that any differences that are observed in the responses between the two groups of mast cell–reconstituted mice do not reflect simply a difference in the success of mast cell reconstitution. If for whatever reason the mast cells with defined mutations are less capable of surviving, locally migrating or maturing than the adoptively transferred wild-type mast cells, this would confound the interpretation of experiments designed to compare the functions of the two adoptively transferred mast cell populations.

Another point needing resolution is whether the mast cell–dependent differences observed in the McLachlan *et al.* study can be associated with differences in the acquired immune response to the bacterial infection analyzed or in its clinical course (such as morbidity and survival). In other words, does the defect in lymph node enlargement correlate with a demonstrable defect in host defense? Finally, by what mechanisms do mast cells orchestrate T cell recruitment and lymph node enlargement in this model? The authors suggest that mast cell– and TNF-dependent enhancement of expression of vascular cell adhesion molecule 1 mediates these effects. But this may not be the entire story, particularly given recent evidence that mast cell–derived leukotriene B_4 may also contribute to T cell recruitment[12].

Although McLachlan *et al.* have focused on lymphocyte recruitment and lymph node enlargement during experimental infections with *E. coli*, studies with mast cell–reconstituted WBB6F1-$Kit^W/Kit^{W\text{-}v}$ mice have linked mast cells with the leukocyte recruitment and/or tissue changes associated with a wide variety of immunological and nonimmunological biological responses, including mouse models of some very important human diseases, such as asthma[1] and rheumatoid arthritis[2] (**Fig. 1**). The prior work as well as the study by McLachlan *et al.* represent early efforts in a large general area for future investigation: defining the importance of mast cells as regulators of remodeling or other tissue changes during immune responses and in many other settings.

In studies such as these, one must first determine whether mast cells are important in those aspects of the response under investigation. One then should have a try at understanding how mast cells exert these effects. Finally, one can consider whether this better understanding of the pathogenetic sequence in the model in question indicates new therapeutic possibilities. Given the large and growing spectrum of disease processes and adaptive responses with which mast cells have been associated, this represents an enormous challenge to the field, as well as a potentially large opportunity to reduce disease and improve health.

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Adjuvants and their signaling pathways: beyond TLRs

Egil Lien & Douglas T Golenbock

TLR ligands mediate adjuvant effects. Unlike lipopolysaccharide, poly(I:C) is capable of using a TLR-Trif–independent pathway to induce costimulatory molecule upregulation on antigen-presenting cells.

Adjuvants enhance the immune response to an antigen with which they are mixed. Thus, they often form an essential part of vaccines. The identification of new adjuvants is therefore an important challenge for vaccine development in the fight against the world's most common infectious killers, including malaria and tuberculosis, for which even natural infection itself is insufficient to induce protective immunity. The development of adjuvants has traditionally been focused on both modified microbial products and synthetic mimics of these molecules. An exceptionally large percentage of these immunologically active microbial molecules are ligands for the family of Toll-like receptors (TLRs), archetypical activators of innate immunity[1]. The most studied TLR ligand is bacterial lipopolysaccharide (LPS; endotoxin), the main component of the outer membrane of Gram-negative bacteria. LPS activates animal cells through TLR4 in cooperation with its coreceptor, MD-2 (ref. 1). Another TLR ligand that has been studied intensively is poly(I:C), considered to be a surrogate of viral double-stranded RNA, which uses TLR3 (refs. 1,2). In this issue of *Nature Immunology*, Hoebe and colleagues[3] address the mechanism behind the adjuvant effect induced by these two compounds, both *ex vivo* and *in vivo*.

This study follows earlier reports[4,5] of mice with a mutation in Trif (also known as TICAM-1), one of five known adaptor proteins containing a Toll–interleukin 1 receptor–resistance domain[6]. Trif functions as an adaptor protein for TLR3 and TLR4 signaling. Trif-deficient mice have a profound defect in activation of the transcription factor NF-κB and the related release of proinflammatory cytokines after exposure to poly(I:C) and LPS[4,5]. Complicating this picture is the finding that Trif is not the sole adaptor associated with TLR3 and TLR4 (**Fig. 1**). MyD88, the first adaptor to be discovered, has been believed to associate with all the known TLRs to induce proinflammatory cytokines[6]. MAL (also known as Tirap), associates with MyD88 to mediate TLR2 and TLR4 signaling[6]. Finally, the most recently discovered adaptor protein, called TRAM, mediates TLR4 signaling. Engagement of TRAM leads to activation of NF-κB, and the subsequent release of inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 6; it also induces cytokines regulated by the transcription factors IRF-3 and IRF-7, such as interferon-β (IFN-β)[7,8].

Egil Lien and Douglas T. Golenbock are at the University of Massachusetts Medical School, Division of Infectious Diseases and Immunology, 364 Plantation Street, Lazare Research Building 308, Worcester, Massachusetts, 01605, USA.
e-mail: egil.lien@umassmed.edu or douglas.golenbock@umassmed.edu

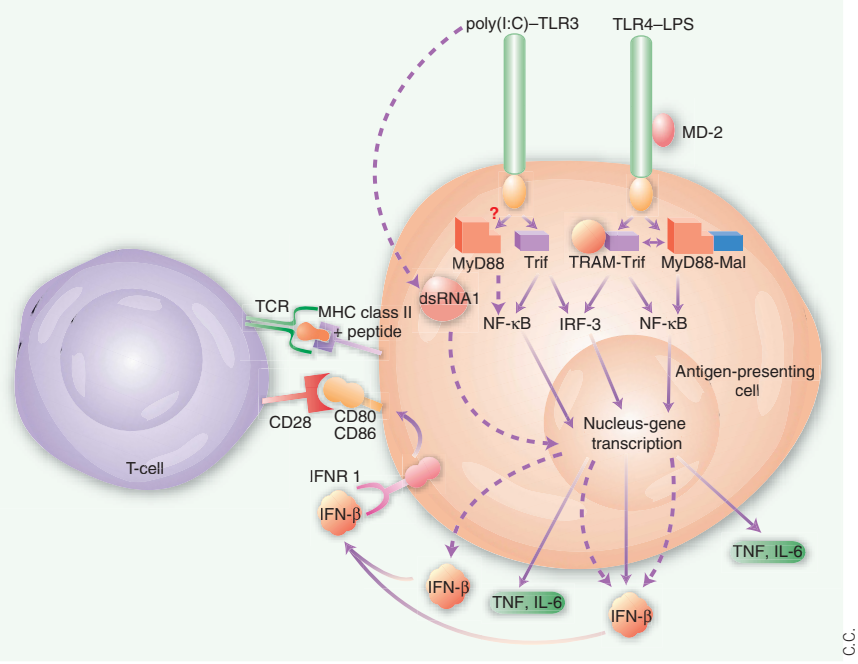

Figure 1 Innate immune signaling pathways that enhance antigen presentation. After LPS or poly(I:C) (double-stranded RNA; dsRNA) challenge, both TLR-dependent and TLR–independent signaling pathways lead to upregulation of costimulatory molecules (such as CD80 and CD86) and adjuvant effects, as suggested by Hoebe[3] and others[9]. In this issue, Hoebe *et al.*[3] suggest Trif (TICAM-1) as the common adapter molecule in TLR3- and TLR4-mediated responses leading to upregulation of CD80 and CD86, whereas a new locus (which they call *dsRNA1*) may be responsible for a non-TLR response to poly(I:C). Furthermore, IFN-RI signaling apparently is central in a feedback loop leading to upregulation of CD80 and CD86. Of the other TLR adapters, MyD88 interacts with MAL (Tirap), mediating the release of proinflammatory cytokines through TLR4, whereas it is unclear whether MyD88 interacts with TLR3 at all. A new adapter called TRAM[7] seems to pair with Trif, mediating MyD88-independent signals through TLR4. Some TLRs may function as intracellular receptors.

It is becoming increasingly apparent that the specificity and fine tuning of responses to TLR ligands are determined by both the relevant receptor and its ability to interact with specific combinations of downstream adaptor molecules.

Hoebe *et al.* delve into the mechanisms mediating upregulation of the costimulatory molecules CD80, CD86 and CD40 after poly(I:C) and LPS stimulation. Their study focuses on the phenotype of Trif mutant mice (which express a dominant negative version of Trif known as *Lps2*), as well as mice deficient in TLR3, MyD88, interferon receptor type I (IFN-RI) and the double-stranded RNA–interacting protein kinase (PKR). Hoebe *et al.* show type I interferon, most likely IFN-β, is crucial in the pathway leading to costimulatory molecule upregulation induced by both LPS and poly(I:C). Distinct differences in mechanisms between the adjuvant activity induced by LPS and poly(I:C) emerged. Most startling was the finding that poly(I:C)-induced upregulation of the costimulatory molecules was mediated in part by a TLR3-Trif-independent pathway. Indeed, Hoebe *et al.* identified a genetic locus on mouse chromosome 7 that is responsible for this. This locus does not correspond to any of the known TLRs. An earlier study[9] supports the idea of a central function for IFN-RI adjuvant effects and agrees with the existence of a TLR3-independent pathway for poly(I:C). However, that earlier study[9] suggests only limited involvement of type I interferon in LPS adjuvant activity.

Despite the clear-cut demonstration of involvement of type I interferons *ex vivo*, the question remains as to whether IFN-RI signaling is also essential for the *in vivo* adjuvant effects of LPS, poly(I:C) and their mimetics. In addition, certain issues will take time to be resolved. For example, the Trif mutant mice are clearly defective in the adjuvant effect mediated by LPS, but so are MyD88-deficient mice, which show normal upregulation of costimulatory molecules. The accessibility of different TLR adaptors to their receptors in different cell types and tissues, and the ability of these adaptors to generate adjuvant signals by TLR ligands, may prove a critical determinant of the subsequent acquired immune response. As four adaptor proteins seem to cooperate to mediate the full response to LPS and other TLR4 agonists[7], additional studies of adjuvanticity in mice with targeted deletions in one or more of each of these adaptor molecules are needed.

As with all important findings, Hoebe *et al.*[3] cover exciting new territory but leave certain issues unresolved. How important is the TLR3-Trif-independent pathway that is suggested? This pathway is not sensitive to inhibition by the drug 2-aminopurine, in contrast to the Trif pathway. Although this drug is thought to be an inhibitor of PKR, PKR-null mice upregulated CD80 and CD86 indistinguishably from wild-type mice, indicating that 2-aminopurine works by an alternative mechanism to suppress Trif signaling. *In vivo* testing of mice that seem to lack the TLR-independent pathway of adjuvanticity should answer questions related to this pathway. Earlier studies showed a severe impairment of poly(I:C)-induced cytokine release in cells from MyD88-deficient mice[2], whereas Hoebe *et al.* provide unequivocal data against this conclusion. Although several lines of evidence indicate that MyD88 has limited, if any, interaction with TLR3, this question needs to be resolved definitively. If MyD88 is dispensable for TLR3 signaling, it will shake the common belief that it is a 'core' adapter for all TLR signaling pathways. It has also been suggested that a TLR3-independent, PKR-dependent pathway induces IFN-α by intracellular (transfected) poly(I:C)[10]. Finally, a mystery well worth addressing is how different TLRs induce similar effects with different sets of adaptor molecules. For example, CpG oligodeoxynucleotides apparently induce all of their known effects through TLR9 and MyD88 alone.

The experiments of Hoebe *et al.* emphasize the potential for TLR ligands as future constituents of human vaccines, but simultaneously warn against simplistic conclusions. Few compounds have been licensed as adjuvant components of human vaccines, and improvements in the pharmacopoeia of adjuvants can be anticipated in the near future. Nevertheless, a key feature for a successful adjuvant is its safety for widespread use. Development of TLR ligands with a reasonable therapeutic index may prove challenging; after all, these are the very same molecules that cause fever and end-organ damage in septic patients.

What is the future for TLR ligands as vaccine adjuvants? Deciphering the complicated signaling pathways for the TLRs will undoubtedly lead to new ideas for how to manipulate this family of receptors for a maximal beneficial response. The

achievement of additional insights into signaling events that are mediated by each of the TLRs and their adaptors, and the delineation of non-TLR-mediated pathways with similar effects, seem a certain road to travel to achieve our goal of developing safe and powerful vaccine adjuvants.

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The NKT cell system: bridging innate and acquired immunity

Masaru Taniguchi, Ken-ichiro Seino & Toshinori Nakayama

Although natural killer T cells are activated during infection, it is not clear how this process occurs. Closer examination indicates that recognition of endogenous ligands and interleukin 12, rather than bacterial products, may drive the activation process.

The innate immune system is characterized by rapid responses to pathogens and is mediated mainly by macrophages, dendritic cells, granulocytes and natural killer (NK) cells. In contrast, the acquired immune system, composed of T and B lymphocytes, is characterized by memory or secondary antigen-specific immune responses. Among the cell types that have been postulated to link the two arms of the immune system, CD1d-restricted NKT cells are compelling candidates, being able to respond rapidly and subsequently to activate other cell types[1,2]. However, the detailed mechanism of NKT cell activation is unclear at present. In this issue of *Nature Immunology*, Brigl *et al.* provide evidence for the important functional involvement of self ligands in the activation of NKT cells during infection, and thus add to our understanding of NKT cells as a 'bridging system' between innate and acquired immunity[3].

Because of their apparent self-reactivity and ability to quickly release large quantities of cytokines such as interferon-γ (IFN-γ), NKT cells are hypothesized to be important in the initiation and regulation of various immune responses[1,2]. Accordingly, the function of self ligand in the activation of NKT cells and the function of NKT cells in the immune system have been subjects of much recent research, debate and speculation. Although the invariant $V_\alpha14$ receptor expressed by NKT cells specifically recognizes an unusual glycolipid, α-galactosylceramide (α-GalCer), in conjunction with CD1d[4], α-GalCer is an exogenous ligand. Thus, the presence of endogenous self ligands and their physiological importance continues to be a source of much speculation. This speculation is fueled by the observation that NKT cells seem to be constantly activated *in vivo*; freshly isolated NKT cells express activation markers such as CD69 and CD44. In addition, the use of the TCR $V_\beta8$ chain by NKT cells is restricted to one or two invariant sequences that are distinct in different tissues[1]. Finally, because no NKT cells develop in the absence of CD1d, it seems that developing NKT cells that recognize self ligands presented on CD1d are positively selected[5].

Although the molecular basis of self ligands for NKT cells remains unclear, their self-reactivity seems to constitute an important step in the initiation of protective immunity. The first cells in the innate system to be activated during an infection are dendritic cells. This activation is mediated by Toll-like receptors (TLRs), which sense bacterial products[6], leading to activation of the transcription factor NF-κB and the production of proinflammatory cytokines (interleukin 1 (IL-1), IL-6 and tumor necrosis factor-α), IL-12 and the upregulation of costimulatory molecules (CD80 and CD86) on dendritic cells (**Fig. 1**). Most importantly, IL-12 is essential for the activation of NKT cells and their subsequent production of IFN-γ during infection[3,7]. Indeed, only NKT cells, but not other cells such as naive T cells or NK cells, express substantial amounts of IL-12 receptor components[1], and NKT cells are primary targets for IL-12 for inducing antitumor activity *in vivo*[8,9]. However, although IFN-γ production by NKT cells is crucial for the induction of efficient protective immunity against pathogens, the precise mechanisms and the initial trigger events are unclear.

Brigl *et al.* now provide an answer to this important question[3]. During the first 2 or 3 days after infection, weak responses by NKT cells to self ligands are amplified by dendritic cell–derived IL-12 in response to TLR activation by microbial products, resulting in the production of IFN-γ. Thus, both IL-12-induced signaling and NKT cell activation by self ligands are necessary to initiate pathogen-specific immune responses. However, the recognition of a pathogen-derived cognate antigen is not required for NKT cell activation. Despite the ability of CD1d to present glycolipid antigen and the recognition of this presentation by $V_\alpha14$ receptor, pathogens do not seem to activate NKT cells directly. Instead, microbial products seem to be important only in stimulating dendritic cells through TLR signaling to produce IL-12.

Although recognition of self ligands and the subsequent weak responses of NKT cells are essential at the initial phase of bacterial infection, this self-reactivity does not elicit any effector functions of NKT cells *in vivo*. In addition, in the absence of dendritic cells, IL-12 alone does not activate NKT cells. Thus, the recognition of self ligand–CD1d is required for IL-12-mediated NKT cell activation. In contrast, the recognition of α-GalCer induces signals to activate NKT cells efficiently even in the absence of IL-12,

Masaru Taniguchi, Ken-ichiro Seino & Toshinori Nakayama are in the Laboratory for Immunoregulation, RIKEN Research Center for Allergy and Immunology, Tsurumi, Yokohama and Department of Molecular Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan.
e-mail: taniguti@med.m.chiba-u.ac.jp