molecules as immunomodulators and vaccine adjuvants. As with the other immunomodulators of microbial origin, the key will be to find a balance between the ability of these molecules to enhance antimicrobial responses and their pro-inflammatory potential.

AHLs as immunomodulators. AHLs are small molecules that are found exclusively in bacteria and are used as second messengers to communicate among organisms within a population in a process known as quorum sensing[76], but they also have other roles, for example, during virulence and biofilm formation. Numerous Gram-negative bacteria, including pathogens, produce AHLs; *P. aeruginosa*, which often infects patients with cystic fibrosis, is one example[77]. Similarly to c-di-AMP and c-di-GMP, AHLs possess immunomodulatory activities in mammalian hosts, although their host receptors are unknown. *In vitro*, AHLs have a range of effects on host cells, from the induction of apoptosis and pro-inflammatory cytokines to potent effects on dampening inflammation (reviewed in REF. 78). Although these effects are perplexing, they might be cell type specific and depend on the concentration of the AHL used in the particular study. For example, the AHL *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL) induces higher levels of the pro-inflammatory cytokine IL-6 in airway epithelial cells from patients with cystic fibrosis than in control cells, a factor that could contribute to the pathogenesis of cystic fibrosis[79]. Consistent with AHLs having a range of effects on host cells, these molecules have been shown to activate the p38 MAPK pathway and to inhibit the pro-inflammatory NF-κB pathway (FIG. 2b). Conversely, their potential interference with PPARγ (peroxisome proliferator-activated receptor-γ), a nuclear receptor protein that functions as a transcription factor to dampen inflammation, would activate the host immune response and thereby modulate inflammation[78].

The potential to use AHLs as an adjunctive anti-infective therapy remains largely unexplored, although recent findings show the potential for using AHLs to treat infectious diseases. One recent study found that AHLs have a protective role: when mice are treated with 3O-C_{12}-HSL before infection with the Gram-negative pathogen *Aeromonas hydrophila*, they have enhanced survival and increased numbers of neutrophils in the blood compared with mice that are not pre-treated with the AHL. *In vitro*, 3O-C_{12}-HSL-treated immune cells exhibit enhanced phagocytic activity, which might lower the bacterial burden and increase survival in mouse infection models[80]. An important direction for the future will be to investigate whether AHLs can also be used as preventive therapy.

Because AHLs have a role in quorum sensing, blocking bacterial communication through AHLs is another potential strategy for the treatment of infectious diseases[81]. Such inhibitors act as antagonists of AHL receptors in bacteria and have the advantage of dampening virulence but not growth, thereby minimizing the potential for the development of resistance. Several screening efforts have focused on identifying small-molecule and natural-product inhibitors that interfere with inter-bacterial communication to suppress biofilm formation and limit the expression of virulence genes. For example, blocking quorum sensing either by immunization[82] or by the use of small-molecule inhibitors[83] reduces mortality in a mouse model of *P. aeruginosa* lung infection, which indicates that quorum sensing inhibitors could be an option for the treatment of patients with cystic fibrosis. Moreover, garlic, which also inhibits quorum sensing, is being tested in clinical trials for the treatment of lung infections in patients with cystic fibrosis[84]. Quorum sensing inhibitors might also be important adjuncts to antibiotic therapy, as their use increases the susceptibility of bacterial biofilms to antibiotics both *in vitro* and *in vivo*[85]. Continued research in this area will probably identify effective quorum sensing control strategies that will establish new avenues for the treatment of patients with cystic fibrosis and *P. aeruginosa* infection, and also for the treatment of other infectious diseases. A caveat to the use of such molecules (or their analogues) as immune modulators is their potential for deleterious modulation of bacterial signalling; for example, they could promote virulence factor production or biofilm formation and/or exert important side effects on the commensal flora.

Perspectives

In this era of emerging pathogens and the re-emergence of many infectious diseases in the setting of rampant antibiotic resistance, the need to develop new antimicrobial strategies is imperative. Here, we have discussed strategies to boost antimicrobial therapy by supporting both innate and adaptive immune responses in the host. The goal in the future will be to explore the potential of some of these anti-infective therapies in combination with antibiotics as a potent way to control infectious diseases. Many of the strategies outlined in this Review have the benefit of being intrinsically unable to engender resistance. The potential caveat to their exploitation in the clinic, however, is the need to find the proper balance that generates an effective immune response and suppresses infection but also limits damaging inflammation. Indeed, immunomodulators are often inherently nonspecific in their boosting of immune responses. It will therefore be necessary to determine the consequences of such boosting when it is overlaid with

Quorum sensing
A microbial cell–cell communication process that uses small signalling molecules to allow bacteria to coordinate population behaviour, in part in response to cell density.

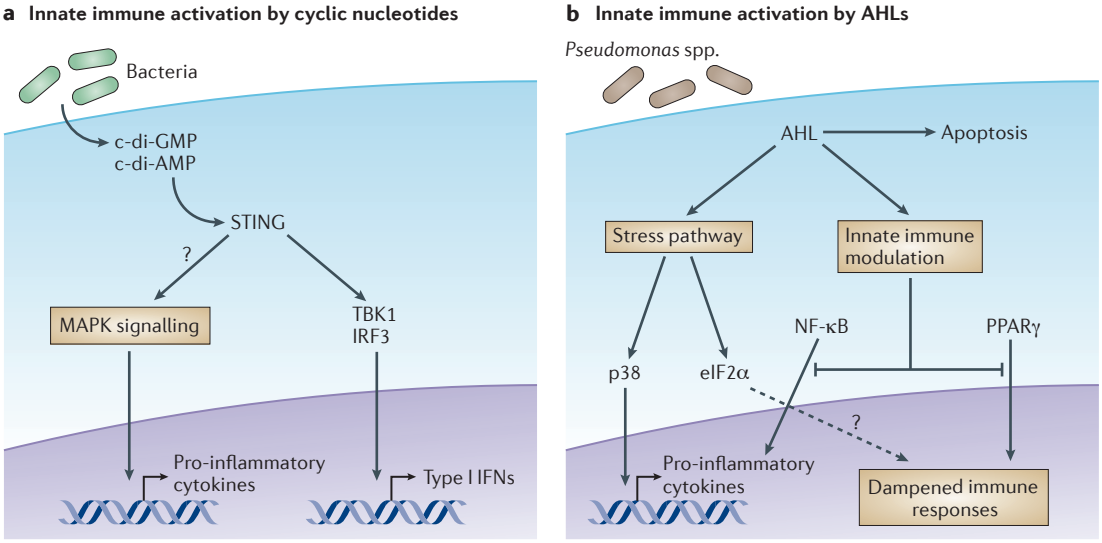

Figure 2 | **Bacterial quorum sensing second-messenger molecules stimulate innate immune activation in mammalian cells. a** | The cyclic nucleotides cyclic di-GMP (c-di-GMP) and c-di-AMP, which are used in interbacterial communication, are released from different bacteria and gain entry into host cells. Stimulator of IFN genes (STING) has been shown recently to directly sense cyclic dinucleotides, leading to activation of the TBK1–IRF3 (TANK-binding kinase 1–IFN-regulatory factor 3) pathway, and the subsequent production of type I interferons (IFNs). STING has also been proposed to upregulate pro-inflammatory cytokines through the induction of mitogen-activated protein kinase (MAPK) signalling, although the mechanism of this induction is currently unknown. **b** | *N*-acyl homoserine lactones (AHLs) are another class of quorum sensing molecule in bacteria. These molecules have been shown to modulate host immune responses in both a positive and negative way. AHLs enter into mammalian cells and trigger apoptosis, modulate immune responses by inhibiting nuclear factor-κB (NF-κB) and the activity of peroxisome proliferator-activated receptor-γ (PPARγ), and trigger stress pathways that result in the activation of p38 and eukaryotic translation initiation factor 2α (eIF2α). These events culminate in attenuation of innate immune responses (by inhibiting pro-inflammatory pathways) or enhancement of immune responses (by inhibiting anti-inflammatory pathways).

the natural perturbations of innate immunity that occur during human diseases (for example, whether TLR agonists reinforce the pro-inflammatory effects of microbial signature molecules or DAMPs). This will probably require detailed mechanistic studies at a systems level in appropriate animal models.

The development of TLR-targeting therapies, which have enjoyed the greatest success to date, will be increasingly facilitated by an appreciation of the massive complexity of the immune system, which will lead to the expanded application of bioinformatics and systems biology to models of host–pathogen interactions and the use of such models in the development of immunomodulatory therapeutics[86]. In addition, structural data showing the molecular interactions of TLRs with their ligands will be useful for defining the specific structural requirements for optimal ligand activity[87–89]. A better understanding of the mechanisms of immunological memory will result in optimized applications of TLR agonists and the design of adjuvants that can specifically stimulate innate immunity to induce long-term adaptive immune responses. Interestingly, it was recently shown that primary immune responses to many common adjuvants are independent of TLR stimulation[90], but that the combined stimulation of several TLRs leads to synergistic improvements in the induction of immunological memory and in the long-term protective activity of a vaccine[91]. The methods of formulation and delivery of such vaccines and of immunomodulatory antimicrobial drugs are also continually being improved[92]. The safety of all new TLR agonists and administration protocols will need to be carefully examined, as acute TLR stimulation can induce inflammation and immune-mediated damage to the host, and chronic TLR stimulation can result in endotoxin tolerance and immune paralysis, potentially impairing defences against infections[93]. Nevertheless, the clinical use of the TLR4 agonist and adjuvant monophosphoryl lipid A (MPL) and of other TLR agonists (TABLE 1) indicates that safe application of TLR agonists to stimulate immune responses is feasible.

It is worth mentioning that many of the studies cited here have focused on the prophylactic use of immunomodulatory compounds to boost immunity against subsequent infections. The results from these studies suggest that certain immunomodulators will be useful in preventing infections (for example, when administered to immunodeficient individuals or on admission of patients to hospitals to avert nosocomial infections), although many of the agents highlighted in TABLE 1 (such as imiquimod) are used or designed as therapeutics. Future studies must address the potential therapeutic uses of PRR agonists, IDR peptides and quorum sensing molecules in helping to clear established bacterial infections, either alone or in combination with antibiotics.

One of the exciting future approaches will be the development of IDR peptides. These peptides show great promise owing to their selective action on innate immunity[63], their potential for both prophylactic and therapeutic usage[56,65] and their ability to enhance the performance of vaccine adjuvants[94,95]. The optimization of peptide sequences for protective activity against infections and for other *in vivo* applications is an important goal of ongoing research. This requires identifying a peptide activity that is easily assessed *in vitro* and that correlates with *in vivo* protection against infection, and then finding the optimal peptide sequence that enhances this *in vitro* activity but retains low toxicity and other properties that are compatible with *in vivo* application. The direct antimicrobial activity of peptides against a range of bacterial pathogens has been successfully optimized using similar approaches involving high-throughput production of iterative peptide libraries combined with *in vitro* screening for inhibition of bacterial growth[96]. This work has been further advanced by computer-aided approaches to predict the antimicrobial activity of a range of peptide sequences on the basis of a small experimental data set of structurally diverse peptides and mathematical modelling[97,98]. Optimization of immunomodulatory peptides using similar approaches is more challenging, as the precise combination of biological activities required to aid in the resolution of a particular infection has proved difficult to establish. However, significant progress has been made (TABLE 2). Sequence modification of natural peptides with known immunomodulatory activities has demonstrated major promise. For example, truncated derivatives of human cathelicidin family peptide LL-37 have been created and found to have enhanced chemokine induction activity[99], and antimicrobial and anti-inflammatory therapeutic peptides have been generated from human bactericidal permeability-increasing protein (for example, XOMA-629). A fragment of human lactoferricin (hLF1-11) with immunostimulatory and anti-infective properties has been identified and has entered clinical trials. Immunomodulatory peptide IDR1 (REF. 63) was generated through iterative sequence modifications and truncations of the natural bovine peptide bactenecin. In addition, enhanced *in vitro* chemokine induction activity in the peptide libraries of synthetic bactenecin derivatives has been shown to correlate with improved protection against infection in animal models, suggesting that optimization of peptide sequences for chemokine induction *in vitro* is a feasible approach for improving the *in vivo* activities of anti-infective peptides[100]. Furthermore, the *in vivo* stability of peptides can be enhanced through the use of D-amino acid peptides, retro-inverso peptides or synthetic peptidomimetics that are resistant to protease degradation[101].

Better methods for the formulation and delivery of immunomodulatory peptides, and approaches taking advantage of the synergistic activities of these peptides with other immune mediators, will continue to facilitate their clinical development. For example, IC-31, comprising a synthetic cationic peptide administered together with an immunostimulatory oligonucleotide, is currently in clinical trials as an adjuvant for a novel *M. tuberculosis* vaccine[94,95], and the synergistic activities of cationic peptides with oligonucleotides in type I IFN induction[102,103] may have a role in the immunogenicity of such formulations. Finally, a class of immunomodulatory therapies designed to boost defences against infection by stimulating the production of natural peptides is an exciting new avenue and might provide a cheaper alternative to the administration of synthetic peptides. As an example, vitamin D is an effective inducer of natural cathelicidin peptide LL-37 in human skin and in innate immune cells, and this is thought to underlie the deleterious effects of vitamin D deficiency on immunity to *M. tuberculosis*[104,105]. In addition, butyrate administration in animal models was shown to improve the outcome of *Shigella flexneri* infection through induction of LL-37 in the colon[106].

We are rapidly approaching the antibiotic resistance era, which is coupled to a dearth of antibiotic discovery. A new generation of immunomodulatory therapeutics for infectious diseases has enormous potential to compensate for this major health care challenge.

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Competing interests statement
The authors declare competing financial interests; see web version for details.

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