Cell. Mol. Life Sci. 65 (2008) 3231 – 3240
1420-682X/08/203231-10
DOI 10.1007/s00018-008-8228-6

Cellular and Molecular Life Sciences

Review

Putting endotoxin to work for us: Monophosphoryl lipid A as a safe and effective vaccine adjuvant

C. R. Casella[a] and T. C. Mitchell[a, b, *]

[a] Institute for Cellular Therapeutics and [b]Dept. of Microbiology and Immunology, 570 S. Preston St., Donald Baxter Bldg., 4th floor, University of Louisville School of Medicine, Louisville, KY 40202 (USA), Fax: +502-852-2085, e-mail: tcmitc01@louisville.edu

Received 25 April 2008; received after revision 05 June 2008; accepted 10 June 2008
Online First 1 August 2008

Abstract. The development of non-infectious subunit vaccines greatly increases the safety of prophylactic immunization, but also reinforces the need for a new generation of immunostimulatory adjuvants. Because adverse effects are a paramount concern in prophylactic immunization, few new adjuvants have received approval for use anywhere in the developed world. The vaccine adjuvant monophosphoryl lipid A is a detoxified form of the endotoxin lipopolysaccharide, and is among the first of a new generation of Toll-like receptor agonists likely to be used as vaccine adjuvants on a mass scale in human populations. Much remains to be learned about this compound's mechanism of action, but recent developments have made clear that it is unlikely to be simply a weak version of lipopolysaccharide. Instead, monophosphoryl lipid A's structure seems to have fortuitously retained several functions needed for stimulation of adaptive immune responses, while shedding those associated with pro-inflammatory side effects.

Keywords. Endotoxin, monophosphoryl lipid A, vaccine adjuvant, immunity, inflammatory toxicity, MD-2, Toll-like receptor 4.

Introduction: The need for new vaccine adjuvants with little or no toxicity

Until recent years, vaccine design relied exclusively on infectious-attenuated or inactivated-whole viral particles or bacteria to establish prophylactic immunity to human pathogens. In the broad context of public health, these vaccines have made, and continue to make, contributions to the eradication of damaging or life-threatening diseases that are unprecedented in human history. However, some of these vaccines have adverse side effects such as local reactions, fever and joint pain, and in some rare cases death or contraction of the illness vaccinated against [1]. Rare complications that are real, or perceived risks that are not, coupled with the now low prevalence of many once common illnesses has lessened the publics' appreciation of the necessity of vaccination. This ironic outcome of the success of vaccination has brought an increased emphasis on safety to the governmental regulatory agencies that must approve new vaccines, as well as to the pharmaceutical concerns producing them. As a result of these many faceted trends in public opinion, public policy, and market pressures, completely non-infectious vaccines consisting of recombinant protein subunits from pathogens have come to be favored.

* Corresponding author.

A major complication of subunit vaccine development is that most recombinant proteins lack intrinsic immunostimulatory activity. Adjuvants are accordingly used to stimulate the immune system further, a benefit first used to boost the efficacy of inactivated-whole pathogen vaccines and now recognized as a virtual necessity in the context of subunit vaccination. In the USA, the only adjuvant compounds approved for use by the federal government continue to be aluminum salt precipitates (alum) that are used to aggregate immunogens. Alum has been used for over 70 years, is found in 80 % of all vaccines, and has been used in hundreds of millions of doses [2–4]. This extensive experience demonstrates that alum is safe with few side effects [2]. However, it promotes predominantly a Th2-type antibody response [3, 5] consisting of production of IgG4 and IgE isotypes, which are best suited for responses against extracellular pathogens and parasites rather than killing or phagocytosing pathogen-infected host cells. The specific bases for alum's Th2-bias are beginning to be elucidated. In experimental animals, alum stimulates a Gr-1^+ subpopulation of leukocytes to produce large amounts of IL-4 [6], a canonical Th2 cytokine and Gr-1^+ eosinophils are recruited within six hours of alum and antigen injection [7]. This early recruitment of IL-4 producing eosinophils could explain the Th2 bias associated with use of alum.

Two considerations have combined to create the need to go beyond alum in the field of adjuvant design. First, for infectious diseases that are recognized as global health threats but against which vaccine development has been so far unsuccessful or only partially effective, such as HIV/AIDS, malaria and tuberculosis [2, 8, 9], a need for Th1-type immunity is widely acknowledged. Th1 immunity is marked by production of antibodies whose isotypes in humans, IgG1 and IgG3, are better able to opsonize and kill pathogens and pathogen-infected host cells (as opposed to rendering them non-infectious through neutralizing activity), as well as by generation of cytolytic $CD8^+$ T cell responses, which directly kill infected host cells. These latter cytocidal properties are critical for protection against intracellular pathogens. Second, even for "historical" vaccines that are already known to be effective, improvements brought by new adjuvants may have important public health benefits. For example, an ability to establish protective immunity with much less antigen than had previously been required might have greatly mitigated the effects of the worldwide influenza vaccine shortage of 2004–2005.

Even as subunit vaccine development has been limited by lack of access to a clinically acceptable adjuvant other than Th2-biased alum, the immunological research community has made tremendous strides in learning how to boost immune outcomes in laboratory settings. Chief among these advances is recognition that families of receptors, the Toll-like receptors (TLR), the Nod-like receptors (NLR), the C-lectin receptors (CLR), and complement, are responsible for innate recognition of a wide variety of microbial components (reviewed recently in [10–13]). Many of these components have therefore been widely studied as candidate adjuvants whose rapid stimulation of immune responses could be used to foster long-term adaptive responses to recombinant proteins in subunit vaccines. Unfortunately, the need for the lowest possible risk of adverse side effects excludes some of these microbial compounds from being used in prophylactic immunization of healthy subjects, and for others extensive testing is needed to ensure they would be safe for use. An important exception is monophosphoryl lipid A (MPLA), which is derived from the lipopolysaccharide fraction of the cell walls of gram-negative bacteria such as *Salmonella minnesota* and which boosts adaptive immunity via TLR4. The remainder of this review discusses the reasons that MPLA is poised to become the first of a new generation of TLR-stimulatory vaccine adjuvants to achieve widespread use in human populations.

Monophosphoryl lipid A: a thirty year journey from the laboratory to widespread use in humans.

Lipopolysaccharides (LPS) from numerous bacteria have been studied to understand their endotoxic and immunomodulatory properties (reviewed in [14]). In the 1970's, Edgar Ribi systematically subjected LPS to chemical modification in order to determine if its desirable immunostimulatory properties could be separated from its endotoxic effects [15]. Ribi eventually created a hydrolytic process in which LPS from *Salmonella minnesota* (which has up to seven acyl chains, three phosphates, and polysaccharides of varying length attached to a di-glucosamine head group, Figure 1A) was converted into a mixture of acylated di-glucosamines, the major species of which possesses six acyl side chains, no polysaccharide side chains and one phosphoryl group (Fig. 1B). This monophosphorylated mixture is widely known as MPL, but is abbreviated here as MPLA to distinguish it from the clinical-grade version manufactured by GlaxoSmithKline and trademarked as MPL adjuvant™. Toxicity and immunomodulatory functions were tested by Ribi and his co-workers by measuring the amount of MPLA needed for lethal effect in chick embryos and for protection from growth of an intradermally implanted tumor cell-line in a guinea pig

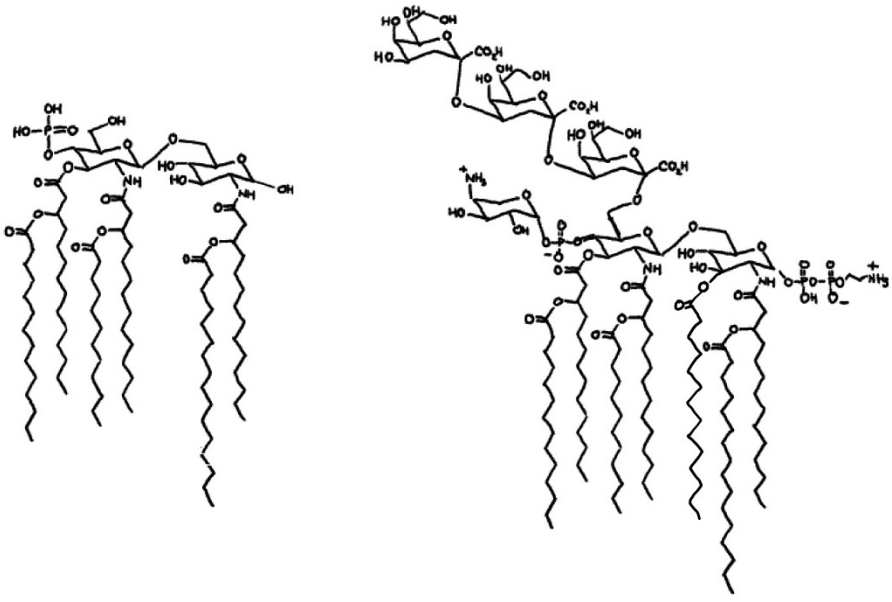

Figure 1. Structures of the major components of MPL adjuvant and LPS as prepared from S. minnesota. Monphosphoryl lipid A, left, is derived from lipopolysaccharide, right, by removal of one or more acyl chains, polysaccharide side groups, and two of three phosphates, as described elsewhere by Evans et al. [17].

tumor model, which showed that MPLA was at most 0.08 % as toxic as its LPS parent while functioning as well, if not better than LPS, in the tumor protection assay [16]. Ribi and colleagues concluded that MPLA was a detoxified version of LPS that retained most or all of the parent compound's beneficial immunomodulatory activities.

The most compelling evidence of MPLA's simultaneous safety and efficacy may be the degree to which it is being incorporated into new commercial vaccines. The company founded by Ribi to commercialize MPLA, Ribi Immunochemicals, for many years sold MPLA as part of its Ribi Adjuvant System, a formulation consisting of MPLA, trehelose, and oil, which is used extensively to generate monoclonal antibodies from experimental animals. Ribi Immunochemicals was acquired successively by two other commercial entities, first Corixa Corporation, and then GlaxoSmithKline Biologicals (GSK Biologicals), which purchased Corixa primarily to gain ownership of what it considers to be a key component of its next generation of vaccines [2]. The clinical grade form of MPLA, called MPL adjuvant^{TM} was approximately 0.1 % as toxic as LPS when tested in pre-clinical rabbit pyrogenicity assays [17], which is in strikingly good agreement with Ribi's early estimates using lethal chick embryo assays [16]. It is important to stress that MPL adjuvant^{TM} is generally added to, rather than used to replace, alum and other vaccine additives that improve 'mechanical' delivery of antigen. GSK Biologicals presently uses MPL adjuvant^{TM} in several vaccine formulations. The most widely used are the three "adjuvant systems": ASO1, ASO2, and ASO4 [2]. ASO4 is associated with the least risk of adverse events, and is a formulation of MPL adjuvant adsorbed onto either aluminum hydroxide or aluminum phosphate. AS04 is used in FENDrix and Cervarix vaccines, which confer protective immunity against hepatitis B virus and human papilloma virus, respectively. AS02 is an oil-in-water emulsion containing MPL adjuvant^{TM} and QS21, a water soluble triterpene glucoside with saponin detergent properties, and has been used to achieve notable protection against malaria in field trials of the RTS,S/AS02a vaccine [18, 19]. AS02 boosts $CD8^{+}$ cytolytic T cell responses to a greater degree than is true of AS04, while AS01 (liposomes mixed with MPL adjuvant and QS21) is better still, although at the potential cost of creating a somewhat higher risk of side effects [2, 20]. In these formulations, MPL adjuvant^{TM} has been delivered in more than 90 000 doses to human subjects with overall frequencies of adverse events that are as low as alum alone [21].

Numerous other studies have addressed the quality of the immune response fostered by both clinical and non-clinical grade forms of MPLA and in most was found to result in a Th1 or a blended Th1 and Th2-type response [22–30]. In some of these studies, the degree of Th1-associated immune responses depended on both the type of antigen being given as well as on the route of administration (intravenous vs. intranasal vs. subcutaneous injection), which indicates that MPLA has a strong but not overwhelming ability to promote Th1 responses. Vaccines containing MPL adjuvant have been registered for use in Europe (FENDrix) and Australia (Cervarix) and approval to use Cervarix is currently being sought of the USA's Food and Drug Administration. It is thus likely that MPL adjuvant^{TM}

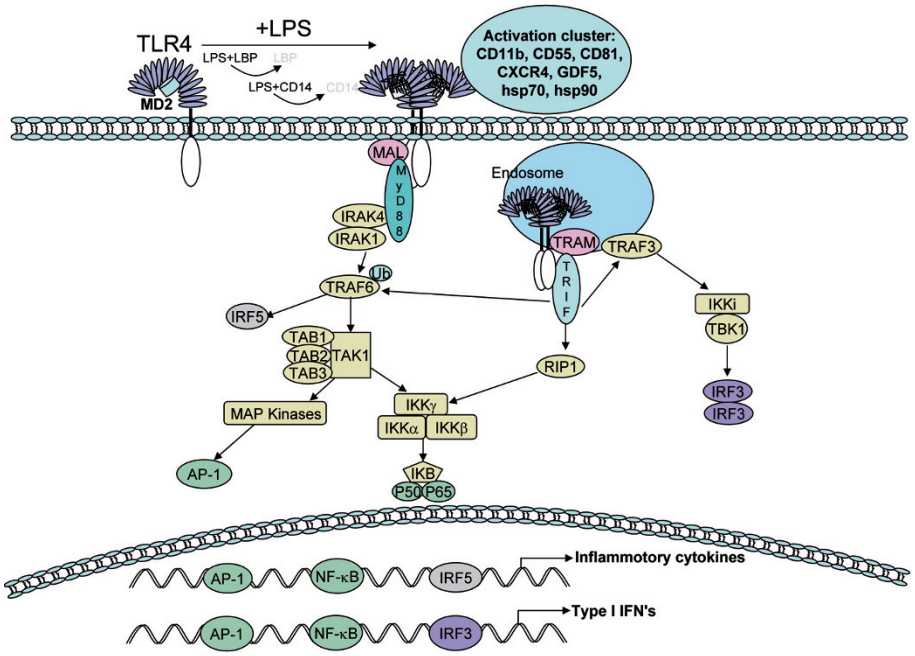

Figure 2. LPS-induced TLR4 signaling pathways. MD2 binding by LPS is achieved in coordination with LBP and CD14, after which the MD2-LPS complex binds TLR4 to form a higher order structure that includes components of an activation cluster. MyD88-dependent and Trif-dependent signaling are now thought to occur sequentially. Mal recruits MyD88 to TLR4 where IRAK4 binds MyD88. IRAK1 then binds IRAK4 leading to the activation and ubiquitinylation (Ub) of TRAF6. TRAF6 in turn activates the transcription factor IRF5. Ubiquitinated TRAF6 also interacts with the TAB1, 2, and 3 complex which activates TAK1 leading to MAP kinase and AP-1 activation, as well as nuclear mobilization of NFkB following phosphorylation of the IKK complex. These transcription factors lead to production of inflammatory cytokines such as IL-12, IL-1, IL-6, and TNFα. Trif-dependent signaling occurs after the MD2-LPS-TLR4 complex is endocytosed. In the endosome, Tram recruits Trif to TLR4 leading to TRAF3 and IKKi/TBK1 activation and the activation of transcription factor IRF3. Trif can also activate TRAF6 and RIP1 leading to active AP-1 and NF-κB, respectively. AP-1, NF-κB and IFR-3 together stimulate production of type 1 interferons such as INFβ. For more detailed description of these pathways, see reviews [10, 61, 78, 79].

will become the first adjuvant since the introduction of alum 70 years ago to be approved for wide use in prophylactic vaccination. If so, this development will represent the first TLR agonist to be used intentionally and expressly for its immunostimulatory properties. The approximately 30 years it has taken since Edgar Ribi first described generation of detoxified, immunoactive MPLA species to the beginnings of widespread use of MPL adjuvant in human populations illustrates the complexity of a necessary confluence of scientific advancement, requirements by public health and commercial entities for extremely low risks of vaccination, and by the pharmaceutical industry for marketable products likely to be accepted by the public. For the time being, MPLA offers a unique combination of efficacy and low toxicity that will serve as a model for future adjuvant development for many years to come. As a pioneer compound, what is learned about the mechanism of action of MPLA's low toxicity adjuvant effects will influence development of many, if not all, of its successors. This is especially true of synthetic versions of MPLA that are under development and which have so far shown an intriguingly wide range of immunostimulatory effects [31–34] and whose future use will benefit from a full understanding of the means by which MPLA functions as a low toxicity adjuvant.

Structure of the endotoxin receptor, MD2/TLR4

Both LPS and MPLA require TLR4 for adjuvant function [17, 35–38], indicating that MPLA retains its parent compound's binding affinity for at least some of the components of the endotoxin-recognition system (Fig. 2). Recognition of LPS is normally initiated by extraction of LPS monomers from aggregates by LPS-binding protein (LBP) in the serum. CD14 catalyzes transfer of LPS from LBP to MD2, the LPS-binding component of the receptor system, and MD2 then stimulates the signaling activities of TLR4, a class I transmembrane protein. Two crystal structures of MD2 have recently been reported, the first structure showing human MD2 loaded with lipid IVa, a tetra-acylated LPS antagonist, and the second structure showing mouse MD2 loaded with eritoran, another tetra-acylated antagonist, in complex at a 1:1 ratio with the extracellular domain of TLR4 [39, 40]. Both structures show that MD2 has an elongated pocket structure whose inner face is lined with hydro-

phobic residues and which binds the acyl chains of its lipid A ligands. An interesting discrepancy in these reports is that the positively charged residues present on the rim of the pocket of mouse MD2 were described as forming ionic bonds with the phosphate groups of eritoran [39], while similar lysine residues in human MD2 were not aligned well with phosphates in lipid IVa [40]. The latter dis-orientation was noted by Fitzgerald and Golenbock [41] as possibly providing the basis for the different biological activities of LPS and of MPLA, because the exposure of the mono- vs multiple phosphate groups to solvent meant that they could interact differently with TLR4. Another interesting possibility is that these exposed phosphates are left free to interact with yet other molecules. Indeed, the Triantafilou sisters have shown that MD2/TLR4 forms an "activation cluster" with several other cell surface molecules such as heat shock proteins 70 and 90, CXCR4, CD55 and others upon engagement by LPS [42]. It was determined that a synthetic monophosphoryl liped A (compound 505) initiated the same clustering of proteins in response to TLR4 interaction as did LPS [42]. However, it is possible that MPLA, while able to cluster similar proteins, is unable to fully engage members of the cluster resulting in different signaling outcomes.

Of course, the complexity of the MD2/TLR4 interaction alone is substantial and could be sufficient to explain low vs. high toxicity signaling outcomes. Kim et al. [39] reported that MD2/TLR4 heterodimers formed heterotetrameric structures upon addition of LPS, and proposed that LPS binding induced a conformational change in MD2 that caused each to bind simultaneously to two TLR4 molecules. A heterotetramer consisting of two LPS-loaded MD2 molecules, each of which was bound to two TLR4 molecules, could thus be assembled. Such a dimerizing effect on MD2/TLR4 would likely promote interaction of adapter proteins bound to the cytoplasmic tail of TLR4, which are discussed below. It is possible that the monophosphate structure of MPLA somehow makes this heterotetramerization, or the higher order aggregates described by Triantafilou et al., occur differently than the more charged structure of LPS or its "toxic core", diphosphoryl lipid A (hereinafter referred to as lipid A).

The mechanism of low toxicity signaling by monophosphorylated lipid A

The number of studies in which MPLA and LPS or lipid A have been compared directly is surprisingly small. This is in contrast to the many reports that involve tests of LPS alone, in endotoxin receptor research, or of MPL adjuvant™ in pre-clinical and clinical vaccine or immunotherapy trials. Could MPLA simply have very low affinity for the MD2/TLR4 endotoxin receptor, with weak signal strength explaining its low toxicity? MPLA's comparatively simple structure and low charge density make this plausible, but a handful of experimental observations suggest the issue is more complex. Direct comparisons of MPLA to LPS or lipid A show that MPLA has at least some functions that are of similar potency as those of its toxic counterparts. For example, Salkowski et al. showed that MPLA and LPS stimulate with equal efficiency the production of anti-inflammatory products such as IL-1 receptor antagonist and glucocorticoid receptor [43] from mouse macrophages. Okemoto et al. first reported [44], and Mata-Haro et al. [45] confirmed that IL-1β transcription is induced by MPLA as efficiently as it is by synthetic lipid A or LPS. Thompson et al. found similar potencies of MPLA and LPS in terms of adjuvant effects on T cell priming in a mouse model using the antigen ovalbumin. At higher doses, MPLA was actually more potent than LPS at boosting the clonal expansion of $CD4^+$ T cells responding to ovalbumin [31]. Demonstrations of MPLA's weak activity relative to LPS are also present in the literature [43, 46, 47]. In one study, Ismaili et al. found very little IL-12 production by human dendritic cells responding to MPLA relative to LPS [46]. But the fact that some immune outcomes can be induced with equal potency by the two compounds indicates that something more complicated than a simple strength-of-signal deficiency is at work. To understand how weak vs. potent activities of MPLA can be reconciled, it is necessary to consider in greater detail the signaling events that follow TLR4 clustering.

LPS-induced signaling via MD2/TLR4

Stimulation of the MD2/TLR4 complex by LPS generates signaling activity through two distinct pathways which have come to be known by the names of the TLR4-proximal adapter proteins, MyD88 and Trif (Fig. 2). Requirements for these adapters in TLR4-mediated signaling have been defined primarily in knock-out and induced mutant studies [48–53]. Of all 13 TLRs that have been identified to date, only TLR4 activates both signaling pathways; as discussed further below, this full-spectrum signaling activity downstream of TLR4 may account for the powerfully inflammatory effects of LPS.

MyD88 is widely viewed to be a "pro-inflammatory" branch of TLR4

LPS does not generate inflammatory shock in *myd88-/-* mice [48], but it can still induce 'slow' MAP kinase activity and NFκB mobilization to the nucleus, and can increase expression of major histocompatibility complex II (MHCII) and costimulatory B7 by antigen-presenting cells (APC) [48, 51, 52]. The failure of LPS to drive inflammatory toxicity in these mice while at the same time inducing stimulatory or co-stimulatory molecules associated with adaptive T cell immunity was one of the observations that caused us to begin testing LPS in *myd88*-/- mice. Our experiments indicated that MyD88 was not required for adjuvant effects on T cell priming [45]. Interestingly, MyD88 is reported elsewhere to be needed to allow adjuvants to inhibit the suppressive activity of $CD25^+$ Treg cells [54] and for long-term retention of previously primed T cells [55]. Hence, some level of MyD88-associated signaling downstream of TLR4 is likely to be needed for robust adaptive immune responses such as those generated by T cells.

The MyD88-dependent pathway of TLR4 signaling is frequently depicted as inducing pro-inflammatory cytokine production, which is associated with NFκB. In fact, both the MyD88 and the Trif-dependent pathways stimulate NFκB activity, although MyD88-induced stimulation is characterized as 'rapid' while Trif-induced stimulation is 'slow', reflecting the fact that NFκB is activated within 10 min of LPS stimulation in wild-type cells, but takes up to 20 min in MyD88- deficient cells [48, 51, 52]. The 'slow' activation of NFκB in MyD88- deficient cells has been proposed to result from the sequential activation of Mal/MyD88 and Tram/Trif pathways (Fig. 2). In this model, MyD88 signaling is initiated from the inner face of the plasma membrane almost immediately after TLR4 activation. The TLR4 complex then undergoes endocytosis, with the Tram/Trif pathway stimulated via interactions with the endosomally associated signaling molecule TRAF3 [53]. This sequence of events likely explains the delay in Trif-dependent signaling events, as compared to those of MyD88.

Given the generalized depiction of MyD88-dependent signaling as the "pro-inflammatory" pathway, it is perhaps surprising that genetic deficiency in components of the MyD88-*in*dependent pathway leads to endotoxin-resistant phenotypes that are similar to those of *myd88-/-* mice. Notably, both MyD88- and Trif-deficient mice are resistant to LPS-induced septic shock and fail to make maximal levels of IL-6, TNF and IL-12p40 in response to LPS *in vitro* [48–51]. This presumably means, as has been concluded elsewhere [49, 50], that both the MyD88-dependent and -independent pathways are required for production of some of the cytokines associated with inflammatory shock. Hoebe and Beutler [50] describe the pathways as being "superadditive" for expression of some genes, with MyD88 and Trif each mediating, say, 10% of maximal expression when signaling alone and 100% when signaling together. Other gene products do appear to be specifically dependent on signaling through one or the other pathway. Examples of these, for Trif-specific signaling, are the chemokines CXCL10 (aka IP-10), and RANTES, and interferon-associated gene products Ifit1 and Ifit2 [49, 50, 56] which are not expressed by Trif-deficient macrophages upon stimulation, but are expressed by *myd88-/-* cells [52, 56, 57]. Especially important is the observation that TLR4/Trif pathway activates interferon response factor-3 (IRF-3), which is involved in production of type I IFNs ([49, 50, 58, 59], reviewed in [10, 60, 61]). Conversely, expression of IFNγ, Cox-2, MIP-1β, CXCL-1, and the serine protease inhibitor serpine 1, among others, are abrogated or greatly reduced in *myd88-/-* cells [48, 52, 62, 63].

Monophosphoryl lipid A as a Trif-biased agonist of MD2/TLR4

In our recent attempts to understand more about how MPLA could function so potently as an adjuvant for T cell priming [31], while having so little inflammatory effect, we performed gene expression profiling on tissues from mice 6 h after they were given immunizing antigen and LPS or MPLA as adjuvant [45]. Because our original intent was to understand the inflammatory environments experienced by T cells, we performed the gene expression analysis on whole spleens and recorded the expression levels of several cytokines, chemokines and other secreted factors. Intracellular products were initially ignored in this approach because splenic populations have such complex cellularity. Two major conclusions were reached from this analysis. First, MPLA had induced transcription of several secreted products to the same levels as those induced by LPS, while others were markedly lower; hence, MPLA did not produce merely a weaker pattern of gene expression when compared to LPS but a discrete subset. Second, it became apparent that the weaker expression levels generally were of genes associated with the MyD88-dependent pathway of TLR4, while those induced to similar levels as LPS were more likely to be associated with the Trif-dependent pathway. Measurement of the levels of proteins secreted into the peripheral blood of immunized mice generally supported these trends,

which prompted further tests of intracellular signaling events *in vitro*. For signaling experiments, cultured macrophages derived from bone marrow were selected because they can be prepared as more homogeneous populations in comparison to whole splenic populations, and because macrophages are of primary importance in mediating LPS-induced septic shock [14]. These experiments again supported the idea that MPLA and LPS were of equal potency in terms of inducing Trif-associated signaling events (activation of IRF-3, secretion of IFNβ, and phosphorylation of Stat1 in response to autocrine/paracrine exposure to type I interferons), while the MyD88-associated 'rapid' stimulation of NFκB was both delayed and reduced. Finally, our study showed that neither LPS nor MPLA required MyD88 to have robust adjuvant effects on T cell priming, whereas expression of Trif was more important. Put together, these patterns caused us to propose that MPLA is an agonist of TLR4 that is functionally biased to Trif-associated signaling intermediates and endpoints because MyD88-associated outcomes were markedly weaker.

Beutler and colleagues and others have previously noted that TLR4 signaling is capable of signaling in different "modes", thanks to the complexity of its adapter usage [41, 64]. For TLR4, MyD88-dependent signaling occurs through yet another adapter known as Mal, which plays a critical role in recruiting MyD88 to the inner face of the plasma membrane and ultimately to the cytoplasmic tail of TLR4 [57, 65–68]. Similarly, Trif is assisted by a co-adapter named Tram which recruits TLR4 to early endosomal compartments where it helps initiate signaling through Trif and TRAF3 [53, 58, 59, 69, 70]. Depending on the agonist used to stimulate TLR4, these four adapter proteins are required to different extents, some that involve primarily Mal-MyD88 [71] and others that require primarily Tram [72]. Thus, MPLA may be the latest example of a TLR4 agonist whose stimulatory activity is selective for one adapter set or another.

An important alternate view of MPLA's low toxicity was offered recently by Okemoto et al., who proposed that MPLA's lack of pro-inflammatory activity is due to its inefficient activation of caspase-1 [44]. Production of some inflammatory cytokines such as IL-1β and IL-18 are tightly controlled, with regulatory mechanisms governing expression at both transcriptional and post-transcriptional levels. Caspase-1, also known as interleukin-1 converting enzyme, plays an important role in maturation of these cytokines by cleaving the precursor forms pro-IL-1β and pro-IL-18. The mature forms are then exocytosed via a non-classical protein secretion pathway [73]. Using mouse macrophages and monocytic cell-lines, Okemoto et al. found that MPLA potently stimulated transcription of IL-1β mRNA, as well as its translation into pro-IL-1β protein, but failed almost completely to induce secretion of mature IL-1β into the culture medium [44]. Very similar patterns were found in our subsequent study: IL-1β transcription was strong in splenocytes of MPLA-treated mice, but production of circulating IL-1β in serum was very low as compared to that seen in LPS-treated mice [45]. Stimulation of IL-1β transcription via TLR4 is known to be MyD88-dependent [48, 63], which indicates either that MPLA has no impairment in terms of MyD88 stimulation (in opposition to our model), or that IL-1β transcription is induced *in vivo* through the secondary effects of MyD88-independent cytokines, or that MPLA is capable of inducing sufficient levels of MyD88-dependent signaling so as to stimulate some MyD88 transcripts (*il-1β*) but not others. Understanding the cause-and-effect relationship, if any, that exists between MPLA's Trif-biased signaling and its ability to prime Il-1β maturation awaits further definition, which can be done by testing caspase-1 and/or IL-1 receptor-deficient cells for the extent to which Trif-biased outcomes occur.

Yet another explanation for MPLA's low toxicity adjuvant function is that it stimulates higher levels of IL-10, a cytokine with anti-inflammatory effects [43]. Such a gain-of-function, relative to LPS, would elegantly explain the low toxicity adjuvant effects that MPLA has on antibody production because increased IL-10 production both limits the extent to which pro-inflammatory factors such as IFNγ are expressed, and contributes directly to B cell responses [43]. Moreover, it has been reported that MPLA can stimulate TLR2 and TLR4, both of which contributed to MPLA's ability to induce IL-10 production by human monocytes [74]. Perhaps stimulation of TLR2, in addition to TLR4, produces higher levels of IL-10 than LPS, leading to a diminished inflammatory immune response. Alternatively, stimulation of TLR2, a receptor that is thought to be strictly MyD88-dependent, at the same time as TLR4 could lead to competition for the MyD88 signaling branch such that MPLA cannot signal completely from either TLR2 or TLR4. A problem with the IL-10 component of these hypotheses, however, is that we saw no differences in the levels of IL-10 produced in mice treated with LPS or MPLA [45]. It is possible that there are small differences in IL-10 levels that were missed in our study, or that human cells react differently than mouse cells.

Other evidence exists that MPLA has a gain-of-function ability to restrain inflammatory environments, even if not mediated by IL-10. An important example is the effect that MPLA has on pro-inflammatory complications unexpectedly caused by a

vaccine against respiratory syncytial virus (RSV) [75]. In the 1960 s, children given formalin-fixed RSV as part of a vaccine trial were found to suffer dramatic lung pathologies upon subsequent exposure to live infectious RSV. More recently, in a cottontail rat model of lung pathology, co-administration of MPLA with formalin-fixed RSV was shown to prevent the excessively pro-inflammatory reaction to challenge infection [76]. MPLA-dependent effects in this important study included a diminution of a broad array of cytokines, both Th1 and Th2-associated, suggesting that MPLA had not merely re-directed the type of immune response but had instead suppressed many of its pro-inflammatory components. Preferential induction of IL-10 by MPLA was not evident, indicating either that its expression was not detected because it was temporally restricted, or confined to anatomical sites, or that other anti-inflammatory mechanisms were responsible. Indeed, the authors of the report suggested that MPLA was successful for another reason altogether: that when paired with formalin-fixed RSV, MPLA desensitized TLR4 to further stimulation by the RSV fusion protein (also known as F protein), a strong pro-inflammatory agonist of TLR4 [77]. Whatever the mechanism, this study is an important indication that MPLA might actively moderate inflammation in some contexts, as opposed to simply failing to cause it to occur.

Concluding remarks

The success of MPL adjuvant in clinical trials, and its acceptance as a safe vaccine additive by regulatory agencies in Europe and in Australia, is a dramatic and pioneering example of safe immunostimulation via alterations in TLR signaling. In the case of MPLA, low toxicity was selected for, and not designed on a rationale basis because its discovery pre-dated that of the TLR family by 18 years. With the ever increasing need for safe ways to improve vaccine efficacy, however, rational manipulation of adjuvants must improve to the point that useful signaling pathways can be kept while harmful ones are left unstimulated or are actively suppressed. Several competing ideas have now appeared to explain the low toxicity function of MPLA; which idea will 'win' is less important than achieving a true understanding of how it occurs so that improvements to both efficacy and safety can continue to be made in future rounds of adjuvant development.

Acknowledgements. The authors are grateful for support received from the Barnstable-Brown Foundation, the W.M. Keck Foundation, the Commonwealth of Kentucky Research Challenge Trust Fund, the Jewish Hospital Foundation, and United States Public Health Service awards AI071047 and AI059023.

1 Jacobson, R. M. (2003). Vaccine safety. Immunol. Allergy Clin. North Am. 23, 589–603.
2 Garcon, N., Chomez, P., and Van, M. M. (2007). GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. Expert. Rev. Vaccines. 6, 723–739.
3 Brewer, J. M. (2006). (How) do aluminium adjuvants work? Immunol. Lett. 102, 10–15.
4 Glenny, A. T., Pope, C. G., Waddington, H., and Wallace, U. (1926). Immunological notes. XXIII. The antigenic value of toxoid precipitated by potassium alum. pp. 38–45.
5 McKee, A. S., Munks, M. W., and Marrack, P. (2007). How do adjuvants work? Important considerations for new generation adjuvants. Immunity 27, 687–690.
6 Jordan, M. B., Mills, D. M., Kappler, J., Marrack, P., and Cambier, J. C. (2004). Promotion of B cell immune responses via an alum-induced myeloid cell population. Science 304, 1808–1810.
7 Kool, M., Soullie, T., van, N. M., Willart, M. A., Muskens, F., Jung, S., Hoogsteden, H. C., Hammad, H., and Lambrecht, B. N. (2008). Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J. Exp. Med. 205, 869–82.
8 Golkar, M., Shokrgozar, M. A., Rafati, S., Musset, K., Assmar, M., Sadaie, R., Cesbron-Delauw, M. F., and Mercier, C. (2007). Evaluation of protective effect of recombinant dense granule antigens GRA2 and GRA6 formulated in monophosphoryl lipid A (MPL) adjuvant against Toxoplasma chronic infection in mice. Vaccine 25, 4301–4311.
9 Pulendran, B. and Ahmed, R. (2006). Translating innate immunity into immunological memory: implications for vaccine development. Cell 124, 849–863.
10 Lee, M. S. and Kim, Y. J. (2007). Signaling pathways downstream of pattern-recognition receptors and their cross talk. Annu. Rev. Biochem. 76, 447–480.
11 Franchi, L., Park, J. H., Shaw, M. H., Marina-Garcia, N., Chen, G., Kim, Y. G., and Nunez, G. (2008). Intracellular NOD-like receptors in innate immunity, infection and disease. Cell. Microbiol. 10, 1–8.
12 Robinson, M. J., Sancho, D., Slack, E. C., LeibundGut-Landmann, S., and Reis e Sousa (2006). Myeloid C-type lectins in innate immunity. Nat. Immunol. 7, 1258–1265.
13 Kemper, C. and Atkinson, J. P. (2007). T-cell regulation: with complements from innate immunity. Nat. Rev. Immunol. 7, 9–18.
14 Beutler, B. and Rietschel, E. T. (2003). Innate immune sensing and its roots: the story of endotoxin. Nat. Rev. Immunol. 3, 169–176.
15 Ribi, E., Parker, R., Strain, S. M., Mizuno, Y., Nowotny, A., von Eschen, K. B., Cantrell, J. L., McLaughlin, C. A., Hwang, K. M., and Goren, M. B. (1979). Peptides as requirement for immuno therapy of the guinea-pig line-10 tumor with endotoxins. Cancer Immunol. Immunother. 7, 43–58.
16 Qureshi, N., Takayama, K., and Ribi, E. (1982). Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of Salmonella typhimurium. J Biol. Chem. 257, 11808–11815.
17 Evans, J. T., Cluff, C. W., Johnson, D. A., Lacy, M. J., Persing, D. H., and Baldridge, J. R. (2003). Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529. Expert. Rev. Vaccines. 2, 219–229.
18 Alonso, P. L., Sacarlal, J., Aponte, J. J., Leach, A., Macete, E., Milman, J., Mandomando, I., Spiessens, B., Guinovart, C., Espasa, M., Bassat, Q., Aide, P., Ofori-Anyinam, O., Navia, M. M., Corachan, S., Ceuppens, M., Dubois, M. C., Demoitie, M. A., Dubovsky, F., Menendez, C., Tornieporth, N., Ballou, W.

R., Thompson, R., and Cohen, J. (2004). Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. Lancet 364, 1411–1420.

19 Alonso, P. L., Sacarlal, J., Aponte, J. J., Leach, A., Macete, E., Aide, P., Sigauque, B., Milman, J., Mandomando, I., Bassat, Q., Guinovart, C., Espasa, M., Corachan, S., Lievens, M., Navia, M. M., Dubois, M. C., Menendez, C., Dubovsky, F., Cohen, J., Thompson, R., and Ballou, W. R. (2005). Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. Lancet 366, 2012–2018.

20 Pichyangkul, S., Gettayacamin, M., Miller, R. S., Lyon, J. A., Angov, E., Tongtawe, P., Ruble, D. L., Heppner, D. G., Jr., Kester, K. E., Ballou, W. R., Diggs, C. L., Voss, G., Cohen, J. D., and Walsh, D. S. (2004). Pre-clinical evaluation of the malaria vaccine candidate P. falciparum MSP1(42) formulated with novel adjuvants or with alum. Vaccine 22, 3831–3840.

21 Baldridge, J. R., McGowan, P., Evans, J. T., Cluff, C., Mossman, S., Johnson, D., and Persing, D. (2004). Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. Expert. Opin. Biol. Ther. 4, 1129–1138.

22 Johansen, K., Schroder, U., and Svensson, L. (2003). Immunogenicity and protective efficacy of a formalin-inactivated rotavirus vaccine combined with lipid adjuvants. Vaccine 21, 368–375.

23 Qiao, M., Murata, K., Davis, A. R., Jeong, S. H., and Liang, T. J. (2003). Hepatitis C virus-like particles combined with novel adjuvant systems enhance virus-specific immune responses. Hepatology 37, 52–59.

24 Coler, R. N., Skeiky, Y. A., Bernards, K., Greeson, K., Carter, D., Cornellison, C. D., Modabber, F., Campos-Neto, A., and Reed, S. G. (2002). Immunization with a polyprotein vaccine consisting of the T-Cell antigens thiol-specific antioxidant, Leishmania major stress-inducible protein 1, and Leishmania elongation initiation factor protects against leishmaniasis. Infect. Immun. 70, 4215–4225.

25 Zhang, P., Yang, Q. B., Marciani, D. J., Martin, M., Clements, J. D., Michalek, S. M., and Katz, J. (2003). Effectiveness of the quillaja saponin semi-synthetic analog GPI-0100 in potentiating mucosal and systemic responses to recombinant HagB from Porphyromonas gingivalis. Vaccine 21, 4459–4471.

26 Brandt, L., Elhay, M., Rosenkrands, I., Lindblad, E. B., and Andersen, P. (2000). ESAT-6 subunit vaccination against Mycobacterium tuberculosis. Infect. Immun. 68, 791–795.

27 Richards, R. L., Rao, M., Wassef, N. M., Glenn, G. M., Rothwell, S. W., and Alving, C. R. (1998). Liposomes containing lipid A serve as an adjuvant for induction of antibody and cytotoxic T-cell responses against RTS,S malaria antigen. Infect. Immun. 66, 2859–2865.

28 Sasaki, S., Tsuji, T., Hamajima, K., Fukushima, J., Ishii, N., Kaneko, T., Xin, K. Q., Mohri, H., Aoki, I., Okubo, T., Nishioka, K., and Okuda, K. (1997). Monophosphoryl lipid A enhances both humoral and cell-mediated immune responses to DNA vaccination against human immunodeficiency virus type. Infect. Immun. 65, 3520–3528.

29 Samuel, J., Budzynski, W. A., Reddish, M. A., Ding, L., Zimmermann, G. L., Krantz, M. J., Koganty, R. R., and Longenecker, B. M. (1998). Immunogenicity and antitumor activity of a liposomal MUC1 peptide-based vaccine. Int. J. Cancer 75, 295–302.

30 Drachenberg, K. J., Wheeler, A. W., Stuebner, P., and Horak, F. (2001). A well-tolerated grass pollen-specific allergy vaccine containing a novel adjuvant, monophosphoryl lipid A, reduces allergic symptoms after only four preseasonal injections. Allergy 56, 498–505.

31 Thompson, B. S., Chilton, P. M., Ward, J. R., Evans, J. T., and Mitchell, T. C. (2005). The low-toxicity versions of LPS, MPL adjuvant and RC529, are efficient adjuvants for CD4+ T cells. J. Leukoc. Biol. 78, 1273–1280.

32 Baldridge, J. R., Cluff, C. W., Evans, J. T., Lacy, M. J., Stephens, J. R., Brookshire, V. G., Wang, R., Ward, J. R., Yorgensen, Y. M., Persing, D. H., and Johnson, D. A. (2002). Immunostimulatory activity of aminoalkyl glucosaminide 4-phosphates (AGPs): induction of protective innate immune responses by RC-524 and RC-529. J. Endotoxin. Res. 8, 453–458.

33 Johnson, D. A., Sowell, C. G., Johnson, C. L., Livesay, M. T., Keegan, D. S., Rhodes, M. J., Ulrich, J. T., Ward, J. R., Cantrell, J. L., and Brookshire, V. G. (1999). Synthesis and biological evaluation of a new class of vaccine adjuvants: aminoalkyl glucosaminide 4-phosphates (AGPs). Bioorg. Med Chem. Lett. 9, 2273–2278.

34 Cluff, C. W., Baldridge, J. R., Stover, A. G., Evans, J. T., Johnson, D. A., Lacy, M. J., Clawson, V. G., Yorgensen, V. M., Johnson, C. L., Livesay, M. T., Hershberg, R. M., & Persing, D. H. (2005). Synthetic toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. Infect. Immun. 73, 3044–3052.

35 Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P., and Malo, D. (1999). Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J. Exp. Med. 189, 615–625.

36 Muta, T. and Takeshige, K. (2001). Essential roles of CD14 and lipopolysaccharide-binding protein for activation of toll-like receptor (TLR)2 as well as TLR4 Reconstitution of. Eur. J. Biochem. 268, 4580–4589.

37 Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11, 443–451.

38 Hirschfeld, M., Ma, Y., Weis, J. H., Vogel, S. N., and Weis, J. J. (2000). Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor. J. Immunol. 165, 618–622.

39 Kim, H. M., Park, B. S., Kim, J. I., Kim, S. E., Lee, J., Oh, S. C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O. J., and Lee, J. O. (2007). Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell 130, 906–917.

40 Ohto, U., Fukase, K., Miyake, K., and Satow, Y. (2007). Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. Science 316, 1632–1634.

41 Fitzgerald, K. A. and Golenbock, D. T. (2007). Immunology. The shape of things to come. Science 316, 1574–1576.

42 Triantafilou, M., Brandenburg, K., Kusumoto, S., Fukase, K., Mackie, A., Seydel, U., and Triantafilou, K. (2004). Combinational clustering of receptors following stimulation by bacterial products determines lipopolysaccharide responses. Biochem. J. 381, 527–536.

43 Salkowski, C. A., Detore, G. R., and Vogel, S. N. (1997). Lipopolysaccharide and monophosphoryl lipid A differentially regulate interleukin-12, gamma interferon, and interleukin-10 mRNA production in murine macrophages. Infect. Immun. 65, 3239–3247.

44 Okemoto, K., Kawasaki, K., Hanada, K., Miura, M., and Nishijima, M. (2006). A potent adjuvant monophosphoryl lipid A triggers various immune responses, but not secretion of IL-1beta or activation of caspase-1. J. Immunol. 176, 1203–1208.

45 Mata-Haro, V., Cekic, C., Martin, M., Chilton, P. M., Casella, C. R., and Mitchell, T. C. (2007). The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 316, 1628–1632.

46 Ismaili, J., Rennesson, J., Aksoy, E., Vekemans, J., Vincart, B., Amraoui, Z., Van, L. F., Goldman, M., and Dubois, P. M. (2002). Monophosphoryl lipid A activates both human dendritic cells and T cells. J. Immunol. 168, 926–932.

47 Saha, D. C., Barua, R. S., Astiz, M. E., Rackow, E. C., and Eales-Reynolds, L. J. (2001). Monophosphoryl lipid A stimulated up-regulation of reactive oxygen intermediates in human monocytes in vitro. J. Leukoc. Biol. 70, 381–385.

48 Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11, 115–122.

49 Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301, 640–643.

50 Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., and Beutler, B. (2003). Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature 424, 743–748.

51 Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K., and Akira, S. (2001). Endotoxin-induced maturation of MyD88-deficient dendritic cells. J. Immunol. 166, 5688–5694.

52 Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P. F., Sato, S., Hoshino, K., and Akira, S. (2001). Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J. Immunol. 167, 5887–5894.

53 Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat. Immunol. 9, 361–368.

54 Pasare, C. and Medzhitov, R. (2004). Toll-dependent control mechanisms of CD4 T cell activation. Immunity 21, 733–741.

55 McAleer, J. P., Zammit, D. J., Lefrancois, L., Rossi, R. J., and Vella, A. T. (2007). The lipopolysaccharide adjuvant effect on T cells relies on nonoverlapping contributions from the MyD88 pathway and CD11c+ cells. J. Immunol. 179, 6524–6535.

56 Weighardt, H., Jusek, G., Mages, J., Lang, R., Hoebe, K., Beutler, B., and Holzmann, B. (2004). Identification of a TLR4- and TRIF-dependent activation program of dendritic cells. Eur. J. Immunol. 34, 558–564.

57 Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., Takeda, K., and Akira, S. (2002). Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature 420, 324–329.

58 Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Visintin, A., Latz, E., Monks, B., Pitha, P. M., and Golenbock, D. T. (2003). LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J. Exp. Med. 198, 1043–1055.

59 Oshiumi, H., Sasai, M., Shida, K., Fujita, T., Matsumoto, M., and Seya, T. (2003). TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J. Biol. Chem. 278, 49751–49762.

60 Takeda, K. (2005). Evolution and integration of innate immune recognition systems: the Toll-like receptors. J. Endotoxin. Res. 11, 51–55.

61 Kawai, T. and Akira, S. (2006). TLR signaling. Cell Death. Differ. 13, 816–825.

62 Kaisho, T., Hoshino, K., Iwabe, T., Takeuchi, O., Yasui, T., and Akira, S. (2002). Endotoxin can induce MyD88-deficient dendritic cells to support T(h)2 cell differentiation. Int. Immunol. 14, 695–700.

63 Bjorkbacka, H., Fitzgerald, K. A., Huet, F., Li, X., Gregory, J. A., Lee, M. A., Ordija, C. M., Dowley, N. E., Golenbock, D. T., and Freeman, M. W. (2004). The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. Physiol. Genomics 19, 319–330.

64 Beutler, B. (2005). The Toll-like receptors: analysis by forward genetic methods. Immunogenetics, 57:385–92.

65 Kagan, J. C. and Medzhitov, R. (2006). Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell 125, 943–955.

66 Horng, T., Barton, G. M., Flavell, R. A., and Medzhitov, R. (2002). The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature 420, 329–333.

67 Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A., and O'Neill, L. A. J. (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature 413, 78–83.

68 Dunne, A., Ejdeback, M., Ludidi, P. L., O'Neill, L. A., and Gay, N. J. (2003). Structural complementarity of Toll/interleukin-1 receptor domains in Toll-like receptors and the adaptors Mal and MyD88. J. Biol. Chem. 278, 41443–41451.

69 Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat. Immunol. 4, 1144–1150.

70 Rowe, D. C., McGettrick, A. F., Latz, E., Monks, B. G., Gay, N. J., Yamamoto, M., Akira, S., O'Neill, L. A., Fitzgerald, K. A., and Golenbock, D. T. (2006). The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc. Natl. Acad. Sci. USA 103, 6299–6304.

71 Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M., and Beutler, B. (2005). CD14 is required for MyD88-independent LPS signaling. Nat. Immunol. 6, 565–570.

72 Georgel, P., Jiang, Z., Kunz, S., Janssen, E., Mols, J., Hoebe, K., Bahram, S., Oldstone, M. B., and Beutler, B. (2007). Vesicular stomatitis virus glycoprotein G activates a specific antiviral Toll-like receptor 4-dependent pathway. Virology 362, 304–313.

73 Delaleu, N. and Bickel, M. (2004). Interleukin-1 beta and interleukin-18: regulation and activity in local inflammation. Periodontol. 2000. 35, 42–52.

74 Martin, M., Michalek, S. M., and Katz, J. (2003). Role of innate immune factors in the adjuvant activity of monophosphoryl lipid A. Infect. Immun. 71, 2498–2507.

75 Kim, H. W., Canchola, J. G., Brandt, C. D., Pyles, G., Chanock, R. M., Jensen, K., and Parrott, R. H. (1969). Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J Epidemiol. 89, 422–434.

76 Boukhvalova, M. S., Prince, G. A., Soroush, L., Harrigan, D. C., Vogel, S. N., and Blanco, J. C. (2006). The TLR4 agonist, monophosphoryl lipid A, attenuates the cytokine storm associated with respiratory syncytial virus vaccine-enhanced disease. Vaccine 24, 5027–5035.

77 Kurt-Jones, E. A., Popova, L., Kwinn, L., Haynes, L. M., Jones, L. P., Tripp, R. A., Walsh, E. E., Freeman, M. W., Golenbock, D. T., Anderson, L. J., and Finberg, R. W. (2000). Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat. Immunol. 1, 398–401.

78 Krishnan, J., Selvarajoo, K., Tsuchiya, M., Lee, G., and Choi, S. (2007). Toll-like receptor signal transduction. Exp. Mol. Med. 39, 421–438.

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