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Educating CD4 T cells with vaccine adjuvants: lessons from lipopolysaccharide

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Abstract

Toll-like receptor (TLR) adjuvants are capable of driving T cell immunity. The TLR4 agonist LPS activates antigen-presenting cells through MyD88- and TRIF-dependent signaling pathways, which initiates CD4 T helper cell clonal expansion and differentiation. Lipopolysaccharide (LPS) supports the development of diverse T helper (Th) lineages, depending on the tissue microenvironment. For instance, peripheral immunization with LPS drives Th1 priming in lymphoid tissue and Th17 priming in the gut. This could be to the result of commensal bacteria inducing Th17-stabilizing cytokines within the intestinal lamina propria. Here, we detail how the response to LPS stimulates CD4 T cell priming in lymphoid tissue and the intestinal mucosa. How this knowledge might be exploited to target specific features of T cell immunity by vaccine adjuvants is also considered.

Using lipopolysaccharide (LPS) to break T cell tolerance

Vaccines are useful for controlling infectious threats, and contain a combination of antigenic peptides and adjuvants. Injection of animals with antigen alone often results in tolerance or non-responsiveness to subsequent exposures, therefore, adjuvants are necessary to stimulate the immune system to generate adaptive responses against the target antigen. LPS is a natural adjuvant that is synthesized by Gram-negative bacteria, and has contributed greatly to our understanding of how vaccines work. Like many natural adjuvants, LPS stimulates Toll-like receptor (TLR)4, which activates signaling pathways mediated by the adaptor proteins MyD88 and TRIF (reviewed in [1]). LPS signaling occurs in diverse cell types including non-hematopoietic cells, but has been best studied in antigen-presenting cells (APCs). Signaling through both adaptors activates nuclear factor (NF)- κ B, which leads to release of inflammatory cytokines. In addition, TRIF induces type I interferons (IFNs) through interferon regulatory factor 3, which stimulates dendritic cells (DCs) to express the co-stimulatory molecules CD40, CD80 and CD86 [2]. TLR-mediated activation of DCs is thought to be a major mechanism that explains how adjuvants divert away from tolerance towards immunity.

CD4 T cells are important for adjuvant effects since they orchestrate adaptive immune responses. DCs present antigen to specific T cells in lymphoid tissue, which results in their clonal expansion and functional differentiation. The activation state of DCs as well as the

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cytokine milieu determines if T cells develop into T helper (Th) 1, Th2, Th17, follicular helper T (Tfh), or regulatory T (Treg) cell lineages. LPS is a potent Th1 adjuvant, because injecting it systemically within a day after antigen exposure increases the level of T cell clonal expansion, long-term survival, IFN- γ production, and migration to non-lymphoid tissues (reviewed in [3]). LPS also promotes the accumulation of memory CD4 T cells into bone marrow [4], although the importance of this reservoir for memory T cells is unknown. The mechanism by which LPS drives Th1 immunity is multifactorial, including the induction of interleukin (IL)-12 and IFN- γ , activation of DCs through type I IFNs, and induction of T cell survival factors. These are desirable properties for vaccines, because they ensure the T cell quantity, quality, and localization are sufficient to control newly acquired infections. Here, we review how LPS promotes T cell immunity and its implications for vaccine design. We explore the individual roles of TLR signaling adaptors, how anatomical microenvironments influence the quality of T cell responses, and how the therapeutic potential of LPS might be harnessed while minimizing toxicity.

Using TLRs as co-stimulatory agents

Study of the contribution of individual cell types during immune responses to vaccines can provide new therapeutic targets. For example, distinct subsets of APCs might be responsible for T cell priming versus tolerance as a result of their anatomical localization or expression of co-stimulatory molecules. Depletion of CD11c⁺ DCs has revealed their crucial role in Th1 priming during immunization with LPS [5]. Although this suggests that direct contact between activated DCs and T cells is necessary for Th1 differentiation, the cell type(s) responsible for detecting LPS *in vivo* is another issue. After injection, LPS travels to the subcapsular sinus of lymph nodes [6]. Its molecular structure is thought to prevent it from crossing the subcapsular membrane, and therefore, it does not appear to stimulate cortical-region DCs directly. Rather, macrophages located within the subcapsular sinus are poised to detect LPS, which results in the release of inflammatory cytokines that then activate DCs. In support of this, mice with macrophages that are non-responsive to LPS have reduced serum levels of tumor necrosis factor, IL-6, and IL-12/23p40 after LPS injection [7]. *Ex vivo* isolated macrophages also express higher levels of TLR4 than do DCs [8], and DCs are efficiently activated by LPS in a bystander manner [9]. By this mechanism, activated DCs can present antigen to T cells as soon as 30 min after immunization [10]. At later time points, CD11b⁺ DCs activated in the periphery migrate to draining lymph nodes to sustain antigen presentation [10]. These findings support a model in which, in response to LPS, subcapsular macrophages initiate immune responses by releasing type I IFNs, which causes DCs to upregulate co-stimulatory molecules that promote Th1 differentiation. In support of this, induction of type I IFNs with the TLR3 ligand polyribinosinic–polyribocytidylic acid (poly I:C) drives Th1 responses indirectly through DC activation [11]. However, in another experimental model, direct DC activation by the TLR9 ligand CpG was required for Th1 priming [12]. Thus, the role of bystander activation versus direct DC activation in vaccine-induced immunity might depend on the TLR adjuvant.

TLR agonists are attractive candidates for vaccine adjuvants due to their ability to activate APCs, which results in the initiation of adaptive immunity. Recent studies have clarified that TLR agonists can also directly stimulate T cells, which adds to the mechanisms by which vaccines work. Although it is generally accepted that human T cells express TLR4 [13], whether murine T cells directly respond to LPS is controversial. Some studies have reported that mouse T cells, including Treg cells, are stimulated by LPS [13–15], whereas others demonstrate a more prominent role for other TLRs. The TLR ligands Pam3CysSK4 (TLR1 and TLR2 ligand), poly I:C (TLR3), flagellin (TLR5), R-848 (TLR7/8) and CpG-containing oligodeoxynucleotides (TLR9) can directly promote the survival, proliferation, cytokine production, and cytolytic activity of cultured mouse T cells [16–20]. *In vivo*, CpG acts on T cells to enhance specific antibody production following immunization [21]. During infection

with *Toxoplasma gondii*, MyD88 expression by T cells is required to control parasite burden [22]. MyD88 also mediates responsiveness to IL-1 and IL-18 in addition to TLR ligands. However, T cells that lack the IL-1 and IL-18 receptors, but possess a functional TLR pathway, control parasite burden [22], which demonstrates that MyD88 functions downstream of TLRs in this context. After infection with lymphocytic choriomeningitis or vaccinia virus, MyD88-deficient CD8 T cells are impaired in clonal expansion, possibly because of reduced survival after proliferation [23,24]. Finally, a tumor model has shown that TLR2 ligation on CD8 T cells augments antitumor activity *in vivo* [25], which demonstrates that MyD88 signaling in T cells supports differentiation.

Overall, TLR ligands stimulate T cell immunity through a complex process that includes the activation of subcapsular macrophages, peripheral DCs, and even T cells themselves. TLR expression in non-hematopoietic epithelial cells could also augment vaccine responses, and targeting specific cellular compartments could be a strategy to maximize vaccine efficacy.

Using the LPS adaptor pathways to stimulate Th1 differentiation and migration

To assess the role of MyD88 expression in non-T cells during immunization, LPS-induced responses have been examined after adoptive transfer of wild-type T cell receptor (TCR) transgenic CD4 T cells into MyD88^{-/-} recipient mice. After immunization with antigen plus LPS, T cell clonal expansion and long-term survival were significantly reduced in MyD88^{-/-} mice [5], which demonstrated an indispensable role for this pathway in LPS adjuvanticity. This result is consistent with stimulation of MyD88 through other TLRs (reviewed in [26]). To test if the impaired response of wild-type T cells primed in the absence of MyD88 was caused by a lack of co-stimulation, mice were injected with an OX40 (CD134) agonist to stimulate T cells directly via activation of mitogen-activated protein kinases and NF- κ B signaling (reviewed in [27]). The OX40 agonist restored clonal expansion, but long-term T cell survival was still impaired, which suggested that the MyD88-dependent survival factors were not co-stimulatory molecules [5]. Unexpectedly, in the setting of LPS stimulation, Th1 differentiation was MyD88-independent, which indicates that cooperation between the MyD88 and TRIF pathways is necessary for the transition of naïve T cell populations to effector T cells. This ability of LPS to stimulate MyD88 and TRIF signaling pathways, which is not observed for other TLR ligands, might explain the strong adjuvanticity of LPS [1].

In contrast to MyD88, much less is understood about the influence of TRIF signaling on adaptive immunity, although a positive impact on T cell priming has been suggested [2,28]. Adoptive transfer experiments with wild-type CD4 T cells and TRIF-deficient recipient mice directly tested the contribution of MyD88-independent signaling in LPS adjuvanticity. After immunization, antigen-specific T cell numbers in lymph nodes and spleen were similar between wild-type and TRIF-deficient mice. However, fewer T cells were recovered from the lungs and liver of TRIF-deficient recipient mice, which suggests that this TLR signaling adaptor potentiates T cell migration to non-lymphoid tissues [29]. Consistent with this, upregulation of the chemokine receptor CXCR3 on T cells is also TRIF-dependent [29]. LPS synergizes with IFN- γ to induce CXCR3 ligands [30] and CXCR3 recruits T cells to the liver during infection [31,32]. However, chemokine receptors have tissue-dependent functions, because LPS induces Th1 cell migration to the lungs through CCR5, and not CXCR3 [33]. More studies are needed to characterize how adjuvants, [or pathogen-associated molecular patterns (PAMPs)] such as LPS can direct T cell migration to mucosal sites that serve either as a port of entry or reservoir for pathogens. This knowledge could be exploited by vaccine design to target memory T cell trafficking to mucosal sites, which allows for a rapid response to infection before dissemination (reviewed in [34]).

Optimal Th1 differentiation through LPS is also TRIF-dependent, because the percentage of T cells that produce IFN- γ is reduced in the spleen, liver, and lungs of TRIF-deficient mice [29]. In response to LPS, DCs upregulate the co-stimulatory molecules CD40 and CD86 through TRIF [2], which bind to the cognate receptors CD154 and CD28 on T cells, respectively. CD28 and CD154 both contribute to IFN- γ production by T cells [35,36], which provides a possible link between TRIF stimulation and Th1 differentiation. Immunization with antigen, anti-CD40 agonist and LPS generates a large pool of endogenous CD8 T cells that are able to synthesize IFN- γ in wild-type and TRIF-deficient mice, which demonstrates that the MyD88 and CD40 pathways synergize to produce antigen-specific effector T cells [29]. This also suggests that some TRIF-dependent effects can be explained by CD40 signaling. However, enforced CD40 stimulation does not fully restore the CD4 Th1 response in TRIF-deficient mice [29]. It is possible that TRIF-dependent type I IFNs directly bind to CD4 T cells *in vivo*, which results in Th1 differentiation. This has not been tested in the context of LPS immunization; however, CD4 T cells use the type I IFN receptor during clonal expansion, but not during Th1 differentiation, after infection with lymphocytic choriomeningitis virus [37]. More studies are needed to assess physiological roles for type I IFN receptor signaling in CD4 T cells.

Most studies of LPS and T cell immunity have focused on LPS-induced Th1 responses to intracellular pathogens; however, immune function also depends on other T cell subsets. Th2 cells are required for protection against extracellular pathogens such as helminths, Th17 cells protect against mucosal pathogens, Tfh cells support antibody responses, and Treg cells are required to maintain self tolerance (reviewed in [38]). Recently, it has become apparent that LPS can support the development and/or function of each major CD4 T cell subset, depending on the situation, which has led to a re-evaluation of its effects on adaptive immunity. For example, mice sensitized intranasally with a low dose of LPS display heightened Th2 responses against allergen [39], whereas intravenous immunization generates Treg cells that limit CD8 T cell responses [40]. In a superantigen model using staphylococcal enterotoxin A to stimulate endogenous T cells that express TCR V β 3, intraperitoneal injection of LPS led to Th17 and Th1 expansion in small intestinal lamina propria [41]. LPS also drives Tfh cell differentiation in mesenteric lymph nodes and spleen [42]. The ability of LPS to support functionally diverse T cell responses could be related to the diverse cytokine microenvironments located within different tissue sites such as lung, spleen, lymph nodes, and gut.

Factors that control intestinal T cell migration and differentiation

The ability to imprint T cells for migration to mucosal sites might become a useful strategy for vaccine development. Oral immunization has been found to increase expression of the intestinal homing integrin α 4 β 7 on T cells, which protects mice from gastric *Helicobacter* infection [43]. More recently, some studies have demonstrated that multiple immunization routes can prime T cells with gut-homing potential [44–46]. Of note, retinoic acid induces the expression of CCR9 and integrin α 4 β 7 on T cells *in vitro*, which promotes their migration from mesenteric lymph nodes to intestinal lamina propria (Refs [47,48], and Figure 1). Although exogenous retinoic acid augments the adjuvant activity of TLR ligands in vaccines (reviewed in [49]), it is not yet clear if this method markedly improves T cell migration to the gut. It is notable that vitamin-A-deficient mice have fewer T cells in the gut lamina propria [47]. Other potential targets implicated in T cell trafficking to the gut include sphingosine 1-phosphate and CCR5 [50,51].

Vaccine efficacy is also determined by the nature of T cell polarization into specialized subsets during initiation of an adaptive immune response. IL-17 has recently gained attention as a mediator of protection against mucosal infections (reviewed in [52]), and it also optimizes vaccine-induced protection from intranasal or intragastric infections [53–56]. Although these data support the use of vaccines to stimulate antigen-specific IL-17 production, many animal

models that are used to generate mucosal Th17 responses incorporate cholera toxin [55–58]. Another consideration is the vaccination route, because antigens given orally often result in tolerance and not immunity [59]. To bypass oral tolerance, we have tested the effects of peripheral immunization on intestinal T cell priming. Intraperitoneal injection of mice with antigen and LPS results in accumulation of specific endogenous T cells in small intestinal lamina propria [41]. One subset of CD4 cells is Th1, based on the ability to produce IFN- γ , whereas another subset present at similar frequency produces IL-17A. This indicates that, in contrast to the spleen, LPS is a potent adjuvant of Th17 responses in the intestine, even when LPS is administered peripherally. In a different study, intranasal immunization with adjuvants that target TLR2, TLR9, or dectin-1 generated Th17-biased responses in the lung [60]. Therefore, the mucosal microenvironment shapes the nature of the immune response generated with distinct adjuvants.

Why are Th17 responses primarily localized at mucosal sites? Our general understanding of T cell activation is that after antigen encounter in lymph nodes, T cells undergo clonal expansion, differentiate into effectors, and migrate towards the source of antigen (reviewed in [61]); thus, T cell homing to non-lymphoid tissue is not necessary for Th1 differentiation [62]. Characterization of Th17 cells has challenged the view that effector differentiation occurs entirely within lymphoid tissue. In healthy naive mice, most Th17 cells are found in the intestinal lamina propria and few, if any, are present in lymph nodes or spleen [63,64], which suggests that differentiation occurs at mucosal sites. Four independent studies have identified a role for commensal bacteria in Th17 cell development [64–67]. Multiple bacteria-derived factors support Th17 differentiation, including bacterial DNA and ATP. Data on the importance of TLRs for generating Th17 responses are varied [64–66,68], which suggests that multiple pathways can trigger intestinal Th17 responses. These pathways appear to converge on lamina-propria-resident DCs that express CD11c, F4/80, CD70, CD80, and CX3CR1, but not CD103 [64,68,69]. Perhaps this DC subset supports Th17 responses by producing IL-23 in response to commensal bacteria (Ref [70] and Figure 1). Conversely, the CD103⁺ DCs in the lamina propria induce Treg cell conversion through retinoic acid [71,72]. DC subsets might influence intestinal inflammation by regulating the balance of Foxp3 and ROR γ t in T cells, which are the transcription factors that are involved in commitment to regulatory and Th17 lineages, respectively.

Recent findings that have shown that transforming growth factor (TGF)- β and IL-6 are not absolute requirements for Th17 differentiation suggest that there is some degree of redundancy [73–75]. When Treg cells are depleted, Th17 cells are able to expand in *Il6*^{-/-} mice because of the presence of IL-21; an IL-2 family cytokine that acts on T cells in an autocrine manner to establish Th17 commitment [73,76]. Further analysis has revealed that although TGF- β and IL-6 stimulate production of the immunosuppressive cytokine IL-10 [77], Th17 cells cultured with IL-23 do not produce IL-10 and are more pathogenic upon transfer. Overall, these data suggest different subsets of CD4⁺ ROR γ t⁺ IL-17A⁺ cells exist and the name Th17 can be broadly applied to all of these. Th17 subsets can be distinguished by their production of IL-10, IL-17F, IL-22, or lineage-specific transcription factors.

Many questions remain about mucosal T cell polarization. Are the Th17 cells direct descendents from naïve T cells or effector Th subsets that are programmed in lymph nodes to traffic to the lamina propria where they receive Th17-polarizing signals (Figure 1)? The acquisition of Th1 phenotype is not as stable as once thought [78], and small T cell populations are often observed to produce IFN- γ and IL-17 *in vivo*. TGF- β is abundant in the intestine and can induce IL-23 receptor gene expression in T cells [79]. The requirement for continual TGF- β stimulation to maintain the Th17 phenotype [80] illustrates the inherent plasticity of effector T cells and reinforces the concept that they are influenced by local cytokines, the concentration of these cytokines, and the lymphoid niche. For instance, high concentrations of TGF- β induce

Foxp3 instead of ROR γ t, whereas low concentrations of TGF- β favor IL-23R expression with subsequent ROR γ t induction and Th17 differentiation [81]. The finding that Treg cells convert into Tfh cells in Peyer's patches where they contribute to antibody responses [82] suggests that modulation of Foxp3 expression through the local cytokine milieu could be exploited for vaccine development. This concept is directly relevant to LPS adjuvanticity since pro- and anti-inflammatory cytokines are produced upon LPS stimulation. Overall, these new data suggest that the outcome of T cell immunity depends upon their interaction with environmentally conditioned APCs, and that mucosal adjuvants that target certain APC subsets might become a strategy to control T cell polarization selectively.

Using LPS derivatives as mucosal adjuvants

Although LPS is a potent mucosal adjuvant [41,42,83], its native form might be too toxic for clinical use. The derivative of LPS, monophosphoryl lipid A (MPL), retains adjuvant function without toxicity, due to preferential stimulation of the TLR4 and TRIF signaling pathway [28,84]. In mice, MPL-containing vaccines activate DCs, which results in their migration to multiple secondary lymphoid organs and subsequent induction of Th1 and Th17 responses [85,86]. A large human study has concluded that adverse events of potential autoimmune etiology do not differ between patients who are receiving MPL or controls [87]. Importantly, MPL is effective at stimulating long-term humoral and cellular immunity against hepatitis B virus in humans [88,89]. The detoxified derivative of LPS, MPL, will probably be approved as a vaccine adjuvant for widespread human use (reviewed in [84]). Thus, TLR4 has proven to be a very useful target in human mucosal vaccines; however, more studies are needed to clarify the cellular mechanisms by which MPL stimulates T cell responses.

Concluding remarks

Recent studies of LPS have provided important clues about how vaccine adjuvants can work. It is well established that triggering the MyD88- and TRIF-dependent signaling pathways through TLRs leads to robust adaptive immune responses (reviewed in [26]). In this respect, LPS is a unique bacterial PAMP because through TLR4 it stimulates the MyD88 and TRIF adaptor signaling pathways. Analysis of the role of MyD88 and TRIF during T cell priming suggests that these signaling adaptors can be used to manipulate immunity selectively. For instance, the inclusion of a TRIF agonist in vaccines might ensure that robust Th1 differentiation takes place, and the T cells are capable of migration to specific organs such as the lungs and liver. By contrast, to treat autoimmunity, T cells need to be negatively regulated. Perhaps a MyD88 antagonist could be beneficial in this situation, as it could reduce the accumulation of self-reactive T cells without having a systemic crippling effect on the immune system, because Th1 differentiation can be MyD88-independent [5].

Perhaps the complex response to LPS has evolved in part to fine-tune adaptive immunity. Different LPS structures elicit qualitatively distinct signals through TLR4 that influence T cell polarization [28,90,91], therefore, the strength of TLR4 signaling might define the quality of the immune response. The potential for LPS to induce all four cytokines within the IL-12 family might underlie its ability to support diverse T cell responses [92,93]. This includes the newly discovered IL-35 cytokine that is produced by Treg cells and contributes to their suppressive function [94]. For instance, an adjuvant that produces more IL-12 and IL-27 compared to IL-23 is predicted to drive Th1 polarization at the expense of Th17, whereas increasing IL-35 levels might result in immune suppression. If LPS variants differentially induce the IL-12 family members, they have the potential to treat a variety of conditions. Overall, recent insights into the relationship between TLRs and mucosal T cell immunity can provide a framework for directing Th polarization during vaccination.

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Figure 1. Intestinal lamina propria DCs influence local T cell differentiation

(1) CD103-negative DCs in small intestine lamina propria respond to commensal bacteria by producing IL-23 [48,70,71]. Combination of IL-23 and TGF- β could trigger local T cells to undergo Th17 differentiation [38]. (2) CD103+ DCs support Treg cell differentiation through production of retinoic acid [71,72]. (3) DCs in the mesenteric lymph nodes that are activated by TLR adjuvants could prime Th17 differentiation as a result of their production of IL-6 and the local abundance of TGF- β . Retinoic acid in the mesenteric lymph nodes induces the expression of integrin $\alpha 4\beta 7$ on T cells, which causes their migration to the lamina propria [47,48]. (4) IL-12 might lead to accumulation of Th1 cells in the lamina propria [38]. (5) As reviewed in the text, the inherent plasticity in Th lineages suggests that there could be some conversion between Treg and Th17 cells [73,74]. (6) It is unknown if the presence of TGF- β and IL-23 in the lamina propria instructs Th1 cells to convert to Th17. In summary, vaccine adjuvants might alter the balance of Th subsets in the gut by stimulating DCs to produce lineage-stabilizing cytokines. Furthermore, TLR adjuvants support T cell clonal expansion by augmenting co-stimulatory molecule expression on DCs (not shown).