Abstract: Agents that activate dendritic cells are essential components for vaccines and can be conceptualized as molecular adjuvants. Other molecular adjuvants affect downstream factors that shape the resulting immune response. This review provides a compendium of recently studied molecular adjuvants, focusing on CD8+ T cell responses, which have important roles in HIV vaccines. Reference is also made to CD8+ T cell antitumor responses, where parallel studies of molecular adjuvants are being pursued. Molecular adjuvants can be considered in the following groups: TNF superfamily molecules such as CD40 ligand; agonists for TLRs; agonists for NAIP, CIITA, HET-E, TP-1-leucine-rich repeat pathway receptors, such as nucleotide-binding and oligomerization domain (NOD)1, NOD2, and cryopyrin; chemokines; ILs; CSFs; IFNs; alarmins; and purinergic P2X7 receptor agonists. Complementing these positively acting agents are strategies to reduce the immunosuppressive effects of CD4+CD25+ regulatory T cells and negatively acting factors such as TGF-β, IL-10, suppressor of cytokine signaling 1, and programmed cell death-1 using neutralizing antibodies, antisense, and small interfering RNA. Especially effective are combinations of molecular adjuvants, which can elicit a massive expansion of antigen-specific CD8+ T cells and show unprecedented efficacy in vaccine and tumor models. Taken together, these new approaches provide significant incremental progress in the development of vaccines to elicit cell-mediated immunity against HIV and other pathogens. J. Leukoc. Biol. 80: 000–000; 2006.

Key Words: dendritic cells · monocyte/macrophages · T cells · CD40 · TLR · regulatory T cells

INTRODUCTION

Vaccination is generally believed to be one of the most effective and cost-efficient ways to prevent or treat disease. Nevertheless, several problems in vaccine development have proved difficult to solve: Vaccines are often weak in the very young and the very old; many vaccines need to be given several times, and the protection that they elicit wanes over time, requiring booster administrations; and for some diseases such as HIV, an effective vaccine has yet to be developed. These problems have a common theme: Many vaccines would be enabled or improved if only they could elicit a stronger and more durable immune response. As immune responses are typically initiated by activation of APCs, notably dendritic cells (DCs), there has been significant interest in improving APC-stimulating adjuvants as a key step in constructing better vaccines [1, 2].

In its purest form, an adjuvant has no antigen-specific qualities, and indeed, one can think of an immunological equation where an immunogen = antigen + adjuvant. Adjuvants have several forms, ranging from mineral salts such as alum to oil-based emulsions such as IFA. However, with the avalanche of information about the components of the immune system, there has been great interest in “molecular adjuvants.” This term can be loosely defined as proteins, lipids, nucleic acids, carbohydrates, or chemical compounds for which DCs have a known receptor whose occupancy leads to a defined sequence of intracellular signal transduction and a change in the DC phenotype resulting in an improvement in the quantity or quality of the ensuing immune response. Although the receptors for molecular adjuvants are now known from genomic information, the molecular adjuvants themselves greatly outnumber their receptors. As a case in point, TLR9 is generally thought of as the receptor for unmethylated CpG-containing oligodeoxynucleotides (ODN), but these ODN can be A-class, B-class, or C-class stimulants [3]. Furthermore, nucleotide sequences containing a synthetic cytosine-phosphate-2’-deoxy-7-deazaguanosine dinucleotide (CpR), immunomodulatory oligonucleotides (IMOs) [4], or a dumbbell-like, covalently closed structure (dSLIM-30L1) [5], can also stimulate TLR9. Under some circumstances, even mammalian DNA can stimulate TLR9 [6]. Thus, when referring to a molecular adjuvant, it is usually more inclusive to refer to an entire class of agonists for an adjuvant receptor (e.g., TLR9 agonists) rather than to single out any one agonist species (e.g., B-class CpG ODN).

Six factors greatly complicate our understanding of molecular adjuvants: (1) There are two major subsets of DCs, conventional or myeloid DCs (MDCs) [7, 8] and plasmacytoid DCs (PDCs) [9, 10], and each has different properties [11]. In...
general, PDCs, which express TLR7 and TLR9, respond to imidazoquinolines (imiquimod and R-848) and CpG ODN stimulation, resulting in enhancement of the expression of costimulatory molecules and induction of IFN-α. In contrast, MDCs, which express TLR3, TLR4, TLR7, and TLR8, respond to polyinosinic:polycytidylic acid [poly(I:C)], LPS, and imidazoquinolines with phenotypic maturation and high production of IL-12 p70 without producing detectable IFN-α [12].

(2) Different adjuvant receptors are expressed on different types of APCs in different species. For example, in humans, TLR9 is expressed on PDCs but not on MDCs, whereas both types of DCs express TLR9 in the mouse [13, 14]. (3) Adjuvant receptors can have different functions in different species. For example, single-stranded RNA is an agonist for TLR7 in mice but works through TLR8 in humans [15, 16]. (4) Different adjuvant receptors signal differently and lead to different APC phenotypes. Considering just the TLRs, stimulation of different TLRs can affect Th1 versus Th2 T cell bias and other aspects of the ensuing immune response [17–19]. (5) In some cases, a single molecular adjuvant interacts with entirely different receptors on different cells. For example, poly(I:C), a form of dsRNA, stimulates TLR3 and/or melanoma differentiation-associated gene 5 (MDA5) on MDCs [20] but signals through retinoic acid-inducible gene-I (RIG-I) in PDCs [21]. (6) Combinations of molecular adjuvants lead to quantitatively and qualitatively different outputs [22–24], but the number of these potential combinations is large.

This review aims to highlight some of the newer and less widely appreciated innovations in molecular adjuvants in the context of HIV vaccines. Although antibodies against the viral envelope (Env) can contribute to protection from HIV [25], there is no agreement in the field on what Env immunogen is suitable for eliciting antibodies with potent neutralizing activity [26, 27]. Consequently, this review will focus on CD8 + T cell responses, as CD8 + T cells appear to be crucial for the control of HIV viremia [28–33]. Reference is also made to the considerable body of knowledge in the tumor immunology literature, where CD8 + T cells are also critical for host protection [34–38]. Given the breadth of the subject, this listing is necessarily incomplete, and the decision about what to include is unavoidably somewhat subjective. Also presented is an overview of the emerging field of immunostimulatory combinations, where the rules for achieving synergy are just beginning to become apparent.

**POSITIVELY ACTING IMMUNOSTIMULANTS**

**CD40 and other TNF receptor superfamily (TNFRSF) agonists as molecular adjuvants**

The TNFRSF includes many important receptors on DCs, macrophages, and T cells. Particularly interesting from the point of view of vaccines are OX40, 4-1BB, CD30, herpes virus entry mediator, CD27, and glucocorticoid-induced TNFR-related protein (GITR), whose ligands are OX40L, 4-1BBL, CD30L, LIGHT, CD27L/CD70, and GITRL, respectively. These molecules have significant potential as vaccine adjuvants as reviewed in detail elsewhere [39–42].

The critical role of CD40 stimulation and CD40L (or CD154) in immune stimulation has been reviewed previously [39, 43]. CD40L strongly up-regulates the expression of CD80 and CD86 on DCs and causes CD4 + T cells to differentiate toward Th1 cells [44, 45]. For CD8 + T cells, CD8α + DCs are particularly important APCs [46], and CD40L serves as the molecular embodiment of CD4 + T cell help for activating CD8α + DCs to generate CD8 + T cells [47, 48]. One of the most important aspects of CD40 stimulation is that it promotes the generation of memory CD8 + T cells [49–51]. As shown by Stone et al. [52], the strength of the CD8 + T cell response to a CD40L-containing DNA vaccine depends on clustering of CD40 receptors in the responding APCs. However, besides CD40L itself, other molecular adjuvants have been described that also stimulate the CD40 receptor (Table 1). In addition, adjuvants such as α-galactosylceramide, the CD1d-presented molecule, which is recognized by innate NKT cells, activate immune responses in part by inducing these cells to express CD40L [35, 58].

**TLR agonists as molecular adjuvants**

Substances that bind to and activate TLRs are TLR agonists. A number of excellent reviews of TLRs are available [18, 59–62] so that TLR agonists are only briefly reviewed here. However, several general points are important to note regarding TLR agonists: MDCs, which express TLR3, TLR4, and TLR7, respond to agonists such as poly(I:C), LPS, and imidazoquinolines with up-regulation of costimulatory molecules and IL-12 production without producing IFN-α. In contrast, PDCs, which express TLR7 and TLR9, respond to agonists such as imidazoquinolines and CpG ODN with up-regulation of costimulatory molecules and the production of large amounts of IFN-α [12]. IFN-α is important, as it promotes the development of CD8 + T cells [63, 64]. For murine bone marrow-derived DCs (BMDDCs), TLR3 agonists and TLR9 agonists such as poly(I:C) and CpG ODN, but not agonists for other TLRs, activate these cells to cross-present exogenous antigens to MHC-I-restricted CD8 + T cells [65]. Although TLR agonists promote the generation of CD8 + T cells even in the absence of CD4 + T cell help [66], such CD8 + T cell responses wane over time, unless CD4 + T cell help [67], or a CD40 stimulus is also present when the CD8 + T cells are stimulated initially [68]. TLR agonists produce a short-lived stimulation of DCs [24] and macrophages [69], which declines within 24 h after a single administration in vitro. Unless delivered in a time-release formulation, a single administration of TLR agonists cannot break tolerance in vivo, whereas the repeated administration of LPS (every day for 4 days) led to effective antitumor CD8 + T cell responses.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40L or its derivatives</td>
<td>[52]</td>
</tr>
<tr>
<td>Agonistic anti-CD40 antibodies</td>
<td>[53]</td>
</tr>
<tr>
<td>Heat shock protein (Hsp70)</td>
<td>[54, 55]</td>
</tr>
<tr>
<td>C4BP</td>
<td>[56]</td>
</tr>
<tr>
<td>Synthetic CD40L binding residues mounted on a</td>
<td>[57]</td>
</tr>
<tr>
<td>C3 symmetric peptide scaffold</td>
<td></td>
</tr>
</tbody>
</table>
cell responses even in the presence of tolerogenic regulatory T cells (Treg) [70]. Protein antigens can also be conjugated directly with TLR agonists to elicit an increased antigen-specific response [71–73]. Some of the salient features of TLR agonists are given in Table 2, and details about particular TLRs follow next.

**TLR3 agonists**

TLR3 signals through the Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF) pathway to generate cytokines [95, 96]. Although viral dsRNAs can be used to stimulate TLR3, the best-tested TLR3 agonist is poly(I:C), which is a synthetic form of dsRNA [97]. Although poly(I:C) has been studied for over 35 years and has been tested extensively in humans [98], it is receiving new attention as a key component in many new immunostimulatory combinations (see below). Two caveats regarding poly(I:C) are worth noting: If poly(I:C) enters the cytoplasm, it can interact with RIG-I or MDA5 to stimulate the production of type I IFNs (described below) [99]. Again, if poly(I:C) enters the cytoplasm of nonplasmacytoid, conventional MDCs such as murine BMDMs, it can interact with the dsRNA-dependent protein kinase R (PKR) and an unidentified non-PKR, non-TLR3 pathway to cause these cells to produce type I IFNs. To reach the cytoplasm, however, poly(I:C) must generally be delivered by electroporation or lipofection, bypassing the need to interact with TLR3 on the DC surface [100]. Therefore, how poly(I:C) is formulated for delivery can have an important effect on the cells that it affects and the responses that it induces.

**TLR4 agonists**

TLR4 can signal to cells through the MyD88 and the TRIF pathways [95, 96]. Its special use in activating human MDCs was reported by Napolitani et al. [23]. The classic agonist for TLR4 is bacterial LPS, which refers to a family of substances containing lipid A and its congeners. In an effort to make a less toxic form of TLR4 agonist, monophosphoryl lipid A was developed and was recently shown to be an effective molecular adjuvant in humans as part of the GlaxoSmithKline AS02A adjuvant [101].

**TLR7 and TLR8 agonists**

One species difference to note is that R-848 is a TLR7 agonist in the mouse and a TLR8 agonist in humans. Another distinction is that TLR7 agonists activate human PDCs to produce IFN-α, whereas TLR8 agonists activate human MDGs, monocytes, and monocyte-derived DCs (MDDCs) to make proinflammatory cytokines and chemokines, such as TNF, IL-12, and MIP-1 [84]. 10-Guanosine nucleosides connected by phosphorothioate linkages (Poly-G10) is a TLR8 agonist, which may be especially useful as a substance that shuts off the immunosuppressive functions of CD4+CD25+ Tregs [89].

**TLR9 agonists**

Unmethylated CpG-containing ODN (CpG ODN) are the prototypic agonists for TLR9. More generally, they are called immunostimulatory sequences of ODN, as many immunostimulatory ODN do not contain a CpG motif. Typically, the ODN is a synthetic phosphorothioate-linked compound, where this chemical linkage protects the ODN from nucleases and makes it more cell permeable. Although plasmid DNA-mediated immunostimulation during DNA vaccination may be a result of CpG motifs [102], it is not clear if this is always a result of an effect on TLR9 [103].

As noted above, TLR9 is present on PDCs and MDCs in mice but only on PDCs in humans. This difference has led to

---

**TABLE 2. TLR Agonists as Molecular Adjuvants**

<table>
<thead>
<tr>
<th>TLRs targeted</th>
<th>Representative agonists</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2/1 and TLR2/6 heterodimers</td>
<td>Mycoplasmal macrophage-activating lipoprotein-2 (Mlp2)</td>
<td>[74–79]</td>
</tr>
<tr>
<td></td>
<td>Tripalmitoyl-cysteinyl-seryl-(lysyl)3-lysine (Pam3CSK4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mycobacterial cell wall fractions enriched for lipoarabinomannan</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mycobacteria-derived monoaoylated muramyl dipeptide derivatives</td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>Poly(I:C)</td>
<td>[80]</td>
</tr>
<tr>
<td></td>
<td>Ampligen (poly I poly C12U)</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>Bacterial lipopolysaccharide (LPS)</td>
<td>[91]</td>
</tr>
<tr>
<td></td>
<td>Lipid A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monophosphoryl lipid A (MPL)</td>
<td>[82, 83]</td>
</tr>
<tr>
<td>TLR5</td>
<td>Bacterial flagellin</td>
<td>[15, 16, 84–88]</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded RNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Imidazoquinoline compounds [resiquimod (R-848), imiquimod, and oxoribine]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7-Thia-8-oxoguanosine and 7-deazaguanosine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ANA975, an oral prodrg of isatoribine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SM-360320 S-27609</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3M-01 and 3M-03</td>
<td></td>
</tr>
<tr>
<td>TLR8</td>
<td>Poly-G10</td>
<td>[84, 89]</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG ODN</td>
<td>[4, 5, 90–92]</td>
</tr>
<tr>
<td></td>
<td>Bacterial DNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vertebrate DNA in liposomes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insect DNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpR-containing ODN, i.e., IMOs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dumbbell-like covalently closed ODN (dSLIM-30L1)</td>
<td></td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
<td>[93]</td>
</tr>
<tr>
<td>TLR11</td>
<td>Profilin (Toxoplasma gondii)</td>
<td>[94]</td>
</tr>
</tbody>
</table>
a concern that many of the actions of TLR9 agonists in mice might not translate to clinical use in humans. It is reassuring, therefore, that at least two pathways in humans have been shown to lead from CpG ODN-initiated immunostimulation of TLR9+ DCs to the activation of TLR9− MDCs. Gerosa et al. [104] found that CpG ODN-activated, human PDCs activated NK cells, which in turn matured MDCs for antigen presentation and for IL-12p70 production through a pathway that was at least partially dependent on NK cell-MDC cell-cell contact. In addition, Gautier et al. [105] found that type I IFN production from CpG ODN-stimulated, human PDCs could enhance IL-12p70 production by MDCs (see below). One or both of these pathways may contribute to the efficacy of TLR9 agonists being observed in clinical vaccine trials.

NAIP, CIITA, HET-E, TP-1 (NACHT)-leucine-rich repeat (LRR; NLR) agonists and agonists of caspase activation and recruitment (CARD)-containing RNA helicases as molecular adjuvants

**Definition of the NLR system**

The NLR proteins form an expanding family [106] also called the CATERPILLER family of proteins [107, 108]. Other names for certain members of this family are nucleotide-binding and oligomerization domains (NODs) [109] and NACHT, LRR, and Pyrin domain-containing proteins (NALPs) [110]. NLR agonists are given in Table 3.

**NLR agonists promote IL-1β processing and secretion**

NLR stimulation is relatively unique, as it can lead to activation of caspase-1, also known as IL-1β-converting enzyme (ICE). Although many DC and macrophage stimuli up-regulate the intracytoplasmic precursor form of IL-1β (termed pro-IL-1β), ICE is necessary to convert inactive pro-IL-1β into secreted, fully mature IL-1β. Similarly, ICE is also needed to convert pro-IL-18 to active IL-18. IL-1β and IL-18 are important in activating several types of immune cells, which makes NLR agonists useful components of vaccines and immunostimulators. For example, IL-1β synergizes with CD40L to induce human MDDCs to produce IL-12 [124].

**NLR agonists for NOD1 stimulation**

NOD1 is activated by forms of dianaminopimelic acid, a breakdown product of peptidoglycan in bacterial cell walls [111]. The minimal motif for NOD1 activation is γ-D-Glu-meso-DAP, also called iE-DAP, with an exposed DAP stem [112].

**NLR agonists for NOD2 stimulation**

NOD2 is activated by MDP [114]. The i.v. administration of MDP formulated in liposomes markedly reduced metastases in mice bearing B16-BL6 melanoma tumors [125]. Clinical trials in dogs and humans with osteosarcoma have used a liposomal form of MTP phosphatidylethanolamine (PE) given i.v. [115, 116].

**NLR agonists for cryopyrin**

NALP-3 has several splice variants, one of which is called cryopyrin. MDP interacts with NALP-3 to stimulate the proteolytic processing of pro-IL-1β and pro-IL-18 [117, 118]. Similarly, extracellular ATP (ATP)e, an agonist for the purinergic P2X7 receptor on macrophages and DCs (see below), has also been shown to activate caspase-1 and promote the processing and secretion of IL-1β and IL-18 [119]. The local administration of ATPeS, a nonhydrolyzable form of ATPe, augmented delayed-type hypersensitivity (DTH) to 2,4-dinitrochlorobenzene (DNCB) or tumor antigens in mice [120]. It has recently been shown that ATPe requires NALP-3/cryopyrin to activate caspase-1 and promote IL-1β release [121]. Also, it has been reported that bacterial RNA and R-837 and R-848, which are usually thought to bind TLR7 in the mouse, activate caspase-1 and promote IL-1β release from murine macrophages in a cryopyrin-dependent manner [122].

**Agonists for RIG-I and MDA5**

Many of the NLR proteins contain a CARD domain. A recently described CARD-containing protein, variously called IPS-1, MAVS, VISA, and Cardif, is an adaptor protein involved in transmitting the activating signals from RIG-I, which also contains a RNA helicase domain. MDA5 is another RNA helicase, which signals similarly to RIG-I [123]. Although these proteins are often considered to be in the NLR family, they have recently been grouped into their own family of RIG-like helicases [126]. Like other NLRs, RIG-I and MDA5 also sense intracellular microbial products, in this case, dsRNA, which is often produced during the replication of certain viruses and interacts with RIG-I to activate IFN regu-

<table>
<thead>
<tr>
<th>Table 3. NLR Agonists as Molecular Adjuvants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Receptor</strong></td>
</tr>
<tr>
<td>NOD1</td>
</tr>
<tr>
<td>M-TriDAP</td>
</tr>
<tr>
<td>FK156</td>
</tr>
<tr>
<td>FK565</td>
</tr>
<tr>
<td>NOD2</td>
</tr>
<tr>
<td>MtriLYS</td>
</tr>
<tr>
<td>Liposomal muramyl tripeptide (MTP)</td>
</tr>
<tr>
<td>MDP</td>
</tr>
<tr>
<td>Extracellular ATP (see text)</td>
</tr>
<tr>
<td>Bacterial RNA, R-837 and R-848</td>
</tr>
<tr>
<td>TLR7 agonists (see text)</td>
</tr>
<tr>
<td>NALP3/cryopyrin</td>
</tr>
</tbody>
</table>

Methods to direct antigens to DCs in lymph nodes

To generate an effective CD8$^+$ T cell response, antigen must be taken up and processed by DCs [143]. Ultimately, the antigen-pulsed DCs must arrive in a lymph node, where cellular immune responses are generated, as originally shown in 1968 by Barker and Billingham [144] using transplant rejection as a model. In autoimmune diabetes, for example, anti-islet cell CD8$^+$ T cells only develop in the single lymph node that drains the pancreatic islets and receives islet antigens [145]. Consequently, it is not unexpected that delivering antigen directly to lymph nodes can markedly enhance immune responses. For example, the direct intralymphatic injection of a DNA vaccine [146] or an antigenic peptide [147] increased CD8$^+$ T cell responses by up to 1000-fold or one million-fold, respectively. Although such injections may not always be practical, there are other ways to steer antigen to lymph nodes DCs, such as conjugating them to DEC205, an anti-DC mAb, along with an agonistic anti-CD40 antibody to activate the antigen-loaded DCs [143].

However, in the intercellular space, soluble antigens are naturally taken up by lymphatics and transported to lymph nodes [148], where a conduit system rapidly brings the antigen into contact with DCs and T cells [149, 150]. As long as DC-activating molecules are also administered in a soluble form (e.g., a soluble form of CD40L [52]), such soluble antigens might not need to be combined with chemokines to elicit an effective CD8$^+$ T cell response. In this case, a chemokine is less important, as the antigen goes to the DCs rather than vice versa.

ILs as molecular adjuvants

A large number of ILs and related cytokines such as hematopoietic growth factors have been described [151], but only IL-2 has been tested extensively in humans as a molecular adjuvant or immunopotentiator [152]. The addition of low-dose, continuous IL-2 to a SIV Gag canarypox vaccine in macaques led to an increase in CD8$^+$ T cell responses but paradoxically decreased CD4$^+$ T cell responses [153]. IL-7 and IL-15 have been shown to enhance vaccine-induced CD8$^+$ T cell responses in mice [154, 155]. A plasmid for IL-12 was shown to increase CD8$^+$ T cell responses to HIV DNA vaccination in mice [156] and led to memory CD8$^+$ T cells 6 months after vaccination [157].

However, a major reason why cytokines have not fulfilled their promise as molecular adjuvants is that they have often been incorrectly conceptualized as endocrine-like hormones. This was certainly the case for TNF (“cachectin”), which was originally thought to be a hormone that circulated in the blood and caused weight loss. Under this endocrine paradigm, many cytokines were tested as systemic agents, with the risk of dose-limiting toxic effects, which include the possibility of promoting autoimmunity. In contrast, most cytokines are naturally produced locally at the site of an immune response, in keeping the need for cell-cell contact for many important, immunological processes. As a result, cytokines have been making a reappearance when given nonsystemically by mixing them into vaccines (especially in a slow-release formulation) or...
administering them in DNA vaccines that create a local, immunostimulatory environment. One advantage of cytokine-expressing DNA plasmids over their protein forms is that they lead to sustained cytokine production over many weeks [158] with the highest cytokine concentration occurring in the lymph nodes draining the vaccination site. When given as part of DNA vaccination, cytokines have an important future in DNA vaccines, as described in other reviews [159–161]. As a case in point, a HIV DNA vaccine that includes an IL-2/IgG Fc fusion protein [162] is now entering human clinical trials.

**Hematopoietic growth factors as molecular adjuvants**

GM-CSF was shown to be an adjuvant in mice [163–165] and has potential for tumor vaccines [166]. However, when used as a molecular adjuvant in a SIV Env and Gag DNA vaccine in macaques, a GM-CSF plasmid did not augment protection from SIV challenge [167]. Similarly, as a protein in humans, GM-CSF did not enhance antibody or cellular responses to hepatitis A, influenza, or tetanus toxoid vaccines [168]. However, combinations of GM-CSF with other molecular adjuvants have shown more promise. The combination of GM-CSF with IL-12 or CD40 ligand was synergistic for the induction of CD8+ T cell responses and protection from vaccinia challenge, and the triple combination of GM-CSF, IL-12, and TNF appeared to be even stronger in some mouse models [159]. Plasmids for GM-CSF and IL-12 were also synergistic in an SIV DNA vaccine in macaques [169]. The combination of GM-CSF and CD40 stimulation was synergistic for generating effective antitumor CD8+ T cell responses in mice [170, 171]. GM-CSF was found to up-regulate OX40 expression on CD4+ and CD8+ T cells, and the combination of GM-CSF with OX40 stimulation was reported to break tolerance to tumor antigens [172]. GM-CSF stimulation of DCs was also synergistic with phosphorothioate backbone ODN, but this effect did not depend on a CpG motif in the ODN [173].

**IFNs as molecular adjuvants**

IFNs can be divided into type I IFNs (IFN-α/β species), type II IFN (IFN-γ), and type III IFNs (IFN-λ1 or IL-29, IFN-λ2 or IL-28A, and IFN-λ3 or IL-28B). As described above, IFN-α/β is important for vaccines, as it promotes the development of CD8+ T cells [63, 64]. Accordingly, vaccination with DCs generated from human monocytes using GM-CSF and IFN-α and pulsed with aldrithiol-inactivated HIV elicited strong responses in the human peripheral blood lymphocytes/SCID mouse model [174].

IFN-γ has long been known to facilitate immunological responses. Its originally described, immunological function was to prime macrophages for antitumor and antimicrobial activities, which were then triggered by TLR agonists such as LPS, as reviewed in ref. [175]. IFN-γ primes human monocytes to produce IL-12p70 following LPS triggering [176]. It has also been reported to enhance the survival of CD8+ T cells in vivo, presumably by inducing the production of IL-15 [177]. IFN-γ also works to prevent the generation of CD4+CD25+ Treg [178].

Type III IFNs are still being characterized. These cytokines can be induced in MDCs by intracellular dsRNA (e.g., Sendai virus), especially when the MDCs have been pretreated with IFN-α [179]. LPS and to a lesser extent, poly(I:C) up-regulated the levels of IFN-λ1 (IL-29) mRNA in human MDDCs [105]. However, IFN-λ1 and IFN-λ2 were recently reported to promote the expansion of CD4+CD25+ Treg, resulting in suppression of immune responses in vitro [180].

**Alarmins as molecular adjuvants**

Besides classic immunological mediators, a number of other endogenous substances can augment immunological responses. Oppenheim and Yang [181] have recently grouped several of these agents into the category of “alarmins” on the basis of three properties: They are rapidly released in response to infection or tissue injury; they have chemotactic and activating effects on APCs, particularly DCs; and they have potent immunoenhancing activity in vivo. A fundamental insight provided by this classification is that many effective adjuvants provide chemotactic activity to attract immunologically important cells to the immunization site and then activate these cells once they arrive there. Although other immunostimulants such as LPS and CD40L activate DCs and macrophages to produce chemokines [182, 183] and therefore do not need an added chemokine to be effective molecular adjuvants [52], alarmins provide an immediately available chemotactic stimulus.

Included in the alarmins are human neutrophil protein, β-defensin-1 (BD1), BD2, BD3, cathelicidin, eosinophil-derived neurotoxin (EDN), and high mobility group box protein 1 (HMGB1). It is interesting that all of the β-defensins bind to CCR6, the receptor on immature DCs, which is also ligated by the chemokine CCL20/MIP-3α [184]. Murine BD2 (mBD2) is also an agonist for TLR4, so it can attract immature DCs through CCR6 and then activate them through TLR4. Consequently, a fusion protein of mBD2 with a tumor antigen leads to significant antitumor immunity [185]. Similarly, LL-37, the C-terminal proteolytic fragment of human cationic peptide 18 (hCAP18), can attract immature DCs through CCR6 and activate them through the purinergic P2X7 receptor [186, 187]. Cathelin-related antimicrobial peptide, the murine ortholog of human cathelicidin/LL-37, acted as an adjuvant when mixed with OVA and injected i.p. in mice and was at least as effective as alum for increasing antibody and CD4+ T cell responses [188]. Similarly, the 18 amino acid porcine protegrins (PTG-1 and PTG-3) also induced IL-1β release by creating an ion channel rather than by activating the purinergic P2X7 receptor [189]. EDN and its close relative pancreatic RNase A activated DCs to produce cytokines and chemokines [190]. EDN also accounts for the majority of the soluble HIV-1 inhibitory activity contained in the supernatants of mixed lymphocyte reactions, and also other RNases, such as angiogenin, have strong anti-HIV effects [191].

HMGB1 is an especially interesting alarmin. A component of the cell nucleus, it is released by necrotic cells. Its primary receptor on DCs and macrophages is the receptor for advanced glycation end-products (RAGE), although it may also bind to TLR2 and TLR4 [192]. As a molecular adjuvant, mice vaccinated with HMGB1 mixed with OVA produced five- to tenfold more antibody. Vaccination with a mixture of apoptotic tumor cells and HMGB1 led to prolonged tumor-free survival after challenge with living tumor cells [193]. Using human cells,
HMGB1 activated MDDCs to up-regulate costimulatory molecules, produce cytokines, and augmented their ability to serve as allostimulators in a MLR [194]. Activated DCs have also been reported to secrete HMGB1 after LPS stimulation, and blocking studies indicated that DCs may require HMGB1 and RAGE to become fully stimulatory APCs [195].

**Purinergic P2X<sub>7</sub> receptor agonists as molecular adjuvants**

As noted above, IL-1β is produced as an inactive intracellular precursor, pro-IL-1β, which requires proteolytic processing by caspase-1 to be released from cells. Agents that cause macrophage apoptosis (e.g., silica) activate caspase-1 and lead to the release of active IL-1β. However, a nonapoptotic phospholipase-mediated endosomal pathway may also be involved in IL-1β release [196]. ATPe is another agent that causes macrophage and DC apoptosis by acting on the purinergic P2X<sub>7</sub> receptor (ligand-gated ion channel-type purinergic receptor 7) to stimulate the release of active IL-1β and IL-18 [119]. LPS, a TLR4 agonist that stimulates the synthesis of intracellular pro-IL-1β, is unable to activate caspase-1 and instead, acts as a priming agent for ATPe-triggered IL-1β release in vivo [197] and in vitro [198]. As members of the NLR family also activate caspase-1 (see above), it is not surprising that ATPe stimulation of P2X<sub>7</sub> requires a NALP3/cryopyrin-mediated pathway to activate caspase-1 and promote IL-1β release [121]. Benzoylbenzoic ATP is a nonhydrolyzable P2X<sub>7</sub> agonist that causes macrophage apoptosis [199] and presumably also IL-1β release from primed cells. Yet another P2X<sub>7</sub> agonist that induces IL-1β release is LL-37, the C-terminal proteolytic fragment of hCAP18 described above [186].

NAD is metabolized by an ADP-ribosylating ectoenzyme, ART2, to activate P2X<sub>7</sub> [200]. ATPe and NAD cause T cell death [201], which might be thought to be immunosuppressive. However, it was reported recently that these agents were selectively toxic to CD4<sup>+</sup>CD25<sup>+</sup> Treg in vitro and in vivo [202]. Therefore, ATPe and NAD have two means for promoting immune responses: by stimulating the processing and release of IL-1β and IL-18 and by killing immunosuppressive CD4<sup>+</sup>CD25<sup>+</sup> Treg. As evidence that these agents could be molecular adjuvants, ATPe's role by reducing the immunosuppressive effects of vaccination with DCs pulsed with a tumor cell lysate [234]. Similar attempts have been made to reduce IL-10-mediated immunosuppression. The use of IL-10 small interfering RNA (siRNA) to knock down IL-10 expression in murine BMDDCs in vitro led to increased CD40 expression and IL-12 production after maturation, without affecting CD80, CD86, or CD83. The resulting IL-10 siRNA-treated DCs stimulated Th1 reponses in vitro, as judged by increased IFN-γ and decreased IL-4 production by antigen-specific T cells [235]. Similar immunostimulatory effects were seen using DCs treated with IL-10 antisense oligonucleotides [236]. More recently, Guiducci et al. [237] found that the intratumoral injection of adenovirus-expressed human CCL16 chemokine into murine tumors along with systemic administration of CpG and blocking antibody to the IL-10R (IL-10Ra or CD210) led to the eradication of established tumors by switching intratumoral macrophages from an M2 to an M1 phenotype in mice.

**Inactivators of Treg as molecular adjuvants**

Tregs can be divided into several types, as reviewed in refs. [203, 204]. In particular, CD4<sup>+</sup>CD25<sup>+</sup> Tregs play an important role in limiting immune responses [205, 206]. In addition, the CD25 IL-2 receptor chain Tregs express CTLA-4 and the GITR as surface markers and characteristically express the Foxp3 transcription factor in their unactivated state [206]. Under some circumstances, vaccination can actually increase the number of immunosuppressive Tregs [207]. Conversely, vaccine-generated responses are enhanced if CD4<sup>+</sup>CD25<sup>+</sup> Tregs are eliminated or inactivated prior to vaccination [207–210], in part by reducing the immunosuppressive effects of Tregs on CD8<sup>+</sup> T cell function [211–214]. For example, Toka et al. [208] found that Treg depletion using anti-CD25 antibody led to enhanced CD8<sup>+</sup> T cell responses to a herpesvirus peptide-CpG conjugate vaccine or a plasmid DNA vaccine. Thus, in immunology as in arithmetic, the negative of a negative is a positive. Some of the means to deplete or inactivate Tregs are given in Table 4.

**Blocks of immunosuppressive cytokines as molecular adjuvants**

In one of the earliest reports of Treg depletion as a strategy to enhance vaccination, Ahlers et al. [228] found that CD4<sup>+</sup> T cell depletion of mice led to heightened CD8<sup>+</sup> T cell responses to a HIV Gag antigen, where GM-CSF and a soluble CD40L trimer were used as adjuvants. It is interesting that they then found that the anti-IL-13 antibody was as immunostimulatory as CD4<sup>+</sup> T cell depletion, suggesting that IL-13 is immunosuppressive in the context of vaccination. Although the role of IL-13 has not been defined completely, it is noteworthy that IL-13 can induce macrophages to produce TGF-β [229]. TGF-β in turn can induce Foxp3 expression in CD4<sup>+</sup>CD25<sup>+</sup> T cells and thereby differentiate these effector cells into immunosuppressive CD4<sup>+</sup>CD25<sup>+</sup> Tregs [230]. Thus, anti-IL-13 may lead to stronger vaccine responses by reducing TGF-β production and prevent the generation of Tregs.

Tregs can produce two immunosuppressive cytokines, TGF-β [231, 232] and IL-10 [233]. A strategy to down-regulate TGF-β by transfecting 4T1 mammary tumor cells with antisense constructs combined with neutralizing anti-TGF-β antibody led to strong antitumor effects in mice and augmented the effects of vaccination with DCs pulsed with a tumor cell lysate [234]. Similar attempts have been made to reduce IL-10-mediated immunosuppression. The use of IL-10 small interfering RNA (siRNA) to knock down IL-10 expression in murine BMDDCs in vitro led to increased CD40 expression and IL-12 production after maturation, without affecting CD80, CD86, or CD83. The resulting IL-10 siRNA-treated DCs stimulated Th1 responses in vitro, as judged by increased IFN-γ and decreased IL-4 production by antigen-specific T cells [235]. Similar immunostimulatory effects were seen using DCs treated with IL-10 antisense oligonucleotides [236]. More recently, Guiducci et al. [237] found that the intratumoral injection of adenovirus-expressed human CCL16 chemokine into murine tumors along with systemic administration of CpG and blocking antibody to the IL-10R (IL-10Ra or CD210) led to the eradication of established tumors by switching intratumoral macrophages from an M2 to an M1 phenotype in mice.

Another immunosuppressive factor is suppressor of cytokine signaling 1 (SOCS1), which is a key negative regulator of the JAK/STAT pathway. In the B16 melanoma model, where tyrosinase-related protein 2 (TRP2) is a major tumor antigen, mice that were vaccinated with TRP2-pulsed DCs treated with SOCS1 siRNA followed by three injections of LPS responded with a massive increase (19.7%) in TRP2-tetramer<sup>+</sup> CD8<sup>+</sup> T cells in their spleens 14 days later [238]. Similarly, vaccination using DCs treated with SOCS1 siRNA generated enhanced anti-HIV Env CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses. In a DNA
TABLE 4. Adjuvanting Immune Responses by Countering the Immunosuppressive Effects of CD4+CD25+ Tregs

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism of action</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deletion of CD25+ cells</td>
<td>IL-2 diphtheria toxin conjugate (denileukin diftitox or ONTAK)</td>
<td>[210]</td>
</tr>
<tr>
<td>Blockade of CD25</td>
<td>Anti-CD25 antibody inactivation of Treg without depletion</td>
<td>[215]</td>
</tr>
<tr>
<td>GTR stimulation with agonistic antibody or GITR</td>
<td>Signals through the GTR block the suppressive effects of Treg</td>
<td>[52, 216–218]</td>
</tr>
<tr>
<td>CD40 stimulation</td>
<td>CD40 stimulation releases mouse but not human DCs from the inactivating effects of Tregs</td>
<td>[219, 220]</td>
</tr>
<tr>
<td>OX40 stimulation</td>
<td>OX40 stimulation using agonistic anti-OX40 antibody or OX40 ligand (OX40L) turns off the immunosuppressive effects of Tregs, and also causes CD4+CD25+ effector T cells to become resistant to the immunosuppressive effects of Tregs</td>
<td>[221, 222]</td>
</tr>
<tr>
<td>4-1BB stimulation</td>
<td>4-1BB stimulation turns off the immunosuppressive effects of Tregs</td>
<td>[223]</td>
</tr>
<tr>
<td>TLR stimulation of DCs</td>
<td>TLR stimulation using LPS or CpG ODN stimulates DCs to produce IL-6, which inactivates Tregs</td>
<td>[224–226]</td>
</tr>
<tr>
<td>TLR2 stimulation of Treg</td>
<td>TLR2 (e.g., using Pam3CSK4), unlike TLR4 stimulation with LPS or TLR9 stimulation with CpG ODN, causes CD4+CD25+ Tregs to proliferate but lose their immunosuppressive function</td>
<td>[227]</td>
</tr>
<tr>
<td>TLR8 stimulation of Treg</td>
<td>TLR8 stimulation of Treg with Poly-G10 ODN turns off the immunosuppressive functions of CD4+CD25+ Tregs</td>
<td>[89]</td>
</tr>
<tr>
<td>Combined TLR3 and TLR7 stimulation of DCs</td>
<td>Synergetic stimulation of DCs with TLR3 with poly(I:C) and TLR7 with R-848 prevented the suppressive effects of Treg</td>
<td>[24]</td>
</tr>
<tr>
<td>P2X7 receptor stimulation</td>
<td>NAD, ATPe, and benzoylbenzoyl-ATP (a nonhydrolyzable derivative of ATP) are selectively toxic to CD4+CD25+ Tregs in vitro and in vivo</td>
<td>[121, 202]</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>IFN-γ antagonizes the generation of CD4+CD25+ Tregs</td>
<td>[178]</td>
</tr>
</tbody>
</table>

Programmed cell death 1 (PD-1)/PD-1 ligand (PD-L1) inhibition

PD-1 is a CD28-related protein that interacts with two ligands, PD-L1 and PD-L2, to convey a negative signal on T cells and B cells. PD-1-deficient CD8+ T cells were not susceptible to the tolerance-inducing effects of resting DCs pulsed with a lymphocytic choriomeningitis virus (LCMV) peptide [240]. Mice that were chronically infected by LCMV-Clone 13, which infects macrophages, had “exhausted” antiviral CD8+ T cells, which had activation surface markers and strongly expressed PD-1 yet lacked effector function. By blocking PD-1 or PD-L1 (also known as B7-H1), the exhausted antiviral CD8+ T cells regained their ability to proliferate, secrete cytokines, kill virus-infected cells, and decrease viral load in LCMV-infected mice even in the absence of CD4+ T cells [241]. This suggests that approaches to block PD-1/PD-L1 interactions using antibodies [242, 243], siRNA, antisense oligonucleotides, or even plasmid DNA encoding the extracellular domain of PD-1 as a soluble blocking molecule [244] could enhance vaccine-induced immune responses.

Conversely, engagement of PD-L2 (also known as B7-DC) using a cross-linking antibody (sHiG12M) stimulated IL-12 production by murine BMDDCs and led to the control of metastatic B16 melanoma [245]. PD-L2 cross-linking up-regulated the APC function of these DCs and increased CD8+ T cell responses, and the combination of PD-L2 cross-linking and CpG ODN was synergistic for inducing CD8+ T cell responses [246]. Thus, strategies for blocking PD-1/PD-L1 interactions may need to avoid blocking the positive effects mediated by PD-L2.

Agents prolonging DC survival as molecular adjuvants

Unless fully activated, DCs are rapidly eliminated by apoptosis. For example, one effect of CD40 stimulation on DCs is that it can up-regulate and activate a Fas-mediated apoptotic pathway, which is a plausible explanation for the otherwise paradoxical involvement of CD40L in the induction of tolerance. Using siRNAs to knock down Fas expression by DCs allowed these cells to avoid apoptosis following CD40 stimulation in vitro, which would be predicted to enhance a resulting immune response [247]. In a DNA vaccination model in mice, Kim et al. [248] gave intradermal injections of siRNAs to knock down Bak and Bax, two proapoptotic proteins, in the lymph nodes draining the vaccination site and found an enhancement in vaccine-elicited CD8+ T cell responses.

An alternate way to prolong DC survival is to introduce antiapoptotic genes into them at the time of vaccination. The coadministration of an antigen plasmid along with a plasmid for bcl-xL, bcl-2, cross-linked inhibitor of apoptosis, dominant-
negative caspase-9, or dominant-negative caspase-8 led to stronger CD8⁺ T cell responses and antitumor immunity in a mouse model, and Bcl-xl was the best antiapoptotic factor.

COMBINATIONS OF IMMUNOSTIMULATORY AGENTS

The idea of immunostimulatory combinations is actually more than 25 years old, based on in vivo observations that mice infected with organisms that grow intracellularly in macrophages (e.g., mycobacteria, brucella, and histoplasma) become extremely sensitive to the lethal effects of bacterial endotoxin. An excellent study from that period showed a correlation between macrophage activation in vivo (as determined by the ability to control Listeria infection) and the ability of crude endotoxin to produce rapid shock and death [250]. This two-component system led to the concept that many macrophage effector functions were induced in two stages, priming and triggering. The major priming factor was determined to be IFN-γ, and the triggering factors included LPS (TLR4 agonist) or a cytokine factor that would now be considered to be soluble CD40L [175]. Since then, numerous immunostimulatory combinations have been described. In this section, these combinations will be classified on the basis of the receptors stimulated. In the last portion, some particularly noteworthy combinations will be discussed in more detail.

Synergy between combinations of TLR agonists

Gao et al. [251] provided one of the earliest reports of TLR synergies. They found that the in vitro treatment of murine macrophages with LPS (TLR 4 agonist) + CpG ODN (TLR9 agonist) was synergistic for TNF production compared with each agent individually. It is interesting that the dual stimulation needed to be applied for 24 h for synergy to occur and was not seen when 2–8 h treatments were used. A similar study by Hume et al. [69] found that LPS + CpG ODN or LPS + digested bacterial DNA was synergistic for IL-12 production by murine BMDDCs.

In another in vitro study, poly(I:C) (TLR3 agonist) + CpG ODN (TLR9 agonist) synergistically stimulated murine macrophages to produce IL-12, TNF, IL-6, and NO and up-regulated MHC I on splenic DCs. Although the production of cytokines was not dependent on type I IFNs, the increase in NO production was dependent on the autocrine production of IFN-β. In vivo, this combination of two TLR agonists reduced the number of pulmonary metastases from B16F10 melanoma [252].

In an elegant study, Gautier et al. [105] found that poly(I:C) (TLR3) or LPS (TLR4) synergized with R-848 (TLR8) in murine BMDDCs for inducing IL-12p70 production. In contrast, the combination of poly(I:C) + LPS was not synergistic. This synergy was dependent on type I IFN production, as it was not observed in mice that were deficient for the type I IFN receptor (IFN-α/βR) or its downstream signaling component STAT1. At the mRNA level, the synergistic combinations were also synergistic for IFN-β but not IFN-α mRNA accumulation, where the IFN-β mRNA increase required IFN-α/βR, suggesting an autocrine and/or paracrine loop. Compatible results were obtained using human MDDCs. For human DCs, LPS and less so, poly(I:C) up-regulated the levels of IFN-λ1 (IL-29) mRNA. Individually, LPS and poly(I:C) but not R-848 in human MDDCs led to the phosphorylation and nuclear translocation of IRAF3, a major transcription factor for IFN-β production, suggesting that R-848 (TLR8 in humans) induced IFN-β production through an IRAF3-independent pathway, which might involve IRF7 and IRF8. These authors hypothesized that LPS and poly(I:C), signaling through the TRIF pathway, could activate IRAF3 to generate IFN-β, which in turn induced the synthesis of IRF7 as a substrate for phosphorylation and activation as a result of TLR7 or TLR8 MyD88 pathway-dependent stimulation.

These results were confirmed and extended in an important study by Napolitani et al. [23] using human MDDCs in vitro. These authors found that poly(I:C) (TLR3) or LPS (TLR4) was synergistic with R-848 (TLR8) and caused these DCs to produce 50- to 100-fold more IL-12p70, IL-23, and δ-4 than was induced by any of the agonists used individually. TNF, IL-6, and IL-10 were also synergistically induced. Whereas TLR stimulation normally produces a transient effect on DCs, which is undetectable by 24 h [69, 70, 253], the two TLR combinations led to prolonged expression of IkBα, a transcription factor that mediates TLR signaling, and prolonged phosphorylation of c-Jun, a transcription factor important in cytokine synthesis. The MDDCs activated by these two TLR combinations were superior at stimulating naive allogeneic CD4⁺ T cells to differentiate into IFN-γ-producing Th1 cells. The two TLR combinations also induced a >20-fold mRNA for IFN-λ1 (IL-29), GM-CSF, G-CSF, and LIF when compared with the effect of each TLR agonist individually. Similarly, although each individual TLR agonist up-regulated IL-1β mRNA, the two TLR combinations produced further up-regulation at the mRNA level. It is intriguing that although the single TLR agonists were essentially unable to induce IL-1β processing and release, the two TLR combinations induced the secretion of a large amount of this protein, suggesting that they somehow activated caspase-1, which is needed to process pro-IL-1β into active IL-1β [23]. As an explanation for their results, Napolitani et al. [23] proposed a “combinatorial code”, where TLR3 and TLR4 signal through the TRIF pathway, and TLR8 signals through the MyD88 pathway [95, 96], and the engagement of two DC activation pathways accounts for the synergism. Although the TRIF pathway can lead to the production of IFN-β [254], the addition of exogenous IFN-β did not duplicate the contribution of the TLR3 or TLR4 agonists to the synergism with TLR8 agonists, suggesting that autocrine production of IFN-β was not sufficient to replace TLR3 or TLR4 agonists for the synergistic effects [23].

This strategy has already been applied to vaccination. Hokey et al. [255] stimulated murine BMDDCs with poly(I:C) + CpG ODN in vitro and found a marked synergy for IL-12p70 production and the polarization of antigen-specific to CD4⁺ T cells toward IFN-γ-producing Th1 phenotype. If the DCs were first loaded with B16 melanoma cells and then stimulated with poly(I:C) and CpG ODN, mice vaccinated with these DCs were able to slow the growth of B16 tumors, which became infiltrated with CD4⁺ T cells and macrophages but not CD8⁺ T cells.
More recently, Warger et al. [24] confirmed that murine BMDCs were synergistically stimulated by poly(I:C) (TLR3) + R-848 (TLR7 in the mouse) to produce RANTES, IL-6, MCP-1, and IL-12p70 and also enhanced the surface expression of OX40L, CD27L/CD70, and 4-1BBL. For RANTES and IL-6, this double TLR stimulation led to faster and more prolonged cytokine production. The two TLR-activated DCs led to polarized, antigen-specific Th1 CD4+ T cell responses and almost completely eliminated the immunosuppressive effects of CD4+CD25+ Tregs on CD4+ T cells in vitro. These DCs were strong stimulators for antigen-specific CD8+ T cells and greatly reduced the immunosuppressive effects of CD4+CD25+ Tregs on antigen-specific CD8+ T cell responses in vitro. In mice vaccinated by the i.p. injection of peptide-pulsed BMDCs, the two TLR-stimulated DCs elicited much stronger CD4+ and CD8+ T cell responses, thereby confirming the in vivo relevance of the synergy observed in vitro.

Synergy between TNFRSF agonists and TLR agonists

By itself, CD40L is a weak stimulator of IL-12 production by DCs, although it is sufficient to induce DCs to produce many other cytokines and chemokines [182, 256]. However, when LPS (TLR4 agonist) was added to CD40L stimulation, human MDDCs produced large amounts of IL-12p70 in vitro [257, 258]. Similarly, the addition of dsRNA via influenza virus infection (TLR3 agonist) to CD40L-stimulated human PDCs led to the production of IL-12p70 and type I IFNs, which in turn activated type I Th cell responses [97]. For murine DCs, CD40L + CpG ODN (TLR9 agonist) synergistically activated DCs to make IL-12 [259], and this combination also stimulated macrophage-mediated antitumor effects on established B16 melanoma in mice [260]. For human PDCs, which express TLR9 and can respond to CpG ODN (unlike MDCs [13, 14]), the combination of CD40L + CpG ODN was required for IL-12 production and the stimulation of Th1 cells [261]. Agonistic anti-CD40 antibody plus poly(I:C) had synergistic antitumor effects in mice [262] and enhanced the response to a DC-directed Gag vaccine in mice [263].

In a remarkable study, Ahonen et al. [264] vaccinated mice i.p. with OVA protein, agonistic anti-CD40 antibody, and various TLR agonists: Malp2 (TLR2/6), poly(I:C) (TLR3), LPS (TLR4), 27609 (TLR7), and CpG ODN (TLR9). As single agents, the CD40 stimulus or the TLR agonists only mildly stimulated the generation of anti-OVA CD8+ T cells. However, when the CD40 and TLR agonists were added, in only 6 days, as many as 16% of splenic CD8+ T cells were specific for OVA by tetramer staining, which was a tenfold increase above CD40 or the TLR agonists alone. For most of the TLR agonists, this synergistic effect was absent in mice lacking the type I IFN receptor, except for Malp2 and to a lesser extent, LPS. This report is one of the best examples of how a combination of molecular adjuvants can be used for vaccination.

A subsequent study of OVA vaccination examined the effects of 4-1BB (CD137) stimulation combined with poly(I:C) (TLR3) and found a similarly remarkable expansion of antigen-specific CD8+ T cells (up to 17% of splenic CD8+ T cells were tetramer+) 6 days later. In this study, a large number of functional, antiviral CD8+ T cells were found in lymphoid and nonlymphoid tissues more than 1 year post-vaccination. In this case, it was shown that the CD8+ T cells must carry the 4-1BB receptor, indicating that the stimulus acted directly on these cells and not on an APC [265].

Synergy and antagonism between NLR agonists and TLR agonists

A NOD1 activator, GM-TriDAP, was found to synergize with Pam3CysLys4 (TLR1/2), Malp2 (TLR2/6), LPS (TLR4), and resiquimod (TLR7/8) for cytokine production by human PBMCs [266]. In human monocytes and DCs, a NOD1 activator (M-triDAP) was found to synergize with LPS (TLR4) for cytokine production [267].

A NOD2 activator, MDP, was found to synergize with Pam3CysLys4 (TLR1/2), Malp2 (TLR2/6), poly(I:C) (TLR3), or LPS (TLR4) for cytokine production in mouse macrophages [268, 269]. Conversely, an inhibitory effect of NOD2 was suggested when splenic macrophages from NOD2−/− mice were found to release larger amounts of IL-12 after stimulation with bacterial cell wall peptidoglycan, a TLR2 agonist that also contains MDP as a NOD2 agonist. In this case, the negative effect of NOD2 stimulation on TLR2 was limited to IL-12 production and not to TNF or IL-10 production [270]. Another study found that MDP activation of NOD2 synergized with CpG ODN (TLR9) for cytokine production by human PBMCs [271]. In the human THP-1 monocyte cell line, MDP synergized with Pam3CysLys4 (TLR1/2), lipid A (TLR4), or CpG ODN (TLR9) for IL-8 production [272, 273]. Another study used vitamin D3-differentiated THP-1 cells and likewise found that MDP synergized with LPS (TLR4) or lipoteichoic acid (TLR2) to stimulate IL-8 production [274]. In human monocytes and DCs, NOD2 activators (MDP or MtriLYS) were found to synergize with purified LPS (TLR4) for cytokine production [267]. In mice, MDP synergized with agonists for TLR2, TLR4, or TLR9 for the induction of cytokines and other responses [269, 272–275]. In dogs, the ability of mononuclear blood cells from dogs to control the growth of tumor cells in vitro was increased synergistically by MDP combined with LPS (TLR4) [276].

For human MDDCs in culture, there was a synergistic effect on IL-12p70 production when FK565 (NOD1 agonist) or MDP (NOD2 agonist) was combined with lipid A (TLR4), poly(I:C) (TLR3), or CpG ODN (TLR9) but not with Pam3CSSNA (TLR1/2) [275].

Another example of NLR/TLR antagonism has been reported for Monarch-1 (Pypaf7), a NLR protein, which is expressed by resting monocyctic cells. TNF treatment of these cells reduced Monarch-1 expression. Conversely, Monarch-1 expression reduced NF-κB activation by TLR2 or TLR4 stimulation. Knockdown of Monarch-1 using siRNA (with or without TNF treatment) markedly increased the amount of IL-1β released by THP-1 monocyteid cells in response to Pam3CSK4 simulation (TLR2 agonist) or LPS (TLR4 agonist) [277]. This antagonism between Monarch-1 and TLR2 is reminiscent of the reported antagonism of NOD2 with TLR2 [270], and the relief of this inhibition using siRNA suggests that siRNA to Monarch-1 or NOD2 could be combined with molecular adjuvants designed to stimulate TLR2 and/or TLR4 [277].
Synergy between TNFRSF agonists and cytokine/chemokine receptor agonists

IFN-γ and also IL-3 and GM-CSF up-regulated the CD40 receptor on human monocytes and dramatically enhanced the ability of CD40L to induce these cells to produce IL-6, IL-8, and TNF [278]. It is important that IFN-γ greatly augmented the ability of CD40L to stimulate IL-12p70 production in murine BMDDCs [279] and human MDDCs [124, 257, 280, 281]. Although less active than IFN-γ, IFN-α also augmented CD40L-induced stimulation of IL-12p70 production by human MDDCs [282]. Furthermore, additional cytokines such as IL-4, IL-13, and GM-CSF also synergized with CD40L for DC production of IL-12p70 by DCs [279, 283]. In vivo, agonistic anti-CD40 antibody combined with IL-2 showed synergistic antitumor activity in mice [284].

Synergy between TLR agonists and cytokine/chemokine receptor agonists

IFN-γ primed mouse macrophages to produce IL-12p70 after LPS (TLR4) stimulation [176, 285], and a similar synergy for IL-12p70 production was found using human MDDCs [257]. Similarly, IFN-γ and CpG ODN (TLR9) synergized in stimulating mouse macrophages to produce NO [286]. For human monocytes, GM-CSF synergized with phosphorothioate backbone ODN (whether or not they contained CpG motifs) for the induction of CCL3/MIP-1α and CCL4/MIP-1β. This combination stimulated monocytes to differentiate into DCs, as judged by the loss of the CD14 monocyte surface marker, the gain the CD33 DC surface protein, and an up-regulation of TLR9 mRNA [175]. In vivo, plasmid DNA encoding IFN-β combined with poly(I:C) (TLR3) in a cationic lipid formulation reduced the ability of CD40L to stimulate IL-12p70 production by human MDDCs [279].

Synergy between NLR agonists and cytokine/chemokine receptor agonists

In vitro, the combination of FK565 (NOD1) and IL-2 led to the synergistic activation of lymphokine-activated killer cells, which were capable of killing tumor cells [289]. IFN-γ and MDP (NOD2) liposomes synergistically induced the tumor cell-killing activity of human monocytes by macrophages in vitro [290]. In vivo, GM-CSF and MTP-PE were synergistic in inducing antitumor killing by alveolar macrophages in dogs [291].

Synergy involving three or more molecular adjuvants

Given that various immunostimulants use different signaling pathways, it is reasonable that combinations of three or more molecular adjuvants could drive DCs to an even higher level of activation and thereby generate even stronger vaccine responses. A study by Lapointe et al. [258] provided the first indication that this might be the case. Using human MDDCs, these authors found that the triple combination of CD40L + IFN-γ + LPS led to the production of extremely high levels of IL-12p70 (~200 ng/ml). The same combination led to IL-10 production, but no more than was produced in the absence of IFN-γ.

In another study of human MDDCs, Luft et al. [282] found that CD40L + IL-1β + IFN-γ or CD40L + GM-CSF + IL-4 + IFN-γ led to high level IL-12p70 production and an expansion of IFN-γ-producing Th1 cells in vitro. A related study found that human MDDCs stimulated with CD40L + IL-1β + IFN-α2a or CD40L + IL-4 + IFN-α2a led to high levels of IL-12p70 production, which in turn stimulated allogeneic CD4+ T cells to become IFN-γ-producing Th1 cells. Similarly, Mailliard et al. [140] found that human MDDCs treated with poly(I:C) + IL-1-β + TNF + IFN-γ produced high levels of IL-12p70, and these activated DCs (which they term α-type-1-polarized DCs or αDC1) were especially effective for the in vivo generation of antigen-specific CD8+ T cells. Using murine BMDDCs, Hokey et al. [255] used a combination of poly(I:C) + CpG ODN + IFN-γ, which they refer to as a “DC1 cocktail”, and found that the addition of CD40L resulted in a marked prolongation in the production of IL-12p70 compared with using the DC1 cocktail without CD40L.

More recently, Napolitani et al. [23] found that human MDDCs stimulated with the double combinations of LPS + poly(I:C), LPS + R-848, or poly(I:C) + R-848 produced high levels of IL-12p70 (0.6–6 ng/ml). If IFN-γ were added as a third component, the levels of IL-12p70 increased from three- to 20-fold (reaching 8–20 ng/ml). If CD40L were added as the third component, the levels of IL-12p70 were higher still and increased ~30-fold (reaching 20–300 ng/ml) [23]. Given that IL-12p70 is effective on human cells in vitro in the range of 0.02–1 ng/ml [257], these triple combinations involving CD40L plus TRIF-signaling and MyD88-signaling TLR agonists clearly produced supraphysiological levels of IL-12p70, which would likely diffuse widely through lymphoid tissues to affect a large number of immune cells.

As an example of how triple combinations can lead to superior vaccines, Ahlers et al. [292] studied mice vaccinated with a peptide composed of a MHC-I-binding sequence from HIV Env fused to a MHC-II-binding Th cell sequence. In this system, GM-CSF + IL-12 + TNF was synergistic for CD8+ T cell responses and was required to generate effective CD8+ T cell responses against viral challenge with Env-expressing vaccinia. Similarly, peritumoral injections with the triple combination of CD40L + poly(I:C) + CpG ODN led to strong antitumor effects on established B16F10 melanoma in mice (G. W. Stone, R. S. Kornbluth, submitted).

FUTURE DIRECTIONS

From the above examples, it is evident that this is a golden age in vaccine research, especially for vaccines that elicit CD8+ T cell responses. Combinations of two or more molecular adjuvants are especially potent, but there are a large number of permutations that could be tested. One can envision two pathways for arriving at an optimal molecular adjuvant formulation for CD8+ T cell responses. The first pathway goes from vaccine pathways...
testing in mice to testing in the SIV/macaque model to testing in humans. This requires a means to compare one vaccine against another, which is one of the stated goals of the Global HIV Vaccine Enterprise initiative [293] but will likely take many years to accomplish. A second pathway goes from testing immunostimulators for antitumor effects in mice and then goes directly to human clinical trials. The difference is that testing a HIV vaccine requires testing in relatively healthy volunteers, and the side-effects must be minimal, whereas tumor immunotherapy can be given to the many patients with cancer who have run out of treatment options and who can accept the possibility of some initial toxicity. Thus, testing molecular adjuvants in the tumor immunotherapy setting will require less investment and will generate data about efficacy and toxicity much more rapidly than comparable HIV vaccine studies.

Although there are many differences between the two testing pathways, both promise to advance new formulations of molecular adjuvants, which will reach the top level of CD8+ T cell responses. At that point, the issues of tissue damage (“reactogenicity”) and the possible induction of autoimmunity can be addressed [294]. In this dawn of a new era of vaccinology, a final test of the fundamental assumption of HIV vaccine research, that it is possible to produce a vaccine strong enough to reliably protect humans from the progression of HIV infection to AIDS, can be foreseen. Only then will we know if HIV vaccines will fulfill the hope and effort that has been invested in their development.

ACKNOWLEDGMENTS

We thank Jeffrey Ahlers, Celsa Spina, François Villinger, Eyal Raz, Gregory Daniels, Dennis Carson, Douglas Richman, Joshua Fierer, Roberto Badaro, and Robert Schooley for stimulating discussions. This study was supported by NIH grant R21AI52842, American Foundation for AIDS Research (amfAR) grant 02719-28-RG, Mesothelioma Applied Research Foundation, and the Research Center on AIDS and HIV Infection of the VA San Diego Healthcare System. G.W.S. was supported by an NIH AIDS Training Grant to UCSD (T32A1007384).

REFERENCES


