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Immunomodulators as adjuvants for vaccines and antimicrobial therapy

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A highly effective strategy for combating infectious diseases is to enhance host defenses using immunomodulators, either preventatively, through vaccination, or therapeutically. The effectiveness of many vaccines currently in use is due in part to adjuvants, molecules that have little immunogenicity by themselves but which help enhance and appropriately skew the immune response to an antigen. The development of new vaccines necessitates the development of new types of adjuvants to ensure an appropriate immune response. Herein, we review commonly used vaccine adjuvants and discuss promising adjuvant candidates. We also discuss various other immunomodulators (namely cytokines, Toll-like receptor agonists, and host defense peptides) that are, or have potential to be, useful for antimicrobial therapies that exert their effects by boosting host immune responses rather than targeting pathogens directly.

Keywords: adjuvant; immunomodulator; vaccine; antimicrobial

Immunomodulators as vaccine adjuvants

The discovery of vaccination by Edward Jenner in the 1700s was one of the most important medical discoveries in history. Today, vaccination remains the safest and most cost-effective medical way of preventing infectious diseases. Although vaccines have had tremendous successes, including the eradication of smallpox in 1979 and the substantial reduction in polio incidence, many infectious diseases remain for which effective vaccines have not yet been developed, such as malaria, tuberculosis, hepatitis C virus (HCV), and human immunodeficiency virus (HIV). One major hurdle that stands in the way of generating such vaccines is the need for adjuvants that can promote and sustain immune responses against antigens of interest. Herein, promising advances in adjuvant development are discussed.

History and background on adjuvants

Adjuvants were discovered in the 1920s by a French veterinarian named Gaston Ramon. Ramon noticed that the addition of certain substances, particularly aluminum salts, to vaccines increased their efficacy. Since then, adjuvants have become an increasingly invaluable component in the field of vaccinology. The word "adjuvant" is derived from the Latin *adjuvare*, "to help." Adjuvants, although often not particularly immunogenic by themselves, serve to promote and enhance immune responses to vaccine components, thereby lessening the required dose of said vaccine and prolonging immunological memory.

Vaccines containing live-attenuated or inactivated viruses or bacteria signal through Toll-like receptors (TLRs) and therefore generally induce an appropriate immune response without the use of adjuvants. However, as we move more toward using more purified antigens that lack the immunostimulatory potential of a whole organism, adjuvants have become necessary to achieve an appropriate immune response. Furthermore, commonly used vaccine adjuvants elicit primarily a humoral (antibody-mediated/T_H2) response, which is ineffective at controlling many types of infections, especially those involving intracellular pathogens.

Adjuvants can act at several different stages of the immune response, although they all influence

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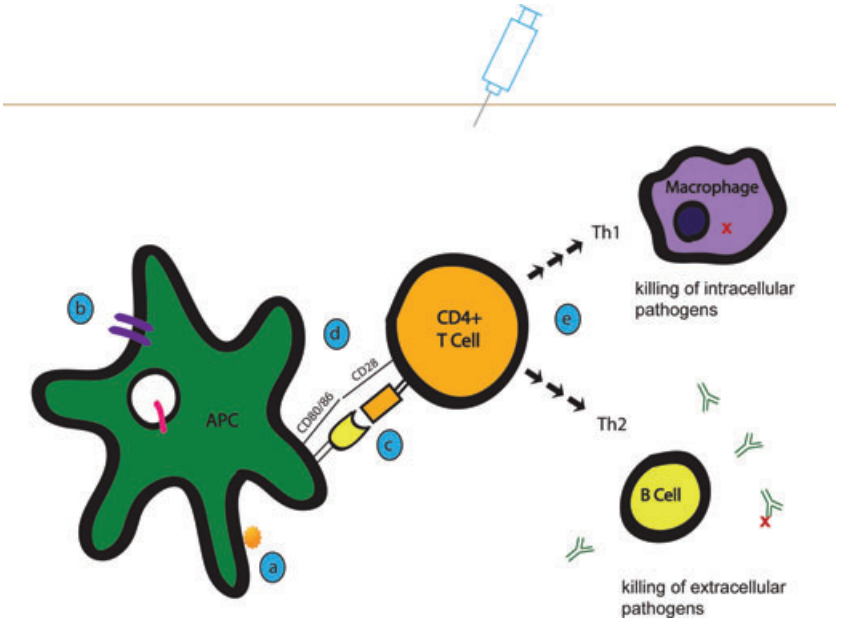

Figure 1. **Adjuvants can act at multiple different stages of the immune response. Some adjuvants, such as alum, aid in antigen capture and uptake (A). They may activate APCs, often via TLR stimulation (B), as is the case with MPL. Some, such as saponins, may aid in antigen presentation to T cells (C) and can enhance costimulation (D), as can be the case for certain cytokines. Ultimately, adjuvants enhance the immune response and polarize the T_H1/T_H2 balance (E). MPL, monophosphoryl lipid A.**

antigen presentation, be it directly or indirectly (reviewed in Ref. 1). They may aid in the recruitment to the site of immunization, antigen recognition, and activation of antigen-presenting and other ancilliary cells (APCs), particularly dendritic cells (DCs), resulting in the production of key cytokines, and can promote antigen presentation.[1] Figure 1 illustrates stages of the immune response where adjuvants typically act.

Qualities of the ideal adjuvant and related challenges

A great challenge in the field of vaccinology is the elucidation of the mechanisms of action of vaccine adjuvants. Many adjuvants have been discovered empirically, and a common function has thus been difficult to determine. For some time, the mechanism of action of vaccine adjuvants was somewhat of a mystery, although the discovery of TLRs and an improved understanding of innate immunity have allowed scientists to begin to elucidate how adjuvants work. Although mechanisms of action of adjuvants are varied, there exist a number of important qualities that must be considered when designing new adjuvants.

Quality and type of immune response

Different types of immune responses are required to effectively clear different types of infections. A humoral (T_H2) response is effective at promoting an immune response that will neutralize bacterial toxins and help combat extracellular infections. Intracellular infections, on the other hand, require a cell-mediated (T_H1) immune response for clearance, although viral infections tend to require a more balanced immune response.[2] The actions of T_H1 cytokines, mainly interleukin (IL)-12 and subsequently interferon gamma (IFN-γ), oppose those of T_H2 cytokines (mainly IL-4 and IL-10), resulting in the polarization toward either a cell-mediated immune response or a humoral response, respectively. Any vaccine must recruit and activate cells appropriately and, ideally, elicit an appropriately balanced T_H1/T_H2 response.

Compatibility with antigen(s)

Because adjuvants often act by increasing uptake of antigens, a physical association between adjuvants and antigens may be necessary. For example, in alum adjuvanted vaccines, antigens are adsorbed onto alum particles, likely via electrostatic

interactions and exchange between phosphate and hydroxyl groups.[3] Clearly, this interaction depends upon the antigen used, and can be further influenced by factors, such as pH, buffer strength, and various *in vivo* factors.[3] In such a case, an inadequate physical association between antigen and adjuvant may cause a vaccine to fail.[3]

Safety

A variety of adjuvants have been created that help elicit a strong immune response, but most of these are far too toxic for use in humans, as it is often difficult to separate immunogenicity from reactogenicity. For example, complete Freund's adjuvant (CFA), an oil-in-water emulsion containing dead mycobacteria, is a potent inducer of cell-mediated responses in mice but causes toxic side effects such that it cannot be used in humans. The ideal adjuvant is sufficiently and appropriately immunogenic, but does not cause excessive inflammation or other immunopathologies.

Stability and cost

In order for vaccines to be administered to large populations, particularly those in developing countries, they must be available at low cost, especially because vaccines are usually administered to prevent prospective disease rather than being used to treat specific known diseases. It is clearly of great importance that potential vaccine adjuvants can be made inexpensively. The use of adjuvants can significantly reduce the amount of antigen required for an effective immune response, presumably reducing production costs, and also allows for antigen sparing in times when demand exceeds supply. Furthermore, it is far easier (and thus more cost-effective) to administer vaccines that are stable at room temperature, and stability is therefore of high importance when considering an adjuvant for use in vaccines. Another factor that affects the cost of vaccination is the enormous potential of vaccines that need a smaller number of doses to achieve protection, with the Holy Grail being single-shot vaccines that would not require boosters; it is felt that adjuvants can help achieve this end.

Adjuvants currently employed in human vaccines

In live and killed vaccines, adjuvanticity is intrinsic, and the addition of exogenous adjuvants is not typically required. However, as there is a greater trend toward the use of recombinant and highly purified antigens, which are generally safer but more weakly immunogenic, the need for effective adjuvants has become quite apparent. Currently, there exist just a handful of adjuvants that are currently employed in vaccines.

Aluminum salts

The usefulness of aluminum-containing adjuvants (typically aluminum phosphate or aluminum hydroxide gels, generally referred to as alum) as adjuvants was discovered empirically when it was observed that contaminants in vaccine formulations could actually enhance vaccine effectiveness. Alum has been used in a variety of human vaccines, including tetanus, diphtheria, pertussis, hepatitis A virus (HAV), and inactivated polio, and is currently the only adjuvant approved for use in the United States.[4]

Alum has several mechanisms of action (discussed in Ref. 5). It was initially believed that the adjuvant activity of alum was due to its retention of immunogenic molecules at specific sites in the body, allowing for their slow release and consequently a prolonged immune response, the so-called "depot effect." Alum also appears to exert its adjuvanticity by keeping antigens in a particulate (rather than soluble) form, thereby enhancing phagocytosis of antigens by APCs.[6] Recently, it has become evident that aluminum salts are capable of directly activating immune cells. Alum can induce maturation of monocytes/macrophages into DC-like cells, and this process is dependent on the activation of NOD-like receptor family, pyrin domain containing 3 (NLRP3), part of the inflammasome, which causes production of pro-inflammatory cytokines.[7,8] The activation of NLRP3 may be direct, or alum may act indirectly by causing release of uric acid, which then activates NLRP3.[9]

Although alum is effective for many types of vaccines and is widely used, it elicits primarily a T_H2 response (including IL-4/5 production and IgG_1 and IgE production by B cells), which is ineffective for vaccination against many types of pathogens. Furthermore, it has been known to cause side effects, such as allergic reactions and granulomas, in certain individuals. Thus, alum will continue to be useful for many vaccines, but other adjuvants will be needed for vaccines that require a more cellular response.

MF59 and AS03

MF59 and AS03 are both squalene-based oil-in-water emulsions. AS03, produced by GlaxoSmith-Kline (London, UK), is used in the influenza vaccine Prepandrix®, which has demonstrated excellent efficacy and safety.[10] MF59 is made by Novartis (formerly Chiron; Basel, Switzerland) and is currently used in Fluad®, an influenza vaccine used primarily in Europe in people aged 65 and over. MF59 was also used in trials of a herpes simplex virus (HSV) 2 vaccine but elicited only mild and transient protection against infection.[11] MF59 promotes monocyte differentiation towards a DC-like phenotype and aids in antigen uptake by DCs and is capable of eliciting both T_H1- and T_H2-type immune responses.[12]

Monophosphoryl lipid A

The TLR4 agonist lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria, is a potent inducer of inflammation and innate immune responses and is an excellent immunological adjuvant, but is far too toxic for use in vaccines. Administration of LPS can lead to the onset of systemic inflammatory response syndrome and septic shock. Thus, substantial efforts have been made to modify LPS in such a way that it will retain immunogenicity but limit its toxic effects mediated by inflammation. The lipid A portion of LPS is responsible for the endotoxicity of LPS, and modification of the lipid A structure can thus result in an LPS-like molecule that is not very endotoxic.

Monophosphoryl lipid A (MPL) is a derivative of *Salmonella Minnesota* R595 LPS. MPL is made by removing a phosphate group and an acyl chain from the LPS molecule. Like LPS, MPL activates TLR4, but it is over 100 times less toxic.[13] This dramatic reduction in toxicity is not merely a reduction in potency. Rather, MPL appears to trigger the Trif-dependent pathway, but its ability to signal through MyD88 is dramatically reduced, compared to LPS.[14]

AS04 is a combination of MPL and either aluminum hydroxide or aluminum phosphate.[15] AS04 is used in the hepatitis B virus (HBV) vaccine FENDrix® and in the human papilloma virus (HPV) vaccine Cervarix, both from GlaxoSmith-Kline Biologicals.[5,15,16] AS04 appears to function by activating DCs, causing the production of cytokines and increase in costimulatory molecules.[17]

Although caution must clearly be used when employing immunostimulants, extensive analyses have revealed no effect of AS04 adjuvanted vaccines on the development of autoimmune disorders.[18] In fact, MPL can be used to treat allergies, due to its ability to dampen T_H2 responses.[19] Various other combinations and formulations of MPL have been used in clinical trials, with promising results (reviewed in Ref. 5).

Virosomes

Virosomes, a type of liposome, are a relatively new option for vaccine adjuvants. Virosomes are empty reconstituted influenza virus envelopes, and because they contain hemaglutinin, they can bind sialic acid on DCs and macrophages, thereby enhancing antigen availability to, and uptake by, these cells.[5,20] Inflexal®, produced by Crucell (Leiden, the Netherlands), is an influenza virus vaccine that is approved for use in many countries.[21] It has also been shown that inactivated HAV adsorbed to the surface of virosomes (Epaxal®, also produced by Crucell) is capable of inducing an immune response that is as effective as the traditional hepatitis A vaccine, but with fewer side effects.[22]

Other vaccine adjuvants with therapeutic potential

Many types of adjuvants are currently at the preclinical and clinical stages of development. Several key vaccine adjuvant candidates are discussed later, and are also listed along with currently used adjuvants in Table 1.

TLR ligands

The discovery of TLRs and an overall greater understanding of innate immunity has allowed for great progress in the understanding and development of adjuvants. The immune system has evolved to react swiftly to the presence of foreign microbes and pathogens. This is accomplished by the host development of a wide array of sentry receptors, collectively termed as the pattern recognition receptors (PRRs), which include TLRs, NOD-like receptors, and RNA helicases such as retinoic acid inducible gene-I. Of these PRRs, the signaling mechanisms and immune functions of the TLR family are the best characterized. TLRs recognize a range of microbe-specific signature molecules, including nucleic acids found in bacterial and viral pathogens, as well as protein and lipid components of microbial cell walls and membranes. Activation of TLRs results in complex signaling cascades that rapidly trigger the onset

Table 1. Types of immune responses triggered by adjuvants

Adjuvant	Type of immune response generated	Vaccine
Adjuvants approved for human use		
Alum	• T_H2	• Used in most vaccines
MF59	• T_H1 and T_H2	• Influenza (Fluad)
AS03	• T_H1 and T_H2	• Influenza (Prepandrix)
AS04 (MPL + alum)	• Good T_H1 response	• HBV (FENDrix)
	• Some T_H2	• HPV (Cervarix)
Virosomes	• T_H1 and T_H2	• Influenza (Inflexal) HAV (Epaxal)
Potential adjuvants		
CpG ODN	• T_H1	• Influenza (Phase I)
	• Good CTL response	• HBV (Phase I)
Montanides	• T_H2	• Malaria (Phase I)
		• HIV (Phase I)
AS01	• Strong T_H1 and T_H2	• Malaria (Phase II)
	• Good CTL response	• HIV (Phase I)
AS02	• Strong T_H1 and T_H2	• Malaria (Phase III)
		• TB (Phase II)
		• HIV (Phase I)
GM-CSF	• T_H1 and T_H2	• HBV (Phase II)
Polyphosphazene	• T_H2	• Influenza (Phase I)
		• HIV (Phase II)
Inulin	• Strong T_H1 and T_H2	• HPV (Phase I)
	• Activates complement	

CTL, cytotoxic T-lymphocytes; TB, tuberculosis.

of inflammation and innate immunity required for microbial pathogen. In addition, the type of reaction elicited depends on the distinct TLRs activated.

TLR ligands are attractive vaccine adjuvant candidates, as they elicit a primarily T_H1-type immune response.[23] The downside to using TLR ligands as vaccine adjuvants is that they can actually work too well and induce toxic levels of inflammation.[24] However, if TLR ligands can be modified in a way that maintains adjuvanticity without producing excessive inflammation, effective vaccine adjuvants can be developed. For instance, as discussed earlier, MPL has shown promise as a vaccine adjuvant, particularly as part of AS04.

Other TLR ligands may also be suitable adjuvants. Unmethylated CpG oligodeoxynucleotides (ODNs) activate immune cells through TLR9, resulting in the production of pro-inflammatory cytokines, and overall inducing a T_H1 response. Studies in various animal models showed that CpG ODNs improve and balance immune responses when administered in combination with currently used vaccine-adjuvant combinations.[25] The adjuvant potential of CpGs is currently being investigated in clinical trials.

TLR7 and TLR8 are activated by single-stranded RNA sequences found in many viral species as well as the small molecules imiquimod and resiquimod, leading to the downstream induction of antiviral responses, including type I IFN production, and enhancement of T_H1-mediated immunity. TLR3 is activated by viral dsRNA and polyinosinic:polycytidylic acid [poly(I:C)], a synthetic RNA that mimics viral RNA. Poly(I:C), imiquimod and resiquimod all exhibit promise as potential vaccine adjuvants (discussed in Ref. 26).

Although TLR agonists show definite promise as vaccine adjuvants, concern does persist regarding the safety of such molecules in that they could exacerbate or even cause autoimmune disorders (discussed in Ref. 27), and/or could exacerbate natural

inflammatory responses to infectious agents leading to hyperinflammation. A Phase III trial of Heplisav, an HBV vaccine containing a CpG sequence, was recently halted due to a study participant developing Wegener's granulomatosis, an autoimmune disease that affects the vascular system.[28]

Montanides

Montanides are squalene-based water-in-oil emulsions; they are similar to incomplete Freund's adjuvant, but are biodegradable and therefore much less toxic.[5] Both ISA 51 and ISA 720 have been used in several clinical trials.[29] Montanide ISA 720 has been used in Phase I clinical trials for malaria vaccines.[30–32] Montanide ISA 51 was also tested for use in a malaria vaccine, but the vaccine was quite reactogenic and the Phase I trial was halted,[33] although another malaria vaccine containing ISA 51 has been tested in nonhuman primates, and efforts are under way to begin clinical trials with this vaccine.[34] One major drawback, however, of using Montanides for large-scale vaccination is their high manufacturing cost.[5]

Saponins

Saponins are natural detergent-like molecules that aid in inducing both humoral and cellular immunity, but are hemolytic and cytotoxic toward human cells.[35,36] Quil A is a heterogeneous enrichment of saponins from the bark of *Quillaja saponaria*, a tree found in South America. Quil A has shown promise in veterinary vaccines, but is too toxic for use in humans.[36,37] The saponin derivative QS-21 is far less toxic than Quil A, and is a good inducer of T_H1 responses. It appears to have adjuvant activity by improving antigen presentation and promote cell-mediated immunity. GlaxoSmithKline has developed two adjuvant formulations, AS01 and AS02, which both incorporate QS-21.[16] AS01 contains liposomes, MPL, and QS-21, whereas AS02 is an oil-in-water emulsion containing MPL.[16] AS02 has been shown to elicit both humoral and cell-mediated immune responses, causing both high antibody titers and IFN-γ levels, whereas AS01 promotes a stronger T_H1 response.[15] A recent detailed review of saponins as adjuvants is provided by Sun *et al.*[37]

Cytokines

Because a major challenge when designing or choosing an adjuvant for a given vaccine is ensuring that the resulting immune response has the appropriate T_H1/T_H2 balance, a logical approach would be to administer appropriate cytokines to skew the immune response in the desired direction. Use of cytokines as vaccine adjuvants has so far been limited. However, granulocyte-macrophage colony-stimulating factor (GM-CSF) has shown promise as a vaccine adjuvant. GM-CSF is known to activate DCs, and studies have shown that the addition or preadministration of GM-CSF to the HBV vaccine improves the response to this vaccine in immunocompromised individuals.[38]

Polyphosphazene

Polyphosphazenes are water-soluble polymers. Poly [di(carboxylatophenoxy) phosphazene] (PCPP) has been shown to enhance antibody responses in mice to an influenza vaccine, while being negligibly reactogenic.[39] Similar results were observed in a Phase I trial of PCPP. CpG ODN and polyphosphazenes are a potent adjuvant combination when administered concomitantly, implying that polyphosphazene may be most useful when combined with other adjuvants.[25]

Polysaccharides

A promising new option is the use of adjuvants based on inulin, a storage polysaccharide found in plants. Such adjuvants can elicit as potent T_H1 and T_H2 immune responses as CFA without the problem of severe toxicity, as has been demonstrated with a range of antigens and animal models.[40,41] Microparticulate inulin (MPI) is a known activator of the alternate complement pathway and is able to promote cell-mediated immunity; MPI-based adjuvants have demonstrated success in a variety of animal models.[42]

Host defense peptides

Host defense peptides (HDPs) are a large family of molecules and are an evolutionarily conserved component of the immune system of many species, including insects, animals, and plants. Although HDPs are diverse in sequence and structure, they share certain characteristics. Generally, HDPs are short molecules, ~12–50 amino acids in length, with a net positive charge, +2–9.[43] Initial interest in HDPs focused on their potent activity against a wide range of microbes, ranging from bacteria to fungi *in vitro*. However, it is now well-known that the HDP family possesses many members with unique

immunomodulatory activity, which will be discussed at greater length later in this review.

The ability of HDPs to regulate aspects of the immune system has made them potential candidates as vaccine adjuvants. HDPs have been shown to regulate cytokine responses, DC and lymphocyte recruitment and maturation, as well as T_H cell polarization, immune functions that play a major role in the development of an adaptive immune response. Animal studies have shown that human neutrophil defensins are able to significantly promote and enhance adaptive, antigen-specific, immunity when used in vaccine formulations.[44,45] Recent studies have investigated the effects on adaptive responses by HDPs used in combination with CpG ODNs. Indolicidin, a bovine HDP, co-formulated with CpG ODN and polyphosphazene, significantly enhanced antigen-specific humoral responses and promoted cell-mediated immunity in cattle, compared to CpG ODN with EMULSIGEN® (MVP Laboratories, Inc., Omaha, NB), an adjuvant frequently used in veterinary vaccines.[46] Similarly, bactenecin derivative innate defense regulator (IDR)-HH2, in complex with CpG ODN within a pertussis toxoid vaccine formulation, synergistically induced the production of chemokines and significantly enhanced the production of toxoid-specific antibodies in mice.[47] This formulation demonstrated responses indicative of a balanced T_H1/T_H2 response. Intriguingly, potent immune responses were observed even after a single application of adjuvanted pertussis toxoid and animals became protected against pertussis infections with this formulated vaccine. These studies demonstrate that HDPs and their derivatives may be used in vaccine formulations to promote an effective, long-lasting, and balanced protective response.

Immunomodulators as anti-infective therapies

The ever-present threat of infectious diseases requires continued development of novel therapeutics to combat microbial pathogens. Traditional antibiotics are becoming increasingly incapable of keeping pace with the development of drug-resistant bacteria. A novel strategy is to manipulate host immune responses to combat infection. Unlike antibiotics, immunomodulation would enhance a preexisting system that is designed to have broad spectrum antimicrobial activity. Furthermore, using an antimicrobial system with multiple mechanisms of action minimizes the development of antimicrobial responses as pathogens try to counter multiple modes of attack against a system that has been effective in keeping them at bay for millions of years. Currently, methods of regulating host immune responses via immunomodulatory molecules are being discovered and developed into candidate therapeutics, some of which are listed inTable 2. Overall these investigations appear to indicate that there are substantial parallels between the molecules used as adjuvants and those that have therapeutic efficacy possibly reflecting the requirement of induction of an appropriate innate immune response.

A major obstacle in the use of immunomodulators is the challenge of manipulating a systemic response to combat infections, many of which are localized, with minimal toxic effects to the host and maximal anti-infective efficacy. Many types of immunomodulators, which are locally used to counter infections, are currently in use, including the topical administration of TLR agonists against dermal infections. However, systemic administration of immunomodulatory agents may lead to systemic activation or suppression of immune responses, which can lead to multiple adverse host effects. Indeed, some systemically administered immunomodulators in use today, including IFNs and TLR ligands, elicit unwanted inflammatory symptoms, such as fever and hypotension. On the other hand, other immunomodulators show minimal toxic effects with great efficacy, such as CSFs or vaccine adjuvants. Although it is possible that immunomodulators can potentially cause widespread toxic effects with low efficacy against localized targets, many factors must be considered. First, many infectious diseases require systemic treatment, including bacteremia or widespread viral infection. Otherwise local infections with systemic inflammatory symptoms may require the systemic use of immunomodulators to rein in immune responses. As well, immune effects during infection, although extensively activated, tend to effectively concentrate at the site of infection via a wide range of mechanisms, including greater recruitment of cells and immune mediators, and increased blood flow to the site of infection. In short, although modulation of the immune response can be extensive and systemic in nature, there is a general priming of immune functions that are activated at the site of infection. As mediators of the

Table 2. Immunomodulators as anti-infectives

Drug	Immunomodulatory function	Anti-infective applications
Cytokines		
Type 1 IFN		
IFN-α	• Antiviral response inducer	• Treatment of chronic HCV and HBV infections • Potential SARS-Coronavirus therapy
IFN-β	• Antiviral response inducer	• Regulation of inflammation in virus-related multiple sclerosis
Type 2 IFN		
IFN-γ	• Antiviral response inducer	• Immune system supplementation for chronic granulomatous disease
	• Macrophage and NK-cell activator • Promotion of cell-mediated immunity	• Potential antifungal therapy
Colony stimulating factors		
G-CSF	• Potential antifungal therapy granulocyte proliferation and differentiation	• Immune system supplementation for neutropenia
	• Promotion of granulocyte antimicrobial responses	• Potential antibacterial and antifungal therapy
	• Enhancement of granulocyte chemotaxis	
GM-CSF	• Granulocyte and monocytes proliferation and differentiation	• Immune system supplementation for neutropenia
	• Promotion of granulocyte and monocytes antimicrobial activity	• Potential antifungal therapy
	• Enhancement of macrophage inflammatory responses	
M-CSF	• Monocyte proliferation and differentiation • Regulation of macrophage inflammatory responses • Promotion of macrophage antimicrobial responses and immunity against extracellular pathogens	• Potential antifungal therapy
TLR agonists		
MPL (TLR4)	• Inflammatory stimulator	• Potential antibacterial, antifungal, and antiviral therapy
	• Activation of leukocyte antimicrobial responses	
Imiquimod (TLR7) Resiquimod (TLR7/8) Isatoribine (TLR7)	• Induction of Type 1 IFNs • Promotion of cell-mediated immunity	• Topical treatment of genital warts • Potential therapy for virally induced skin diseases • Potential therapy for systemic viral infections
CpG ODN (TLR9)	• Induction of Type I IFNs	• Potential therapy against viruses and intracellular microbes
	• NK cell activation • Promotion of cell-mediated immunity	
Host defense peptides		
IMX-942	• Chemokine induction	• Potential antibacterial therapy
IDR-1	• Enhancement of cellular recruitment	• Potential immune system supplement in immunocompromised individuals
IDR-1002	• Regulation of inflammatory responses	
hLF1–11	• Regulation of macrophage differentiation	• Potential immune system supplement in immunocompromised individuals
	• Enhancement of macrophage antimicrobial activity	

immune system, immunomodulators closely mimic this synergistic, and priming activity. A classic example of this is the use of CSFs to bolster immune cell functions that are only used when pathogens are encountered. In essence, the use of immunomodulators in anti-infective therapy is situationally dependent on the function of the immunomodulators and the disease or disease symptoms they are being used to treat. Development of future immunomodulators must take these issues into account and a greater understanding of the immune system will allow for the discovery or creation of modulating agents with increased selective action against infection while minimizing the induction potentially harmful immune responses.

Endogenous immunomodulators: cytokines

The adaptability of immune response functions is dependent on multiple complex regulatory networks. One such network consists of the actions of cytokines, cell-to-cell signaling mediators, which play a major role in the coordination and orchestration of immune system functions. The cytokine family encompasses a range of peptides and proteins that have profound regulatory effects on immunity. Pro-inflammatory cytokines mediate the onset of inflammation, promoting recruitment and activation of immune cells. Cytokines are also essential in the development of the adaptive response in that they determine the T_H1/T_H2 balance. Regulatory cytokines, such as anti-inflammatory mediators IL-10 and tumor growth factor beta, act to regulate and limit the potentially harmful effects of immunity as well as mediating the resolution of the immune response. The actions of cytokines regulate virtually all aspects of the immune system. Thus, they are prime candidates for the development of immunomodulators, which can beneficially boost the immune response.

Interferons

IFNs are a subfamily of cytokines originally noted for their potent antiviral properties. IFNs are also capable of potentiating a number of antimicrobial defenses during an immune response. In humans, IFNs are grouped into three distinct classes: IFN type I, II, and III. Currently, the classification and mechanisms of action of type III IFNs are not well understood. Type I and II IFNs are widely used as anti-infective agents in a wide variety of clinical applications.

Type I IFN. Type I IFNs consist of a number of structurally homologous proteins including 13 IFN-α subtypes, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, and IFN-ω.[48] Type I IFNs are produced in response to microbes by a number of cell types, including macrophages, DCs, and endothelial cells.[49] These IFNs bind the type I IFN receptor of neighboring cells, activating members of the Janus activated kinase (JAK) family. This leads to the downstream dimerization of STAT proteins and the expression of IFN-stimulated genes (ISGs) which mediate type I IFN antiviral functions.[48] The result is the inhibition of protein synthesis, production of RNA digesting enzymes, and the promotion of cellular apoptosis, all serving to inhibit viral replication.[49]

IFN-α and IFN-β have gained prominence as therapeutic agents against viral infections. IFN-α derivatives are widely used to treat patients with infection by HBV and HCV. Conventional IFN-α treatment results in modest improvement to disease outcomes.[50,51] PEGylation, the addition of polyethylene glycol chains, to IFN-α improves its stability and significantly improves its immunomodulatory activity.[52] As such, PEG-IFN-α is commonly used in the treatment of chronic HBV and HCV infections in combination with other antiviral agents, including lamivudine and ribavirin. IFN-β is used in the treatment of multiple sclerosis (MS), due to its reduction of inflammation in the central nervous system. IFN-β also reduces the *in vitro* replication rate of human herpesvirus-6 (HHV-6), a neurotropic virus that may have a role in exacerbating MS pathogenesis.[53] This trend is also seen *in vivo* when monitoring HHV-6 replication in IFN-β–treated MS patients. Thus, it is conceivable that the improvements seen with IFN-β treatment owe a large part to its antiviral properties.

Type II IFN. In humans, the sole member of the type II IFN family is IFN-γ. The production of IFN-γ is limited to a small number of cell types, namely T-lymphocytes (especially helper and cytotoxic T cells), DCs, and NK cells.[49] IFN-γ binds the type II IFN receptor, leading to activation of the JAK-STAT pathway.[48] Differential activation by type I and II IFNs of JAK-STAT family members leads to different downstream gene transcription. Although many ISGs are regulated by both type I and II IFNs, there

exist subsets of genes distinctly regulated by each IFN class, resulting in differences in biological functions between the two.[48] Although IFN-γ displays modest antiviral activity, it has significant regulatory effects on other aspects of immunity. IFN-γ can activate macrophages and NK cells, leading to increased microbicidal activity against intracellular pathogens via enhancement of phagocytosis and oxidative metabolism.[49] IFN-γ also strengthens cell-mediated immunity by promoting T_H1 helper cell responses during infection.

IFN-γ is widely used as an anti-infective agent in patients suffering from chronic granulomatous disease (CGD). Individuals with CGD are unable to mount a proper oxidative burst response, specifically the production of superoxide anion, in their immune cells, and thus suffer from an increased frequency of microbial infection. IFN-γ treatment significantly reduces the frequency of bacterial and fungal infections in CGD patients.[54,55] However, whether the protective effects of IFN-γ are due to the correction of superoxide production or through another mechanism is still poorly understood.[56,57]

IFN-γ is also being considered as a potential antifungal. IFN-γ can enhance the antifungal capabilities of murine macrophages *in vitro*.[58,59] In murine models of systemic cryptoccosis, IFN-γ modestly reduced infection levels of *Cryptococcus neoformans*, and caused an even greater reduction when used in conjunction with antifungal agent amphotericin.[60,61] A Phase II clinical trial of AIDS patients with acute cryptococcal meningitis demonstrated that IFN-γ, in addition to the standard therapy of amphotericin and fluconazole, increased rates of clearance of *C. neoformans* and reduced the levels of fungal antigen in the cerebrospinal fluid.[62]

Colony-stimulating factors

CSFs are a group of cytokines that play an essential role in the proliferation and differentiation of leukocyte effector cells from hematopoietic precursors. There are three major CSFs: granulocyte colony-stimulating factor (G-CSF), which stimulates the differentiation of myeloid precursors into granulocytes, macrophage colony-stimulating factor (M-CSF), which stimulates differentiation into monocytes, and GM-CSF, which stimulates differentiation into both cell types. CSFs also extensively regulate the antimicrobial functions of their target cell types and are currently used as anti-infective immunomodulatory treatments.

G-CSF. G-CSF is primarily produced by endothelial cells, monocytes/macrophages, and fibroblasts in response to host stressors, including microbial infections and physical trauma.[63] G-CSF binds receptors on myeloid precursor cells in the bone marrow, resulting in cellular proliferation and differentiation into mature granulocytes.[64] G-CSF also enhances neutrophil phagocytic activity, the respiratory burst response, and the expression of surface adhesion molecules required for effective chemotaxis.[64] The ability of G-CSF to generate antimicrobial responses has led to its investigation as a potential anti-infective agent.

G-CSF is commonly used to combat microbial infections in neutropenic individuals. Neutropenia can have a variety of causes including cytotoxic chemotherapy, viral infections, immunosuppressive agents, and hereditary defects, resulting in an increased frequency of microbial infections. The ability of G-CSF to stimulate the production of granulocytes has been exploited to treat neutropenia with great success and minimal adverse effects. Treatment with filgrastim or lenograstim, both recombinant forms of human G-CSF, results in increased neutrophil counts in neutropenic individuals and a decrease in microbial infections.[65,66] In addition, filgrastim has been shown to improve neutrophil antimicrobial functions that are compromised in HIV infected individuals, including neutrophil respiratory burst, chemotaxis, and expression of cellular adhesion molecules.[67]

Efforts to develop G-CSF as treatments outside of neutropenic conditions have had mixed success. In rodent models of bacterial pneumonia, G-CSF decreased bacterial clearance and increased lung injury in *Escherichia coli* and *Klebsiella pneumoniae* infected animals but improved clearance and decreased lung injury in *Staphylococcus aureus* infected animals.[68,69] A systematic review of clinical studies investigating the efficacy of recombinant G-CSF products as an adjunctive therapy for pneumonia showed no significant change in mortality rates in G-CSF–treated individuals.[70] In contrast, numerous animal models demonstrate that G-CSF, either alone or in conjunction with an antibiotic, leads to improved survival and bacterial clearance in bacterial-induced sepsis models.[71,72]

G-CSF is also being considered as a potential antifungal therapy. In numerous animal models, G-CSF exhibits protective effect against a range of fungal diseases, including candidiasis and aspergillosis.[73,74] A Phase II clinical investigation suggests that filgrastim, in combination with fluconazole, can decrease recovery time when used to treat candidiasis in non-neutropenic individuals, but more clinical studies are required to determine the efficacy of G-CSF as an antifungal therapeutic.[75]

GM-CSF. GM-CSF is produced by a range of cell types, including endothelial cells, fibroblasts, and macrophages, in response to infection or pro-inflammatory mediators, such as IL-1 and tumor necrosis factor alpha.[76] Binding of GM-CSF to its receptor on myeloid precursor cells promotes their differentiation into granulocytes and monocytes. Like G-CSF, GM-CSF also plays a major regulatory role in the functions of immune cells. GM-CSF is thought to prime and enhance pro-inflammatory responses of stimulated macrophages.[76] Due to these abilities, GM-CSF is already used as an adjuvant in vaccine therapy. However, the ability of GM-CSF to enhance the antibacterial and antifungal activity of immune cells *in vitro*, via priming of the oxidative and phagocytic responses, has led to its investigation as a potential anti-infective immunomodulatory therapeutic.[77]

GM-CSF has been used to raise leukocyte counts in immunosuppressed individuals, including those suffering from leukopenia, while decreasing the frequency of infectious complications.[78,79] In non-neutropenic settings, GM-CSF has displayed mixed preclinical results as a potential anti-infective therapy. Animal studies investigating the effects of GM-CSF on various infection models have demonstrated that GM-CSF has little effect on *Listeria monocytogenes* clearance and may actually worsen infection by mycobacteria.[80,81] However, numerous *in vitro* and animal studies have demonstrated that GM-CSF, in combination with antifungal drugs, can boost immune protection against fungal diseases, including candidiasis, suggesting its potential as an antifungal therapeutic.[82,83]

M-CSF. M-CSF is constitutively produced by macrophages, fibroblasts, and endothelial cells, and acts on myeloid precursors to promote their differentiation into the monocyte/macrophage lineage.[76] M-CSF is also a regulator of macrophage-lineage cells, modulating survival, and proliferation responses. In contrast to GM-CSF, M-CSF does not augment pro-inflammatory responses in macrophages. Rather, it promotes M2-macrophage-like responses that include the dampening of the inflammatory response, increased phagocytic activity, and promotion of T_H2 cytokines.[76] M-CSF also potentiates macrophage functions, including chemotaxis, cytokine production, superoxide production, and antimicrobial activity.[84]

Clinical experience with M-CSF as an anti-infective has been limited. Animal studies have shown that M-CSF treatment, in combination with other antifungal drugs, can lead to improved outcomes in acute *Candida albicans* infection and chronic candidiasis.[85,86] A Phase I clinical trial in healthy individuals showed an increase in circulating monocytes counts in response to M-CSF treatment.[87]

Exogenous immunomodulators: TLR agonists

Activation of TLRs leads to the activation of NF-κB, a family of transcription factors that plays an essential role in initiating the inflammatory response. However, certain TLRs, through subtly different signal transduction pathways, can specifically enhance the activity of antiviral and cell-mediated immune responses. The fine balance of TLR activation during infection, and their downstream signaling activity, leads to an immune response that can be tailored to combat specific types of infections. As such, artificial activation and manipulation of TLRs is currently a strategy for the robust stimulation of immune responses for the purpose of anti-infective therapy.

TLR4 agonists

LPS has been shown to boost resistance to viral and bacterial infection in numerous animal models. Although the TLR4 agonist MPL is used as an adjuvant in vaccines, its efficacy as an anti-infective agent in a clinical setting is not yet known. However, numerous animal models demonstrate the potential for TLR4 agonists in combating infectious diseases. MPL has been shown to elicit prophylactic protection against a wide range of infections, including bacteria, viruses, and parasites.[88] Aminoalkyl glucosaminide phosphates, a distinct family of synthetic lipid A derivatives demonstrate protection in murine models of *L. monocytogenes*, influenza, and

Yersinia pestis infections when used as a prophylactic.[88,89] Preliminary trials indicate that these agonists are well tolerated in humans, and clinical trials are under way to determine the ability of TLR4 agonists to counter infection.

TLR7/8 agonists

Synthetic TLR7/8 agonists are used for treatment of numerous skin cancers and topical treatment of various infectious diseases. Imiquimod, a TLR7 agonist, is widely used for the treatment of genital and perianal warts caused by HPV.[90] In addition, imiquimod shows efficacy against other skin diseases caused by viruses, including molluscum contagiosum.[90] Similarly, topical administration of resiquimod, a TLR7/8 agonist, shows efficacy in reducing viral reactivation and shedding in individuals with genital HSV-2 infection.[91] The efficacy of these synthetic agonists against systemic viral infections is still poorly understood and clinical trials have yielded mixed results. The major limitation of using resiquimod or imiquimod systemically is their relatively poor safety profiles. A clinical study investigating the effects of orally administered imiquimod on asymptomatic HIV-infected patients showed variable effects on viral load.[92] However, patients exhibited adverse flu-like symptoms, similar to those observed during IFN therapy, including fatigue, fever, vomiting, and hypotension. A Phase II clinical trial investigating the efficacy of orally administered resiquimod in HCV-infected patients showed similar effects. However, a study demonstrated that intravenously administered isatoribine, a TLR7 agonist, is effective in reducing viral load in chronic hepatitis C patients with mild side effects.[93]

TLR9 agonists

TLR9 activation leads to the initiation of cell-mediated immunity against intracellular pathogens, including the induction of type I IFNs and activation of NK cells.[94] As such, TLR9 agonists are being considered as potential anti-infective therapeutics against a range of intracellular pathogens. Numerous animal models demonstrate the safety and efficacy of CpG ODNs in combating a large range of intracellular bacterial and viral species (reviewed in Ref. 94). A Phase I clinical trial demonstrated the ability of CpG ODN monotherapy to reduce viral RNA levels in HCV-infected patients.[95] However, the Phase III trial for the same indication failed to demonstrate efficacy. CpG ODN, in conjunction with PEG-IFN and ribavirin, significantly decreased viral RNA load and increased the frequency of viral negativity in HCV-infected patients compared to PEG-IFN and ribavirin treatment alone.[94] CpG ODNs may also have potential as antiparasitic agents. Studies have demonstrated that CpG ODN treatment, prophylactically or therapeutically, increased host resistance to *Leishmania* infection in healthy and simian immunodeficiency virus–infected macaques.[96,97] These promising results underscore the potential of TLR9 agonists as antiviral and antiparasitic agents.

HDPs as immunomodulators

Some HDPs, such as human cathelicidin LL-37, can selectively modulate immune responses. HDPs play an essential role in the activation and progression of the immune response. They can promote chemotaxis and induce the production of chemokines, leading to overall enhancement of leukocyte recruitment to the site of infection.[43] HDPs also regulate the production of a range of cytokines, leading to profound downstream effects on immunity, including the regulation of leukocyte activation, DC differentiation, and T_H cell polarization.[43] HDPs also help limit the harmful aspects of immune responses. Many HDPs have been shown to suppress the production of inflammatory mediators induced by microbes or microbial components.[43] Indeed, certain HDPs have been shown to decrease mortality in animal models of microbially induced sepsis. HDPs also play a role in the induction of many wound healing responses, including the promotion of angiogenesis, and the recruitment and proliferation of epithelial cells.[43] These unique regulatory properties of HDPs have made them promising candidates as anti-infective therapeutics, which are potentially safer and more effective than classic immunomodulators.

Currently, numerous HDPs are in various stages of clinical development as direct antimicrobial agents or regulators of inflammatory or immune disorders. The use of immunomodulatory HDPs in clinical anti-infective applications is a more recent field of study. Key studies involving IDRs, HDP derivatives with immunomodulatory properties and minimal antimicrobial activity, have demonstrated that immunomodulation by HDPs represents a viable anti-infective strategy. In one of the first proof-of-principle studies, IDR-1 was

shown to protect mice against infection bacterial pathogens, including methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus*.[98] This protection correlated with the induction of leukocyte chemoattractants, increased recruitment of immune effector cells to the site of infection, as well as the suppression of harmful inflammatory responses with no discernable cytotoxicity as is sometimes seen with natural HDPs. More recently, IDR-1002, a derivative of bovine bactenecin, was shown to exhibit improved protection in mice challenged with *S. aureus* or *E. coli*.[99] Again this protection correlated with the immunomodulatory activities of IDR-1002, including the induction of chemokines and enhancement of leukocyte recruitment. To date, a number of immunomodulatory HDP-derivatives are in the early stages of clinical development. This includes the immunomodulatory peptide IMX942, an IDR-1 derivative developed by Inimex Pharmaceuticals (Burnaby, Canada), which is currently being developed as a potential clinical anti-infective against surgical site infections, chemotherapy-induced neutropenia, and hospital-treated pneumonia. In addition, the human lactoferricin fragment hLF1–11 possesses a range of immunomodulatory activity, including the regulation of macrophage differentiation and potentiation of macrophage antimicrobial activity.[100] Its safety has been established in Phase I clinical trials as a potential anti-infective therapeutic in immunocompromised patients (AM-Pharma, Bunnik, the Netherlands). Although clinical experience with regulatory peptides is still in its early stages, immunomodulatory HDPs represent a reservoir of potential anti-infective agents.

Conclusion

The idea of modulating host responses for clinical benefit is not new. However, it is a concept that is gaining prominence over time, in credit to its successes in many applications listed above. Like any other clinical drug treatment, immunomodulation also comes with its set of dangers and limitations. Nevertheless, as an increasing number of immunomodulators are discovered and their mechanisms of actions elucidated, we steadily gain an understanding of the complex network that makes up the host immune system. This understanding, coupled with the many clinical successes of existing immunomodulators, will lead to the discovery of future immunomodulatory agents that are more effective, safer, and more cost-effective, opening the doors for the development of superior vaccines and anti-infective therapeutics.

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Conflicts of interest

The authors declare no conflicts of interest.

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