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Innate immune mechanisms and the identification of immune potentiators as vaccine adjuvants

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Introduction

Vaccines are considered to be one of the most significant medical interventions against infectious diseases (Hilleman, 2000). Despite this success, major obstacles remain in developing vaccines for pathogens against which vaccines do not exist (such as hepatitis C virus, HCV) or against emerging pathogens (such as severe acute respiratory syndrome, SARS) and improving suboptimal vaccines (such as bacille Calmette-Guerin, BCG) (Kieny et al., 2004). Key elements needed to design effective vaccines include (i) identification of protective antigen(s) against which a robust and durable adaptive response must be generated, (ii) compounds that can stimulate the innate immune responses (Hoebe et al., 2004), e.g., bacterial cell wall components, and (iii) optimal delivery systems, which will carry and dispense the antigenic and immunostimulatory cargo to the appropriate cells of the immune system (O'Hagan, 2004). A key gap in our understanding, but an area of significant future gains, centers on issues relating to recent developments in the field of innate immunity, the mechanistic underpinnings of adjuvant activity, and the use of novel immune potentiators in vaccine formulations.

Innate immunity: a trigger point for the immune system

Upon interaction with pathogens, the innate immune system provides a first line of defense and reacts within minutes to minimize the immediate threat posed by the pathogen. In addition, the early activation of innate immune components serves to create an inflammatory environment that conditions the host for effective generation of adaptive immune responses (Iwasaki and Medzhitov, 2004). This rapid activation of innate immunity is achieved through the recognition of relatively conserved molecules found associated with pathogens. In essence, the innate immune system has evolved to detect broad and conserved structures associated with infectious agents. Conversely, the adaptive immune system, which is comprised of antigen-specific T and B cells, is more focused on the antigenic epitopes found in the proteins and carbohydrates

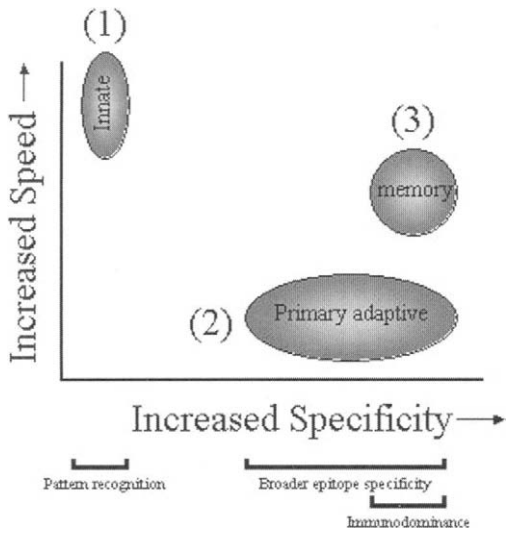

Figure 4.1 Speed and specificity of the immune response. (1) Cells of the innate immune system, such as macrophages, dendritic cells, NK cells, and neutrophils, rapidly respond to microbial patterns through their pattern recognition receptors and are the first line of defense against invading pathogens. Their early activation also provides the inflammatory context for antigen recognition by cells of the adaptive immune response. Despite their ability to respond quickly, the effectiveness of the innate response is limited due to the lack of fine specificity. (2) Although slower, adaptive immune responses are precise because of the incredible diversity of antigen receptors on the surface of T and B cells. (3) Memory T and B cells are activated much more rapidly compared to the initiation of primary adaptive cell responses and their antigen specificity is more focused. Thus memory responses, the goal of vaccination, provide both speed and specificity in the recognition and elimination of infectious agents.

derived from pathogens. This focused response is slower due to the time needed to expand B and T cell clones bearing antigen receptors with the appropriate specificity. The ultimate goal of natural host defense and prophylactic vaccination is to generate adaptive memory responses that are both rapid and highly focused (Figure 4.1). Targeting of both the innate and adaptive arms of the immune systems is critical in achieving this goal.

Pathogen-associated molecular patterns and pattern recognition receptors

In 1989 Charles Janeway postulated that the immune system may be equipped to trigger an innate immune response using pattern recognition receptors (PRRs) that can be engaged by pathogen-associated molecular patterns (PAMPs; Janeway, 1989). At the time it was known that lipopolysaccharide (LPS), lipotechoic acid, double-stranded (ds) RNA, peptidoglycans, and other pathogen components could nonspecifically stimulate immune cells and that some of these components functioned as vaccine adjuvants when added to immunization regimens along with specific antigen. Thus, the addition of such mircobial components to experimental immunizations leads to the development of robust and durable adaptive immune response. The mechanism behind this potentiation of immune responses was not very well understood until recently, when some of the PRRs involved in the innate immune response to PAMPs were identified (Table 4.1). Engagement of these PRRs may lead to the activation of one or more immune cells, such as macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells, and B cells. Some of the PRRs are also expressed on nonimmune cells, such as epithelial and endothelial cells, and activate these cells during infection and possibly vaccination. PRR-mediated activation of cells leads to secretion of inflammatory cytokines and chemokines. In addition, they may lead to maturation and migration of key antigen-presenting cells (APCs), such as DCs (Figure 4.2).

While postulating PAMPs, Janeway perhaps envisioned that the high-order multimeric patterns (lattices) with precise spatial arrangements of these immunostimulatory compounds gave rise to pathogen recognition and subsequent innate immune activation. However, experimental evidence suggests that PRR specificities are not primarily driven by the multimeric patterns of well-known PAMPs, as specific receptor–ligand engagements appear to be governing recognition of pathogenic signatures or patterns (Beutler, 2003). This is important to note in the context of the discovery of novel synthetic compounds that could stimulate PRRs. Nevertheless, as has been recently shown with other receptor–ligand

Table 4.1 Pattern recognition receptors (Akira and Takeda, 2004)

Receptor	Ligand
TLR-1[a]	Bacterial lipoproteins from mycobacterium, neisseria
TLR-2[a]	Zymosan yeast particles, peptidoglycan, lipoproteins, glycolipids, lipopolysaccharides
TLR-3	Viral double-stranded RNA, poly (IC)
TLR-4	Bacterial lipopolysaccharides, plant product taxol
TLR-5	Bacterial flagellins
TLR-6[a]	Yeast zymosan particles, lipotechoic acid, lipopeptides from mycoplasma
TLR-7	ssRNA; immiquimod and R848; other synthetic nucleoside analog compounds such as loxoribine and bropirimine
TLR-8[b]	Single-stranded RNA, R848, immiquimod
TLR-9	CpG oligonucleotides[c]
TLR-10	Unknown
TLR11	Bacterial components from uropathogenic bacteria
Nodl, 2	Peptidoglycan
Scavenger receptors	Acetylated/malelylated proteins; modified low-density lipoproteins and other polyanionic ligands
Macrophage mannose receptors and other c-type lectin receptors	Sulfated sugars, mannose, fucose, and galactose modified polysaccharides and proteins
Type 3 complement receptors and dectin type receptors	Zymosan particles, β-glucan

[a]TLR-1 and 6 can form heterodimers with TLR-2 that further changes their specificities.
[b]TLR-8 is found to be active only in humans.
[c]Different CpG oligonucleotide sequences are optimal for murine and human TLR-9.

pairs such as T cell receptor–major histocompatibility complex (TCR-MHC) interactions, the formation of higher order structures of ligand–receptor pairs may be necessary to transmit potent downstream signals.

Janeway's postulate about PRRs was confirmed years later, when the drosophilla transmembrane receptor protein, Toll, was shown to regulate antifungal responses (Lemaitre et al., 1996). Soon after this discovery, Medzhitov cloned the first mammalian Toll-like receptor (TLR) that was essential for LPS recognition (Medzhitov et al., 1997). Based on sequence homology, many other mammalian TLRs were subsequently cloned and shown to respond to a multitude of pathogenic ligands (Table 4.1). PRRs can be broadly divided into two classes, one that leads to phagocytosis, such as scavenger receptors, mannose receptors, and β-glucan receptors (Gordon, 2002), and others that are nonphagocytic, such as TLRs and nucleotide binding oligomerizing domain proteins (Nods). However, TLR-induced phagocytosis, which may be due to indirect effects of inflammatory response, has been reported (Doyle et al., 2004). As the name suggests, phagocytic receptors upon engagement with their ligands lead to the engulfment into phagocytic cells, such as macrophages. By contrast, nonphagocytic receptors upon engagement with their respective ligands lead to elaborate signal transduction cascades and distinct cellular activation events.

Some of the PRRs have been shown to bind their ligands directly (Sato et al., 2003; Lien et al., 2000; Rutz et al., 2004), but it remains to be seen whether this is a general rule or whether intermediate protein components are involved. One of the key questions in the biology of PRRs is how to justify a large number of often structurally different ligands being recognized by a limited number of receptors. One way to explain the complexity of ligand recognition is to implicate intermediate proteins which may have conserved sites for PRR binding and other distinct sites that bind different PAMPs. For example, drosophilla Toll has been shown to recognize microbial ligands only through its interaction with Spätzle, a host protein that directly interacts with microbial components (Lemaitre et al., 1996). Similarly, mammalian TLR-4 recognizes LPS indirectly through LPS binding protein (LBP) and the surface receptor CD14

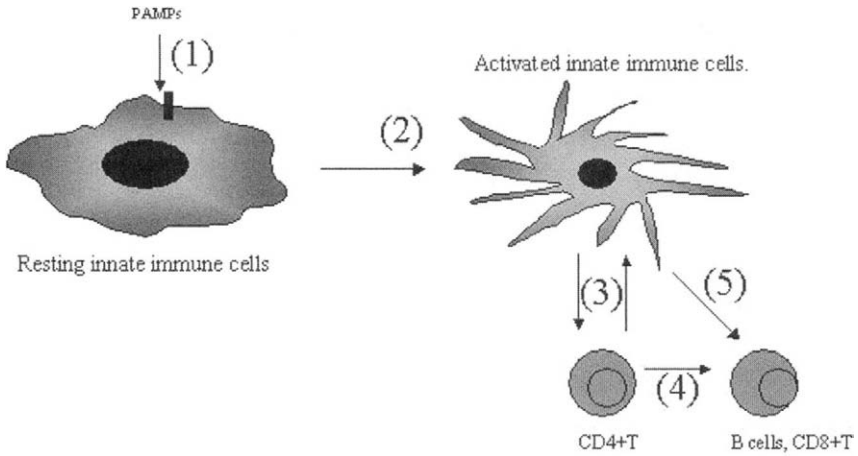

Figure 4.2 Cellular response to PRR-mediated trigger. Upon stimulation through Toll-like receptors (TLRs), tissue resident dendritic cells (I), which bridge innate and adaptive immunity, undergo maturation (2) that leads to their increased expression of costimulatory molecules, increased surface expression of MHC-class II molecules, and their migration to secondary lymphoid tissue, such as spleen and lymph nodes. Priming of antigen-specific naive CD4 T cells (3) takes place in secondary lymphoid organs. Activated CD4+ cells undergo clonal expansion and provide help to antigen-specific B cells to undergo antibody class switching as well as to CD8 T cells (4). In addition, activated dendritic cells may license CD8+ T cells to become cytotoxic. PRR-mediated activation of B cells may contribute to long-term serological memory (Bernasconi et al., 2002).

(Schroder et al., 2000). Moreover, it was shown that LBP also binds to structurally different peptidoglycan moieties from pneumococcal cell wall (PCW) that mainly signal through TLR-2 (Weber et al., 2003). These findings suggests that the addition of a second tier for PAMP recognition by intermediate proteins such as LBP creates greater diversity for ligand recognition by a limited set of receptors like the TLR family.

Innate immunity conditions the host for subsequent adaptive immunity

Goals of innate immune activation are two-fold: to limit the immediate threat by direct action of effector cells such as neutrophils, NK cells, macrophages, etc., and to establish a microenvironment that will be conducive to specific adaptive immunity. Signaling through TLRs as well as Nod proteins results mainly in the activation of transcription factors such as NFkB and IRF3 that provide the inflammatory context for the rapid activation of host defenses (Akira and Takeda, 2004). The NFkB pathway controls the expression of proinflammatory cytokines such as IL-1β and TNF-α, whereas the IRF3 pathway leads to the production of antiviral type I interferons (IFN-α and IFN-β). Along with these cytokines, various chemokines, such as IL-8, monocyte chemoattractant proteins (MCPs), macrophage inflammatory proteins (MIPs), and RANTES are released and their receptors expressed on the surface of activated cells. As a result, vascular endothelial cells may alter surface expression of selectins and intercellular cell adhesion molecules (ICAMs) leading to the extravasation and selective retention of leukocytes at the inflamed site. This cellular infiltrate consists of activated monocytes, neutrophils, basophils, eosinophils, and NK cells, many of which also express TLRs and subsequently become activated by the presence of their respective ligands and secrete a variety of necessary cytokines and chemokines. As a consequence of this inflammatory microenvironment, monocytes that infiltrate the site can differentiate into macrophages and DCs; the latter being crucial in priming of naive CD4+ helper as

well as inducing CD8+ T cell differentiation into killer cells. DC subsets, which express distinct arrays of TLRs, are found at strategic anatomical sites allowing them to respond rapidly to microbial invasion (Kadowaki et al., 2001). Upon capture of antigen and PAMP-induced maturation, DCs migrate to local lymphoid tissues where antigenic peptides are presented to T cells via MHC class I and class II molecules, thus starting the clonal expansion of antigen-specific T cells (Figure 4.2). Besides a direct effect on priming of specific T cells, the innate immune response may have indirect effects, as has been suggested that DCs activated through a TLR may gain an ability to inhibit the suppressive ability of CD4+CD25+ regulatory T cells (Treg) (Pasare and Medzhitov, 2003). Also, it has been suggested that engagement of B cells by some TLRs may be a crucial point in isotype switching as well as antibody secretion capability (Bernasconi et al., 2002; Liu et al., 2003). Hence, innate immune activation is crucial to the robustness of adaptive immunity. Indeed, it has long been the "immunologist's dirty little secret," before the establishment of the PAMP-PRR recognition concept, that the addition of nonspecific microbial components to experimental purified proteins during immunization elicits much stronger specific response (Anderson, 1955). Recent growing knowledge of various components of the mammalian innate immune system can make the process of selection of immune potentiating compounds more informed, thus reducing probable associated toxicity while increasing their usefulness in vaccine formulations.

Adjuvants in vaccine research

Adjuvants historically are defined functionally as vaccine components that enhance the immunogenicity of antigen *in vivo*. Adjuvants can be functionally differentiated as immunopotentiators and delivery vehicles (O'Hagan, 2004). Immune potentiators directly activate the innate immune system (e.g., induction of inflammatory cytokines) whereas delivery systems concentrate and display antigens in repetitive patterns (e.g., cationic microparticles) (O'Hagan et al., 2004), target antigens to APCs, or help co-localize antigens and immune potentiators. Ideal immune potentiators can improve the effectiveness of vaccines by (i) accelerating the generation of robust immune responses, (ii) sustaining responses for a longer duration, (iii) generating antibodies with increased avidity and neutralization capacity, (iv) eliciting cytotoxic T lymphocytes (CTLs), (v) enhancing immune responses in individuals with weakened immune systems (e.g., children and elderly or immunocompromised adults), (vi) increasing a responder to non-responder ratio for a given vaccine, and (vii) reducing the amount of antigen needed, thus reducing the cost of vaccination programs.

Early vaccine research mainly emphasized better humoral responses; as a result currently licensed adjuvants such as aluminum salts are geared towards enhancing antibody responses without significant elicitation of Th1 or CTL responses (Lindblad, 2004). However, the need for effective vaccines that may prevent chronic viral diseases, such as HIV and HCV, has altered a paradigm of vaccination towards the generation of cellular response in addition to neutralizing antibody responses. Moreover, a more detailed and sophisticated understanding of the biology of individual infectious diseases as well as unique characteristics of protective immune responses against them suggests that a few-adjuvants-many-vaccines model will not be an optimal solution. Thus, a battery of specifically tailored adjuvant candidates for next-generation vaccines that can be used to elicit superior (quantitative and qualitative) memory responses is needed. There has been an explosion of information on innate immunity generated in academic research laboratories in the last decade that is slowly being used to generate a new generation of immunopotentiators to tackle conventional as well as emerging infectious diseases. To this end, many new and existing adjuvant formulations are being tested in various clinical and preclinical trials (Table 4.2).

Table 4.2 Adjuvants and delivery systems in preclinical and clinical trials (Aguado et al., 1999; Engers et al., 2003; Kenney et al., 2002; Pink and Kieny, 2004)

Adjuvant category	Representative examples
Mineral salts	Aluminum and calcium salts[a]
Emulsions and surfactant-based formulations	MF59[a], AS02, Montanide ISA-5i and ISA-720, QS21
Particulate delivery vehicles	Microparticles, immunostimulatory complexes; liposomes, virosomes, virus-like particles
Microbial derivatives	Monophosphoryl lipid A, CpG oligonucleotides, cholera toxin and heat labile toxin from *E. coli*, lipoproteins
Cells and cytokines	Dendritic cells; IL12 and GM-CSF

[a] Currently licensed for human use as adjuvants.

Structure and function of TLRs and Nods

TLRs are the largest and best-studied family of PRRs. They are type I integral membrane glycoproteins with considerable cytoplasmic domain homology to IL-1 receptors. Structurally, TLRs consist of an extracellular domain that contains 19–25 leucine-rich repeat (LRR) motifs. LRR domains have been suggested to form a horseshoe-like concave structure that is thought to interact with their ligands either directly or indirectly through intermediate proteins. Intracellularly a conserved, ~200 amino acid cytoplasmic domain, known as TIR (Toll/IL1 receptor domain), is important for its association with adaptor proteins, thus linking these transmembrane receptors to their downstream signaling pathways (Takeda et al., 2003). Nod proteins consists of three main regions: a LRR domain, which may work similar to LRR domain of TLRs; a nuclear oligomerization domain that is essential for Nod clustering and subsequent signaling; and effector motifs such as the caspase recruitment domain that leads to actual signal transduction through binding to signaling kinases (Chamaillard et al., 2003a). In the absence of a canonical oligomerization domain, it is not known whether oligomerization of TLRs takes place and whether it is required for signaling. As transmembrane proteins, it is possible that TLR membrane aggregation may occur through other transient structures such as lipid rafts or caveoli.

Ligands of TLRs and Nods

The TLR family is the single most abundant family of proteins identified to date that recognizes molecular signatures, with TLR-1-11 discovered mostly based on homology searches. Ligands to these receptors were either identified with over expression of these receptors in cells lacking them (such as human embryonic kidney (HEK) cells) or by using mouse strains that do not express particular TLRs because of gene targeting or by natural mutations in TLR genes. TLR-1 and TLR-6 share considerable homology and are shown to have qualitatively different functional cooperation with TLR-2 (Takeuchi et al., 1999). For example, in a HeLa cell system coexpression of TLR-1 and TLR-2 resulted in increased reactivity to *Neisseria meningitides* components (Wyllie et al., 2000), whereas TLR-2 and TLR-6 cooperatively recognize peptidoglycan from *Staphylococcus aureus* (Ozinsky et al., 2000). TLR-6-deficient macrophages respond normally to tri-palmitoyl lipopeptides from other bacteria through TLR-2, but do not recognize di-palmitoyl-derived lipopeptides from mycoplasmal origin. The recognition of the latter is only restored when TLR-2 and TLR-6 are coexpressed. This functional cooperation may be due to a physical interaction between individual TLR molecules (Tapping and Tobias, 2003). Interaction of TLR heterodimers, such as the one described above, or their ability to collaborate with other innate immune receptors may also be helpful in generating qualitative differences in microbial component recognition as well as inflammatory outcomes (Mukhopadhyay et al., 2004). This observation could be useful in devising assays to screen for novel TLR agonists.

A specific blocking monoclonal antibody against TLR-3, as well as TLR-3 transfectants of HEK cells, demonstrated that the recognition of poly-I:C sequence and other dsRNA sequences were TLR-3 dependent (Matsumoto et al., 2002). In addition, TLR-3 gene targeted mice have defective responses to viral RNA, indicating that TLR-3 is involved in viral RNA recognition (Alexopoulou et al., 2001). Immunostaining analysis of monocyte-derived DCs as well as transfected B cell lines showed intracellular localization of TLR-3 and the responses were not blockable with anti-TLR-3 monoclonal antibody. Thus, the expression and localization of TLR-3 may be modulated either by TLR-3 or the maturation state type of expressing cells (Matsumoto et al., 2003).

LPS is a major membrane component of Gram-negative bacteria. TLR-4, which is now shown to be the crucial sensor of LPS, was the first mammalian TLR to be cloned based on homology to drosophilla Toll (Medzhitov et al., 1997). Historically, mouse strains such as C3H/HeJ have been known to be LPS hyporesponsive; subsequent to the cloning of TLR-4, a mutation in TLR-4 cytoplasmic region was found in C3H/HeJ mice that explained defective TLR-4-mediated signaling and LPS hyporesponsiveness (Poltorak et al., 1998; Qureshi et al., 1999). Although TLR-4 has been established as an essential receptor for LPS responsiveness, other molecules, such as CD14, LBP, and MD-2, have been identified in aiding LPS recognition (Haziot et al., 2001; Shimazu et al., 1999; Akashi et al., 2003). Moreover, TLR-4 has also been implicated as a sensor for a variety of other structurally divergent pathogen products, suggesting that LPS recognition is one of many functions for this receptor (O'Hagan and Valiante, 2003).

CHO cells expressing TLR-5 were found to respond to culture supernatants from Gram-positive and Gram-negative bacteria. Purification of culture supernatant led to the discovery that bacterial flagellin is a TLR-5 stimulatory component (Hayashi et al., 2001). Since then flagellin proteins from a variety of bacteria have been shown to be TLR-5 agonists.

The life cycles of infectious agents in their hosts can be largely extracellular (e.g., many bacteria), or intracellular where rapid entry into cells is required for survival and continued proliferation (e.g., most viruses and some bacteria like mycobacterium, leishmania, and salmonella). Thus, optimal recognition of some ligands, such as viral RNA and bacterial DNA, requires intracellular receptors, whereas bacterial cell wall components, such as LPS or lipoproteins, are best detected extracellularly. Some other ligands, such as peptidoglycans, appear to be recognized extracellularly as well as intracellularly. TLR-1, 2, 4, 5, 6, and 11, whose ligands are most probably available extracellularly are surface expressed, while TLR-3, 7, 8, and 9 and Nod family proteins are localized in subcellular organelles or in the cytoplasm and are therefore focused on intracellular pathogen ligands. This latter group of sensors also may require acidic compartments for optimal recognition of their natural ligands (Lee et al., 2003). Based on sequence homology and similarity of ligands (purine derivatives), it is suggested that TLR-7, 8, and 9 belong to a common subfamily of TLRs (Du et al., 2000; Chuang and Ulevitch, 2000). The natural ligand for TLR-9 is bacterial DNA. Moreover, species specificity has been observed in optimal ligand recognition; thus human TLR-9 does not optimally recognize CpG oligonucleotide sequences that are best recognized by murine TLR-9 and vice versa (Bauer et al., 2001). These observations on cellular localization of some TLRs and other distinctive features of PRRs should be considered during the experimental screening for novel immune potentiators. TLR-7 gene targeted mice have revealed that it may recognize G+U-rich single-stranded RNA (Lund et al., 2004). TLR-8, which also recognizes G+U-rich single-stranded RNA, is functional in humans but inactive in mice, probably due to amino acid deletions in the extracellular domain of the murine isoform (Jurk et al., 2002). Loxoribine, a low molecular weight guanosine analog that functions as an immune potentiator, is recognized by TLR-7, but not human TLR-8

(Heil et al., 2003). Contrary to this discrimination in ligand recognition, R-848 (resiquimod) stimulates human TLR-7 as well as TLR-8 (Hemmi et al., 2002; Jurk et al., 2002).

Other intracellular regulators of pathogen-induced inflammation include Nod family proteins. Nod1 has been shown to sense peptidoglycan motifs from Gram-negative bacteria (Girardin et al., 2003a; Chamaillard et al., 2003b). It was suggested that Nod1, which is found in the epithelial cells that line the intestinal tract, is thus a sensor of pathogenic Gram-negative bacteria in the gut. Nod2, which has considerable homology to Nod1, specifically recognizes muramyl dipeptide (Girardin et al., 2003b). Nod2 is expressed in cells of the myeloid lineage as well as in muscosal epithelial cells (Gutierrez et al., 2002). Thus, PRRs are expressed extracellularly as well as intracellularly depending on their ligands of choice. After their sensing of ligands, either directly or indirectly through intermediate proteins, specific signal transduction pathways are initiated that lead to inflammatory outcomes.

Signaling through PRRs

All TLRs, except TLR-3, share a common signaling pathway that utilizes an adapter protein called MyD88 (myeloid differentiation factor 88). In some cases (e.g., TLR-5, 7, and 9), MyD88 has been shown to bind directly via the TIR domain, whereas in others (e.g., TLR-2 and 4) another intermediate adaptor molecule known as TIRAP (TIR domain-containing adaptor protein) is involved. Through its N-terminal death domain MyD88 binds to another family of signal transducers, known as IRAKs (IL-1 receptor-associated kinase), which in turn bind to TRAF6. Subsequent activation of the transcription factors NFkB and AP-1 by TRAF6 involves yet other adaptor proteins known as TAK1 and TAB2 (Figure 4.3). One of the important questions in TLR biology is how the specificity in signal transduction is achieved given the similarity of the cytoplasmic domains. The multitude of adapter proteins involved in TLR signaling suggests that qualitative differences in signal transduction outcomes can be achieved by

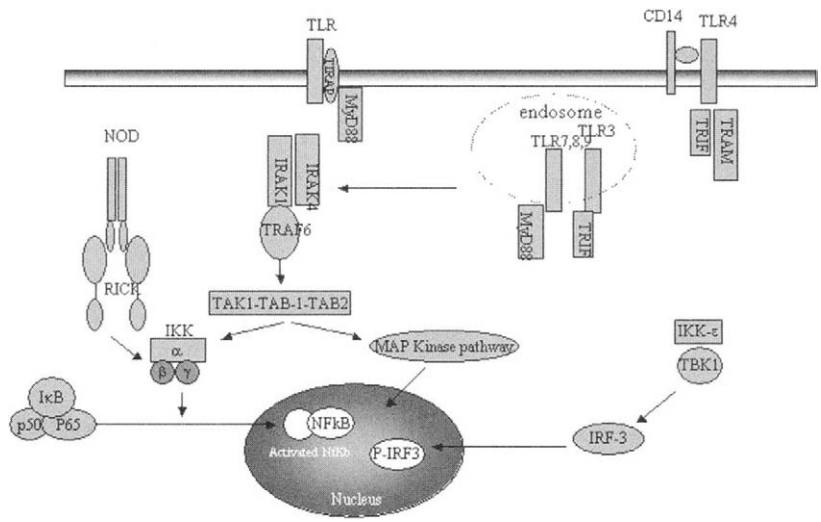

Figure 4.3 Signaling through PRRs. Three kinds of PRRs are shown: cytoplasmic, such as Nod proteins; endosomal, such as TLR-7, 8, and 9; and cell surface receptors, such as TLR-4. Upon interaction with their respective ligands, a signaling cascade is initiated which ultimately leads to the expression of inflammatory genes through activation of transcription factors NFkB or IRF3. A simplified pathway is shown here. Receptors interacting with multiple adapter proteins possibly provide the complexity of outcome. Regulator proteins of PRR signaling pathways provide a rich source of targets for drug discovery.

utilizing various combinations of receptor and adapter proteins (comprehensively reviewed in Akira et al., 2003).

In contrast to TLR signaling pathways, Nod proteins do not signal through a MyD88-dependent pathway. After association with their ligands, Nod1 and 2 activate the NFkB pathway through their CARD domain and the formation of signaling complexes with the receptor-interacting serine/threonine kinase (RICK). Subsequent activation of NFkB and MAPK pathways leads to expression of inflammatory cytokines and chemokines (reviewed in Chamaillard et al., 2003a). Signaling through PRRs thus involves a complex and rapidly growing set of transmembrane and intracellular adaptors and kinases that offer a wide range of potential targets for vaccine adjuvants.

Small-molecule immune potentiators: the future of adjuvants?

Given the plethora of natural products capable of activating innate immune mechanisms, research and development efforts to exploit these immune potentiators as vaccine adjuvants and therapeutics have been aggressive. Although *in vivo* proof of concept has been established for the use of many natural PAMPs as adjuvants and therapeutics and a number of these have been advanced into clinical trials, the trend for the future suggests an increased reliance on synthetic analogs. This is due mainly to the lower manufacturing and regulatory hurdles associated with synthetic immune potentiators, as these are highly defined and standardized. In addition, a synthetic platform allows for a more rational approach to the optimization of next-generation compounds possessing greater potency and decreased toxicity. From this perspective, perhaps the most promising immune potentiator platform recently identified is based on a small-molecule approach. The identification of imidizoquinolines as TLR-7- and/or TLR-8-dependent small-molecule immune potentiators (SMIPs) indicates that more traditional pharmaceutical-based or drug-like molecules can be exploited as vaccine adjuvants and immune modulators (Hemmi et al., 2002; Jurk et al., 2002). Indeed, imidizoquinolines have been shown to enhance antigen-specific responses in mouse models and, therefore, have the potential to be developed as adjuvants for humans (Vasilakos et al., 2000). It is important to note that the first-generation imidizoquinoline imiquimod is licensed as an antiviral as well as an anticancer topical therapy (Aldara®) and not a vaccine adjuvant. This indicates that SMIPs offer a flexible platform for use as both vaccine adjuvants and immune therapies.

A SMIP-based platform holds significant potential for the design and development of improved vaccine adjuvants. There are numerous advantages that can be realized throughout the discovery and development pipeline if SMIPs, rather than other natural or synthetic immune potentiators, are chosen as the platform of the future. For discovery efforts the incredible diversity of scaffolds generated through combinatorial chemistry, the ability to target innate immune mechanisms with exceptional selectivity, and the tried and tested drug discovery engines of high-throughput screening (HTS) and hit to lead (HTL) optimization can now be applied to immune potentiators. Moreover, the small-molecule platform opens up new avenues for manipulating the innate immune response by providing new intracellular targets and signaling pathways (Figure 4.3). Later in development and manufacturing a SMIP-based adjuvant provides a low-cost, highly pure, and standardized alternative to all other existing candidate immune potentiators. Given these advantages and the likelihood that more and diverse SMIP families will be discovered, it appears that the TLR-7/8 agonists, imidizaquinolines, represent only the first in a long line of future small-molecule-based adjuvants and therapeutics.

Drug discovery strategies for immune potentiators

Traditionally, efforts have been devoted in vaccine research towards identifying protective antigens rather than optimizing adjuvants able to change qualitatively the immune response. This probably was due to temporally different peaks of research activity in adaptive and innate immunity, with the latter peaking only recently. As a result, only two delivery systems (aluminum salts (Lindblad, 2004) and oil-in-water emulsions, MF59 (Podda, 2004)) and no direct immune potentiators are approved for widespread use in humans as adjuvants for prophylactic vaccines. This lack of progress in approval of immune potentiators as adjuvants is due to perceived toxicity risks and the lack of a comprehensive approach toward adjuvant discovery. Thus, to increase our portfolio of immune potentiator compounds to be used with new subunit vaccines and tailored to the specific requirements of each particular vaccine, a systematic approach towards novel adjuvant discovery is needed. Because of relatively well-understood outcomes of engaging various PRRs, high-throughput assays can be developed to screen large numbers of chemically defined compounds able to trigger them. HTS has been very successfully established in drug discovery research to search for inhibitors of various intracellular proteins and enzymes as well as to search for ligands of orphan receptors. In these drug discovery programs, often diverse chemical libraries were available to screen and the *in vitro* inhibition of a target was predictive of a compound's *in vivo* activity. If these two criteria are satisfied, HTS can become a tool of choice to screen for novel immunopotentiators. The identification of small drug-like chemicals of imidazoquinoline family as TLR-7 and 8 ligands suggests that HTS of large and diverse libraries of small molecules can be applied to novel adjuvant discovery. A general strategy of such a screening is outlined in Figure 4.4. Assays to identify

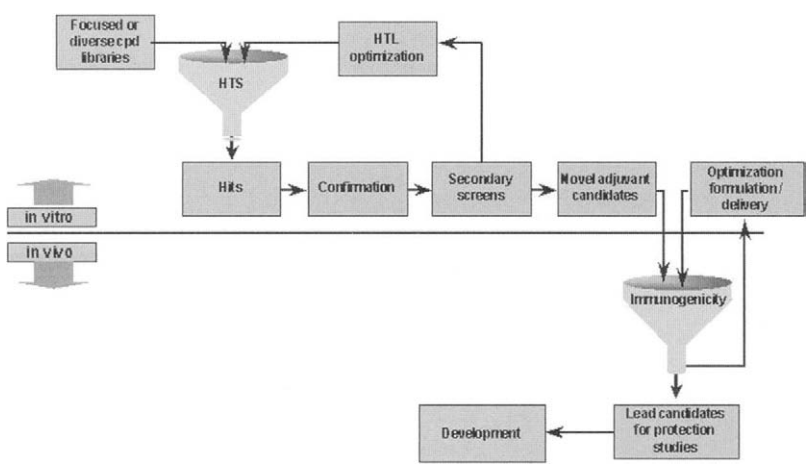

Figure 4.4 Drug discovery approach to immune potentiator discovery and optimization. Libraries of diverse chemical compounds (natural or synthetic) can be mined for immune potentiators using high-throughput screening (HTS). Cell-based assays can be designed to measure general activation of innate immune cells or screens can be targeted towards specific receptors or signaling components of the innate immune system such as Toll-like receptors (TLRs). Active compounds (hits) identified in a primary screen are then confirmed and prioritized based on level of potency and selectivity. In hit-to-lead (HTL) optimization, chemical analogs of the initial hits are generated and tested in an iterative fashion to identify compounds with increased potency and solubility. Secondary screens can be performed to evaluate mechanism of action, toxicity, and other parameters. The HTL-optimized immune potentiators (lead candidates) can then be formulated and tested for increased compatibility with different delivery systems. *In vivo* immunogenicity and toxicity studies using established vaccine candidates are conducted to identify lead immune potentiators, which then are put in development as novel vaccine adjuvants.

compounds can be open ended with functional outcome, such as cytokine secretion, without any bias towards a target as a scoring system. In contrast, well-established assays, such as TLR engagement-driven NFkB activation and cytokine secretion, can be used specifically to identify compounds targeting a particular pathway. Regardless of which approach is used, novel compounds and their analogs identified in HTS will need to be optimized for further development. Again drug discovery engines can be applied to the search for new immune potentiators. HTL optimization is an iterative process of structural modification and creating analogs followed by screening for improved activity to arrive at a lead/optimized compound. Lead compounds, thus identified, may then be tested for their efficacy in experimental vaccine formulations using established animal models and benchmarking against known immune potentiators. The quantity and quality of immune responses can be judged using functional antibodies, CD4+ and CD8+ T cells and protection against an infectious challenge.

Random screening based on functional outcome

The observation that most known immune potentiators are capable of inducing the secretion of inflammatory cytokines *in vitro* and that these compounds also act as effective adjuvants in vaccine formulation can be used as a basis of screening compound libraries for families of molecules satisfying these criteria. The methodology for such a screen is straightforward; it relies on established immunological assays, such as ELISA and multiplex cytokine analysis, to measure cytokines and chemokines produced by immune cells such as primary cells or cell lines. Random screening also eliminates any prior assumptions about the mode of action, and thus it can yield compounds for which no known receptors or targets have yet been identified. Because of this unbiased screening approach, once a compound is optimized, a thorough investigation will need to be carried out to ascertain its target and mode of action. This can be done using available genomics and proteomics approaches, which may aid in identification of previously unknown targets of immunological relevance.

Targeted screening

Recent developments in our understanding of PRR-mediated signaling pathways and subsequent molecular outcomes can be used in targeted screening. Thus, libraries of natural (such as pathogen products or peptide sequences) or synthetic compounds can be screened using cells expressing specific TLRs and appropriate reporter genes to identify agonistic compounds. Specifically, cells that do not naturally express TLRs (such as HEK cells) can be cotransfected with an NFkB or IRF-3-driven reporter gene and a TLR cDNA for which a novel agonist is sought. As has been outlined above, TLR ligands can be well-defined immunopotentiators for subunit vaccines. Various TLR ligands, such as CpG (TLR-9 ligand) and imidazoquinolines (TLR-7 and 8 ligands), have been shown to stimulate *in vitro* cytokine production and to activate NFkB-driven reporter genes. Recently, a peptide ALTTE isolated from bacterial fimbriae has been shown to be a TLR-2 ligand (Ogawa and Uchida, 1995). Also, another peptide isolated from insects was able to stimulate NK cells to produce IFN-γ (Chernysh et al., 2002). Thus, immunomodulators can also be based on peptides. Combinatorial peptide libraries, consisting natural or modified amino acids, can be screened in defined TLR-readout assays. Strategies are being explored as to whether linking peptide immunostimulators in frame with antigenic protein will provide a necessary innate immune stimulation along with mounting an adaptive response to the selected antigen (Jackson et al., 2004). Besides selecting agonists for PRR, targeted approaches of identifying inhibitors of known immunological antagonists (such as negative regulators of TLR signaling) or compounds that potentiate positive regulators

of the immune system, such as activators of NFkB pathway, can be undertaken.

Future directions

Because of intense research activity leading to theoretical and mechanistic understanding of innate immunity, a foundation is provided for systematic approaches towards immunopotentiator discovery. We envision that the future of vaccine adjuvant research will increasingly employ the tools and platforms of modern drug discovery. The key challenges and opportunities will be found in linking systems biology of the immune system with our growing molecular understanding of adjuvant mechanisms (Figure 4.5). The ultimate goal is to become predictive of how manipulation of the innate immune response at the molecular level gives rise to distinct protective responses in vaccinees. To achieve this goal critical gaps in our understanding of innate immune potentiators will need to be filled. In particular, it will be important to identify the structure–function relationships between innate immune agonists and their molecular targets. The identification of novel and structurally diverse immune potentiators from small molecule libraries could not only provide a portfolio of vaccine adjuvant candidates but also powerful tools for mechanistic studies of innate immune activation.

Subunit antigens that do not inherently possess structures that stimulate the innate immune system are often shown to be poor immunogens. Vaccines against important but difficult disease targets such as HIV, HCV, herpes simplex virus (HSV), and *Neisseria meningitides* are either in clinical trials or in planning stages. These diseases may need both cellular as well as humoral responses for effective resolution. Also, for some diseases, such as tuberculosis, a greater understanding is available about the quality of T helper responses needed to resolve the infection. These recent breakthroughs in immunology of infectious diseases also allow for a better application of improved antigen discovery and their formulation with novel immune potentiators and delivery systems. Thus, in academic institutions, the vaccine industry, as well as governmental agencies, research and development efforts are converging towards developing next-generation adjuvants. Several of these first-generation candidates (such as CpG, MPL, and imidazoquinolines) have shown some

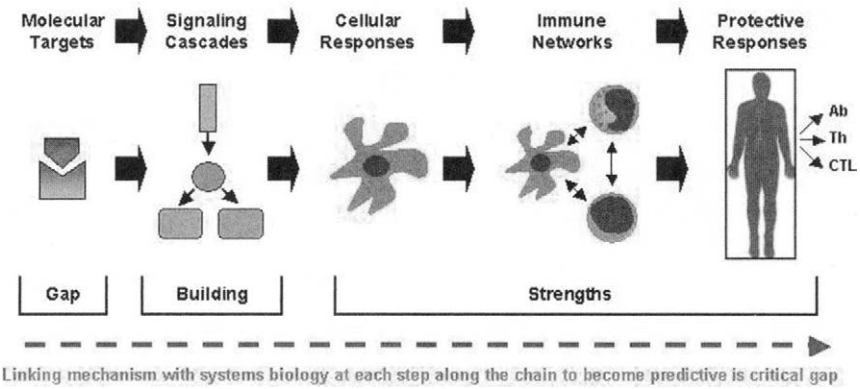

Figure 4.5 Linking molecular mechanisms to systems biology in adjuvant design and development. The long-term goal of adjuvant and immune potentiator research should become increasingly more predictive and rational. Before the appreciation of innate immunity and its mechanistic underpinnings, adjuvant research was almost exclusively empirical. Today, armed with new tools, molecular targets, and platforms, the ability to connect molecular mechanisms to *in vivo* outcomes is possible. The critical gap in our understanding right now lies in the incomplete knowledge surrounding target–ligand binding. However, the overall gaps in connecting each level of interaction (molecular mechanism – signal transduction – cellular responses – immune networks – systems biology) need to be filled to become truly predictive. (See Color Plate Section.)

efficacy in experimental animals and in I clinical studies in humans (Cooper et al., 2004; Mason et al., 2005; Hengge and Cusini, 2003).

Besides use in vaccines to initiate and sustain robust adaptive immune responses, these next-generation immune potentiators may be suitable for use against acute bioterrorism threats, where pathogenic organisms may be modified to have broad antibiotic resistance or evade prophylactic vaccines (Dennis, 2001). In these cases relative toxicity may be acceptable in the face of probable mass casualties because of bioterror weapons. Governmental agencies have a direct stake in the exploration of novel immune potentiators in this national security area. As has been true for most governmental/noncivilian investments in emerging technologies, a byproduct of national security research is usually of use for nongovernmental purposes. Immune potentiator platforms are ideally suited for this dual civilian/governmental use model given their potential utility in prophylactic vaccines, immune-based therapies, and bioweapons countermeasures.

Although there is a heightened acceptance, both by regulatory agencies as well as commercial vaccine manufacturers that improved vaccine adjuvants are needed to meet challenges of the future infectious diseases, there is a skepticism due to new technology carrying unknown risks that are still unevaluated. At present, the safety and regulatory hurdles that will be encountered with the addition of novel immune potentiators and delivery systems to final vaccine formulations may be significant and are still largely ill defined. The key focus should be on separating the potential increases in immune toxicity from improved immunogenicity of adjuvanted vaccines. Currently, our understanding of this "trade-off" is rudimentary but the tools and models are being developed to approach the question rationally. It is likely that improved formulation and controlled release of potent immune potentiators will limit toxicities while increasing efficacy. In addition, the growing number of immune potentiators, targeting diverse innate immune mechanisms, should allow for the identification of candidates with improved therapeutic indices. Thus, selecting the optimal platform(s) for development and identifying the key cellular and molecular targets of the innate immune system that will trigger the safest and most effective immune responses against diverse pathogens should be the long-term goal. At this early stage, the hurdles appear high and public/private partnerships to fuel research and development may be required to overcome them. Such partnerships have already been established in the areas of HIV and biodefense vaccine research and it is likely that government/industry cooperation on these particularly difficult vaccine problems will lead to breakthroughs in adjuvant development. Overall, despite the aforementioned uncertainties, we are entering a dynamic period in vaccine research driven largely by our increased understanding of innate immunity. The mechanistic understanding and the tools to manipulate this system are growing and it is likely that novel immune potentiators and delivery systems will make a significant impact on vaccine development in the near future.

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References

Aguado, T., Engers, H., Pang, T., and Pink, R. (1999). Novel adjuvants currently in clinical testing November 2–4, 1998, Fondation Merieux, Annecy, France: a meeting sponsored by the World Health Organization. *Vaccine* 17, 2321–2328.

Akashi, S., Saitoh, S., Wakabayashi, Y., Kikuchi, T., Takamura, N., Nagai, Y., Kusumoto, Y., Fukase, K., Kusumoto, S., Adachi, Y., Kosugi, A., and Miyake, K. (2003). Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J. Exp. Med.* 198, 1035–1042.

Akira, S. and Takeda, K. (2004). Toll-like receptor signalling. *Nature Rev. Immunol.* 4, 499–511.

Akira, S., Yamamoto, M., and Takeda, K. (2003). Role of adapters in Toll-like receptor signalling. *Biochem. Soc. Trans.* 31, 637–642.

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413, 732–738.

Anderson, J.R. (1955). Iso-immunisation of rabbits by means of Freund-McDermott adjuvants. *Br. J. Exp. Pathol.* 36, 137–142.

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., and Lipford, G.B. (2001). Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA* 98, 9237–9242.

Bernasconi, N.L., Traggiai, E., and Lanzavecchia, A. (2002). Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 298, 2199–2202.

Beutler, B. (2003). Not "molecular patterns" but molecules. *Immunity* 19, 155–156.

Chamaillard, M., Girardin, S.E., Viala, J., and Philpott, D.J. (2003a). Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. *Cell Microbiol.* 5, 581–592.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M.A., Foster, S.J., Mak, T.W., Nunez, G., and Inohara, N. (2003b). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nature Immunol.* 4, 702–707.

Chernysh, S., Kim, S.I., Bekker, G., Pleskach, V.A., Filatova, N.A., Anikin, V.B., Platonov, V.G., and Bulet, P. (2002). Antiviral and antitumor peptides from insects. *Proc. Natl. Acad. Sci. USA* 99, 12628–12632.

Chuang, T.H. and Ulevitch, R.J. (2000). Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur. Cytokine Netw.* 11, 372–378.

Cooper, C.L., Davis, H.L., Morris, M.L., Efler, S.M., Krieg, A.M., Li, Y., Laframboise, C., Al Adhami, M.J., Khaliq, Y., Seguin, I., and Cameron, D.W. (2004). Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. *Vaccine* 22, 3136–3143.

Dennis, C. (2001). The bugs of war. *Nature* 411, 232–235.

Doyle, S.E., O'Connell, R.M., Miranda, G.A., Vaidya, S.A., Chow, E.K., Liu, P.T., Suzuki, S., Suzuki, N., Modlin, R.L., Yeh, W.C., Lane, T.F., and Cheng, G. (2004). Toll-like receptors induce a phagocytic gene program through p38. *J. Exp. Med.* 199, 81–90.

Du, X., Poltorak, A., Wei, Y., and Beutler, B. (2000). Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *Eur. Cytokine Netw.* 11, 362–371.

Engers, H., Kieny, M.P., Malhotra, P., and Pink, J.R. (2003). Third meeting on novel adjuvants currently in or close to clinical testing. World Health Organization-Organization Mondiale de la Sante, Fondation Merieux, Annecy, France, 7–9 January 2002. *Vaccine* 21, 3503–3524.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zahringer, U., Coyle, A.J., DiStefano, P.S., Bertin, J., Sansonetti, P.J., and Philpott, D.J. (2003a). Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* 300, 1584–1587.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J., and Sansonetti, P.J. (2003b). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869–8872.

Gordon, S. (2002). Pattern recognition receptors: doubling up for the innate immune response. *Cell* 111, 927–930.

Gutierrez, O., Pipaon, C., Inohara, N., Fontalba, A., Ogura, Y., Prosper, F., Nunez, G., and Fernandez-Luna, J.L. (2002). Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J. Biol. Chem.* 277, 41701–41705.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410, 1099–1103.

Haziot, A., Hijiya, N., Gangloff, S.C., Silver, J., and Goyert, S.M. (2001). Induction of a novel mechanism of accelerated bacterial clearance by lipopolysaccharide in CD14-deficient and Toll-like receptor 4-deficient mice. *J. Immunol.* 166, 1075–1078.

Heil, F., Ahmad-Nejad, P., Hemmi, H., Hochrein, H., Ampenberger, F., Gellert, T., Dietrich, H., Lipford, G., Takeda, K., Akira, S., Wagner, H., and Bauer, S. (2003). The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *Eur. J. Immunol.* 33, 2987–2997.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. (2002). Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nature Immunol.* 3, 196–200.

Hengge, U.R. and Cusini, M. (2003). Topical immunomodulators for the treatment of external genital warts, cutaneous warts and molluscum contagiosum. *Br. J. Dermatol.* 149 (Suppl. 66), 15–19.

Hilleman, M.R. (2000). Vaccines in historic evolution and perspective: a narrative of vaccine discoveries. *Vaccine* 18, 1436–1447.

Hoebe, K., Janssen, E., and Beutler, B. (2004). The interface between innate and adaptive immunity. *Nature Immunol.* 5, 971–974.

Iwasaki, A. and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nature Immunol.* 5, 987–995.

Jackson, D.C., Lau, Y.F., Le, T., Suhrbier, A., Deliyannis, G., Cheers, C., Smith, C., Zeng, W., and Brown, L.E. (2004). A totally synthetic vaccine of generic structure that targets Toll-like receptor 2 on dendritic cells and promotes

antibody or cytotoxic T cell responses. *Proc. Natl. Acad. Sci. USA* 101, 15440–15445.

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

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A.M., Wagner, H., Lipford, G., and Bauer, S. (2002). Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nature Immunol.* 3, 499.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R.W., Kastelein, R.A., Bazan, F., and Liu, Y.J. (2001). Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* 194, 863–869.

Kenney, R.T., Regina Rabinovich, N., Pichyangkul, S., Price, V.L., and Engers, H.D. (2002). Second meeting on novel adjuvants currently in/close to human clinical testing. World Health Organization–Organization Mondiale de la Sante Fondation Merieux, Annecy, France, 5–7 June 2000. *Vaccine* 20, 2155–2163.

Kieny, M.P., Excler, J.L., and Girard, M. (2004). Research and development of new vaccines against infectious diseases. *Am. J. Public Health* 94, 1931–1935.

Lee, J., Chuang, T.H., Redecke, V., She, L., Pitha, P.M., Carson, D.A., Raz, E., and Cottam, H.B. (2003). Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* 100, 6646–6651.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86, 973–983.

Lien, E., Means, T.K., Heine, H., Yoshimura, A., Kusumoto, S., Fukase, K., Fenton, M.J., Oikawa, M., Qureshi, N., Monks, B., Finberg, R.W., Ingalls, R.R., and Golenbock, D.T. (2000). Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J. Clin. Invest.* 105, 497–504.

Lindblad, E.B. (2004). Aluminium adjuvants: in retrospect and prospect. *Vaccine* 22, 3658–3668.

Liu, N., Ohnishi, N., Ni, L., Akira, S., and Bacon, K.B. (2003). CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nature Immunol.* 4, 687–693.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., Iwasaki, A., and Flavell, R.A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* 101, 5598–5603.

Mason, K.A., Ariga, H., Neal, R., Valdecanas, D., Hunter, N., Krieg, A.M., Whisnant, J.K., and Milas, L. (2005). Targeting Toll-like receptor 9 with CpG oligodeoxynucleotides enhances tumor response to fractionated radiotherapy. *Clin. Cancer Res.* 11, 361–369.

Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A., and Seya, T. (2003). Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J. Immunol.* 171, 3154–3162.

Matsumoto, M., Kikkawa, S., Kohase, M., Miyake, K., and Seya, T. (2002). Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem. Biophys. Res. Commun.* 293, 1364–1369.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity [see comments]. *Nature* 388, 394–397.

Mukhopadhyay, S., Herre, J., Brown, G.D., and Gordon, S. (2004). The potential for Toll-like receptors to collaborate with other innate immune receptors. *Immunology* 112, 521–530.

O'Hagan, D.T. (2004) In Levine, M., Kaper, J.B., Rappuoli, R., Liu, M., and Good, M.F. (Eds) *New Generation Vaccines*, 2nd edn. Marcel-Dekker, New York, pp. 259–270.

O'Hagan, D.T. and Valiante, N.M. (2003). Recent advances in the discovery and delivery of vaccine adjuvants. Nat Rev Drug Discov, 2, 727–735.

O'Hagan, D.T., Singh, M., Dong, C., Ugozzoli, M., Berger, K., Glazer, E., Selby, M., Wininger, M., Ng, P., Crawford, K., Paliard, X., Coates, S., and Houghton, M. (2004). Cationic microparticles are a potent delivery system for a HCV DNA vaccine. *Vaccine* 23, 672–680.

Ogawa, T. and Uchida, H. (1995). A peptide, ALTTE, within the fimbrial subunit protein from Porphyromonas gingivalis, induces production of interleukin 6, gene expression and protein phosphorylation in human peripheral blood mononuclear cells. *FEMS Immunol. Med. Microbiol.* 11, 197–205.

Ozinsky, A., Underhill, D.M., Fontenot, J.D., Hajjar, A.M., Smith, K.D., Wilson, C.B., Schroeder, L., and Aderem, A. (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc. Natl. Acad. Sci. USA* 97, 13766–13771.

Pasare, C. and Medzhitov, R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299, 1033–1036.

Pink, J.R. and Kieny, M.P. (2004). Fourth meeting on novel adjuvants currently in/close to human clinical testing. World Health Organization–Organization Mondiale de la Sante Fondation Merieux, Annecy, France, 23–25 June 2003. *Vaccine* 22, 2097–2102.

Podda, A. and Del Giudice, G. (2004). In Levine, M., Kaper, J.B., Rappuoli, R., Liu, M., and Good, M.F. (Eds) *New Generation Vaccines*. Marcel Dekker, New York, pp. 225–235.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282, 2085–2088.

Qureshi, S.T., Gros, P., and Malo, D. (1999). Host resistance to infection: genetic control of lipopolysaccharide

responsiveness by TOLL-like receptor genes. *Trends Genet.* 15, 291–294.

Rutz, M., Metzger, J., Gellert, T., Luppa, P., Lipford, G.B., Wagner, H., and Bauer, S. (2004). Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. *Eur. J. Immunol.* 34, 2541–2550.

Sato, M., Sano, H., Iwaki, D., Kudo, K., Konishi, M., Takahashi, H., Takahashi, T., Imaizumi, H., Asai, Y., and Kuroki, Y. (2003). Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappa B activation and TNF-alpha secretion are down-regulated by lung collectin surfactant protein A. *J. Immunol.* 171, 417–425.

Schroder, N.W., Opitz, B., Lamping, N., Michelsen, K.S., Zahringer, U., Gobel, U.B., and Schumann, R.R. (2000). Involvement of lipopolysaccharide binding protein, CD14, and Toll-like receptors in the initiation of innate immune responses by Treponema glycolipids. *J. Immunol.* 165, 2683–2693.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp. Med.* 189, 1777–1782.

Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. *Annu. Rev. Immunol.* 21, 335–376.

Takeuchi, O., Kawai, T., Sanjo, H., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Takeda, K., and Akira, S. (1999). TLR6: a novel member of an expanding toll-like receptor family. *Gene* 231, 59–65.

Tapping, R.I. and Tobias, P.S. (2003). Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. *J. Endotoxin Res.* 9, 264–268.

Vasilakos, J.P., Smith, R.M., Gibson, S.J., Lindh, J.M., Pederson, L.K., Reiter, M.J., Smith, M.H., and Tomai, M.A. (2000). Adjuvant activities of immune response modifier R-848: comparison with CpG ODN. *Cell. Immunol.* 204, 64–74.

Weber, J.R., Freyer, D., Alexander, C., Schroder, N.W., Reiss, A., Kuster, C., Pfeil, D., Tuomanen, E.I., and Schumann, R.R. (2003). Recognition of pneumococcal peptidoglycan: an expanded, pivotal role for LPS binding protein. *Immunity* 19, 269–279.

Wyllie, D.H., Kiss-Toth, E., Visintin, A., Smith, S.C., Boussouf, S., Segal, D.M., Duff, G.W., and Dower, S.K. (2000). Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. *J. Immunol.* 165, 7125–7132.