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Understanding How Lipopolysaccharide Impacts CD4 T Cell Immunity

Jeremy P. McAleer

Anthony T. Vella

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Department of Immunology, University of Connecticut Health Center

Abstract

Lipopolysaccharide (LPS) is a natural adjuvant synthesized by gram-negative bacteria that has profound effects on CD4 T cell responses. LPS stimulates cells through Toll-like receptor 4 (TLR4), causing the release of inflammatory cytokines and upregulation of costimulatory molecules on antigen presenting cells. The combination of signals from antigen, costimulation, and cytokines allow CD4 T cells to overcome suppressive barriers and accumulate in large numbers. T cells that are primed in an LPS-stimulated environment are programmed for long-term survival following clonal expansion. LPS is well-known for generating Th1 responses, however, under appropriate conditions it can also support differentiation into other T helper lineages, demonstrating its pleiotropic nature. Although molecular analyses have provided insights into how immune responses are controlled by LPS *in vivo*, its powerful adjuvant activity is also associated with toxicity. Research on partial TLR4 agonists such as monophosphoryl lipid A have demonstrated that toxicity and immunogenicity are not always linked, making them useful candidates for human vaccines. In this sense, many years of LPS research have ultimately contributed to vaccine design, and the next generation may involve studying how the balance between different CD4 T cell subsets is controlled.

Keywords

LPS; endotoxin; adjuvant; survival; differentiation

I. INTRODUCTION

The inflammatory milieu has profound effects on how mature lymphocytes respond to antigenic stimulation. Thus, whereas most encounters with antigen (Ag) are tolerated, Ag that are associated with cells that may potentially damage the host often cause the outgrowth and functional differentiation of lymphocytes, leading to elimination of the antigenic source. This situation is experimentally mimicked or induced in order to study lymphocyte activation, or to generate a state of resistance against microbes or tumors that express similar Ag. Injecting Ag in the absence of non-specific inflammatory stimuli results in immunological tolerance characterized by non-responsiveness to subsequent antigen exposures.[1] However, the addition of adjuvants readily converts this tolerogenic signal into a productive immune response during primary and secondary exposures.[1, 2] Lipopolysaccharide (LPS) derived from the cell membrane of gram-negative bacteria is a natural adjuvant that partially functions by generating an inflammatory environment during lymphocyte activation. As reviewed elsewhere, LPS initially gained interest as the component of gram negative bacteria responsible for toxicity and pyrogenicity,[3] but later

Corresponding author: Anthony T. Vella, PhD., Department of Immunology, Room L-3057, University of Connecticut Health Center, Farmington, CT 06030, Phone: 860-679-4364, Fax: 860-679-1868, vella@uchc.edu.

studies revealed that it could generate protection from subsequent infection that correlated with increased antibody levels.[4–6] Findings that LPS-unresponsive mice are susceptible to gram-negative infections further highlighted the importance of this molecule in immune protection.[3] Therefore, a large body of research has been devoted towards investigating the adjuvant effects of LPS on lymphocytes. This review discusses how LPS influences various aspects of CD4 T cell activation, such as long-term survival and effector differentiation.

II. LPS DETECTION AND SIGNALING

A. LPS Detection

The LPS adjuvant effect begins with its recognition by innate receptors, first involving the association of LPS binding protein with LPS molecules followed by their transfer to CD14 receptors, as reviewed elsewhere.[7] CD14 is present in soluble and membrane-bound forms and is required for endotoxicity. However, CD14 lacks a transmembrane domain and does not signal in the presence of LPS. Rather, CD14 directs LPS to TLR4 which does initiate the signaling process (Fig. 1).[7] In the absence of CD14, high concentrations of LPS are required to stimulate TLR4.[8] Another important molecule is MD-2 which is associated with TLR4 on the cell surface. MD-2 directly binds LPS,[9] thereby conferring TLR4 responsiveness.[10] B cells also express the receptors RP105 and MD-1, structurally related to TLR4 and MD-2, which are important for their LPS response.[11, 12] Although immune cells primarily respond to extracellular LPS, intestinal epithelial cells retain TLR4 in the Golgi apparatus and require LPS internalization for its detection.[13] Therefore, non-immune cells can differ fundamentally in the way they respond to LPS and their role in the adjuvant effect on T cells is largely unknown.

B. Signaling through TLR4

Toll-like receptor signal transduction has been reviewed by Kawai and Akira,[14] and this section highlights some basic points about TLR4. The intracellular portion of TLR4 contains a Toll/interleukin-1 receptor (TIR) domain which is common to all members of the TLR family.[14] This domain recruits cytoplasmic adaptors that initiate the signaling process, named myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor protein/MyD88-adaptor-like (TIRAP/Mal), TIR domain-containing adaptor inducing IFN-β/TIR-domain-containing molecule 1 (TRIF/TICAM 1), and TRIF-related adaptor molecule/TIR-domain-containing molecule 2 (TRAM/TICAM 2) (Fig. 1). TIRAP recruits MyD88 to activated TLR4 receptors[15] while TRAM links TLR4 with TRIF, resulting in two distinct signaling pathways that cooperate to induce inflammatory cytokines, and upregulation of costimulatory molecules on antigen presenting cells (APCs).[14] The MyD88 pathway begins with the phosphorylation of IRAK4 and IRAK1, leading to activation of TNF receptor-associated factor 6 (TRAF6).[14] TRAF6 activates transforming growth factor-β-activated protein kinase 1 (TAK1). TAK1 is involved in activating the IKK complex and MAP kinase pathway, leading to degradation of IκB and activation of JNK and p38.[14] LPS also activates ERK through MEK1 and MEK2. MyD88-dependent signaling ultimately results in the activation of NF-κB and AP-1 transcription factors.[14] The TRIF pathway begins with the recruitment of receptor interacting protein 1 (RIP1) and TRAF6, both of which cooperate to activate NF-κB.[14] In addition, TRIF activates TANK-binding kinase 1 (TBK1) and IKKi, resulting in the phosphorylation of interferon regulatory factor 3 (IRF3) and IRF7. IRF3 and IRF7, along with NF-κB and ATF2/c-Jun, are important transcription factors for IFN-β expression.[14] Since IRF3 is constitutively expressed and IRF7 is induced by IFN-β, initial IFN-β production is primarily IRF3-mediated and thereafter IFN-β amplifies type I IFN production in an autocrine and paracrine manner through IRF7. This pathway is important for APC activation since macrophages deficient in either TRIF or the type I IFN receptor fail to upregulate

costimulatory molecules in response to LPS, unlike MyD88-deficient macrophages.[16] TRIF was also found to be important for the upregulation of Ag-bearing MHC class II chains.[17] Taken together, MyD88 and TRIF signaling appear to fully account for the natural adjuvant activity of LPS.[18, 19]

C. Negative Regulation of TLR4 Signaling

TLR4 signaling is negatively regulated at every level, as reviewed,[20] probably to limit overzealous inflammatory reactions. On the cell surface three proteins negatively regulate LPS signaling through different mechanisms (Fig. 1). The RP105 receptor was mentioned as amplifying B cell responses to LPS, however, on non-B cells RP105/MD-1 complexes can prevent the binding of LPS to TLR4 and MD-2.[20] Additionally, ST2L prevents the recruitment of TIRAP/Mal and MyD88 to TLR4, and SIGIRR sequesters IRAKs and TRAF6. Many other factors have been shown to interfere with TRAF6 function such as β-arrestins, A20, and TRAF4.[20] This suggests that downregulation of TRAF6 is important for immune homeostasis and TRAF6 may provide a therapeutic target for patients with TLR-dependent inflammation. The E3 ligases Triad3A and SOCS1 target TLR4 and TIRAP/Mal for degradation, respectively.[20] Several splicing variants of signaling molecules such as IRAK1, IRAK2, and MyD88 have been discovered and may function by competitively blocking interactions between related proteins and their ligands. Negative regulators can be induced by LPS, such as IRAK-M and TRAF1, the latter of which specifically inhibits the TRIF pathway.[20] Downstream of TRIF, IRF3 phosphorylation at serine residue 339 causes its degradation. At the transcription factor level, ATF3 recruits histone deacetylases and thereby restricts the access of NF-κB and AP-1 to promoters for IL-6 and IL-12β .[20] Collectively, the presence of many negative regulators in TLR signaling suggests that individual cells only transiently respond to pathogen-derived products. One interesting physiological question is if T cell activation uncouples negative regulation of TLR4 signaling, leading to enhanced LPS sensitivity such as that seen during sepsis.[21]

III. T CELLS ARE IMPORTANT FOR LPS ADJUVANTICITY

Experiments beginning in the 1950's demonstrated that LPS can generate primary and secondary antibody responses towards non-associated Ag, establishing the concept of LPS as an adjuvant.[22–25] The timing of immunization was critical, with optimal responses observed when LPS was injected within 24 h of Ag.[23, 25] Cell types contributing to this adjuvant effect were of considerable interest and in some models adjuvanticity could be explained by the direct stimulation of B cells. For instance, LPS could substitute for helper cells *in vitro* for generating antibody responses and could stimulate B cell mitogenesis.[26–28] Chiller showed that LPS injection overcomes a requirement for T cells in generating antibody responses *in vivo*, terminating B cell tolerance in mice that had a tolerized T cell compartment.[29] However, numerous reports also cited an indispensable role for helper T cells (Th) in the LPS adjuvant effect. In 1971 Allison reported that thymectomy abrogates the ability of several adjuvants including LPS to enhance antibody responses, and other investigators later confirmed the importance of T cells *in vivo* and *in vitro*.[30–36] It is now appreciated that T cell-independent antibody responses are predominantly IgM-mediated while Th cells promote isotype switching. Therefore, one possibility to explain the apparently conflicting data from Chiller and Allison is that Chiller's model may have been less dependent on isotype switching. In support of this, T cells were found to be required for LPS to enhance a secondary IgG response while there was no effect on IgM production.[35] However, LPS can accelerate the kinetics of a T-dependent IgM response.[37] In addition to antibody models, an autoimmune model that incorporated LPS into sensitization found that T cells transferred Experimental Autoimmune Thyroiditis to naïve mice, while B cells were not required.[38] Thus, it was clear that Th cells are required for some effects of LPS

adjuvanticity, and the types of studies listed above stimulated interest in examining the effects of LPS on Th cell activation.

IV. EFFECTS OF LPS ON CD4 T CELL ACTIVATION

A. Historical View of the LPS-T cell Adjuvant Effect

Mature Th cells express CD4 and are grouped into subsets based on their profile of cytokines generated following stimulation. Functional differentiation from a naïve state results in specific populations with Th1, Th2, or Th17 properties, determined by their ability to produce IFN-γ, IL-4, or IL-17, respectively. Also, some CD4 T cells regulate immune responses by inhibiting immune cells. These regulatory populations either develop in the thymus and constitutively express high levels of CD25, or differentiate in peripheral tissues and secrete IL-10. In addition to effector differentiation, T cell activation causes their proliferation, which is important for amplifying Ag-specific immune responses. Considering the central roles for Th cells in immunity, the effects of adjuvants on CD4 T cell accumulation and functional differentiation are of considerable interest. In 1981 Parks reported that injecting LPS 3 h after a tolerogenic form of Ag interfered with tolerance induction in Th cells, allowing mice to generate a T cell-dependent antibody response following re-exposure to Ag.[39] Now the term "T cell activation" is commonly used in place of "interference with tolerance," and as discussed below, LPS affects CD4 T cell activation by supporting their accumulation and functional differentiation.

In the 1980s and 1990s, studies found that LPS could induce T cell mitogenesis *in vitro* either through direct stimulation or signals derived from APCs, indicating that T cells may also be a direct target *in vivo*.[40–42] Mouse studies provided means to examine CD4 T cell activation under physiological settings, and LPS injection caused the upregulation of CD25, CD69 and slightly increased the percent CD44^{high} CD4 T cells incorporating the proliferation marker BrdU.[43, 44] Since CD25, CD44, and CD69 are upregulated following T Cell Receptor (TCR) stimulation, this suggests Ag may be required for the LPS effect and LPS may potentially activate self-specific peripheral T cells. Indeed, transgenic models directly demonstrated this can occur. Injecting LPS into mice in which the T cell repertoires are restricted to either islet Ag or myelin basic protein (MBP) results in autoimmune diabetes or experimental autoimmune encephalomyelitis (EAE), respectively.[45, 46] The latter study found that inducing EAE in mice with a normal T cell repertoire renders them susceptible to relapse following a future LPS injection, indicating that Ag-experienced T cells may be preferentially targeted by LPS. This effect is not restricted to self-specific T cells, as infecting C57BL/6 mice with *Salmonella typhimurium* causes CD4 T cells to respond to a subsequent LPS injection by producing the effector cytokine IFN-γ.[47] Cytokine production was not impaired when the T cells were transferred to MHC class II-deficient mice prior to LPS injection, demonstrating the Ag-experienced T cells responded to LPS independently of TCR signals. A mechanism for this effect may be the induction and action of IL-12 and IL-18, as shown in this and other systems.[47–49] Overall, antigenic stimulation endows T cells with an ability to respond to LPS-derived signals.

The practice of tracking Ag-stimulated T cells *in vivo* has contributed greatly to our understanding of adjuvanticity. Superantigens have been paramount in this respect since they activate a large pool of endogenous CD4 and CD8 T cells by linking conserved TCR Vβ chains with MHC class II molecules on APCs. For example, injecting Staphylococcal Enterotoxin A (SEA) derived from the pathogen *Staphylococcus aureus* into mice results in the expansion of T cells expressing TCR Vβ3 among other Vβ genes, as reviewed elsewhere.[50] This expansion is followed by extensive contraction during the next few days, resulting in fewer Vβ3 T cells compared to untreated mice.[51] In 1995 it was demonstrated that injecting LPS at the same time or 24 hours after SEA rescues Vβ3 T cells from deletion,

resulting in an approximately five-fold increase in cell numbers.[52] This difference between LPS-treated and untreated mice is maintained for months, indicating that a major effect of LPS on T cells is to enhance their survival following clonal expansion.[53] Subsequent reports further demonstrated that LPS increases the accumulation of peptide-stimulated CD4 T cells.[54, 55] Thus, the ability of LPS to enhance T cell survival *in vivo* appears to be a general phenomenon that occurs with concomitant TCR stimulation.

B. LPS as an Inducer of Th Differentiation

1. Th1 and Th2—Studies on effector differentiation in the 1990s demonstrated that LPS can bias immune responses towards Th1. Culturing human T cells in LPS-free medium for 6 days, followed by non-specific stimulation with PMA plus ionomycin resulted in their production of both IFN-γ and IL-4.[56] However, when the T cells were first cultured with *Brucella abortus* LPS and then stimulated they produced higher levels of IFN-γ and negligible amounts of IL-4, indicating that direct stimulation of human T cells with LPS promotes Th1 differentiation. It was not determined if the cytokine-producing T cells were Ag-experienced, however, as T cell-derived IFN-γ is thought to require previous TCR stimulation. LPS was also found to decrease IL-5 and increase IFN-γ from human peripheral blood T cells stimulated with anti-CD3 plus anti-CD28.[57] This effect was dramatically reduced when the T cells derived from subjects with asthma, implying that LPS did not revert an established Th2 response. Other models have found an inhibitory role for LPS in Th2 differentiation. Stimulating naïve CD4 T cells or Th2 clones with anti-CD3 plus anti-CD28 resulted in IL-4 production that was significantly reduced by lipid A,[58] the component of LPS that activates TLR4. Lipid A did not affect IFN-γ production, indicating its effect was specific for the Th2 cytokine. Furthermore, IL-4 production from transgenic CD4 T cells decreased when Ag-pulsed APCs were cultured with LPS.[59] Although these examples indicate that Th2 differentiation can be inhibited when T cells or APCs are treated with LPS, different doses or routes of administration were not tested and may be instrumental in driving these results. For example, intranasal immunization with a low dose of LPS generated Th2 inflammation characterized by eosinophils, while a high dose elicited a Th1-type of neutrophilic inflammation.[60] Also, LPS exacerbated a Th2 asthmatic response in Balb/c mice that correlated with more CD4 T cells producing IL-5 and IL-13, possibly because a low dose was used.[61] Another factor affecting Th differentiation can be the species from which LPS is derived. Under conditions in which *E. coli* LPS supported Th1 differentiation, LPS from *P. gingivalis* elicited Th2 cytokines from CD4 T cells *in vivo* and *in vitro* (Fig. 2).[62, 63] These results may be explained by the presence of TLR2 ligands in *P. gingivalis* LPS preparations.[64, 65] Overall, although most studies demonstrate Th1 polarization, the effects of LPS on differentiation are influenced by the dose, route of immunization, and LPS species.

2. Th17 and Tregs—Helper T cells that produce IL-17 have recently been identified and are actively being investigated as potentially key cells in immune system regulation. Interleukin-6 and TGF-β are important for the development of Th17 cells from naïve T cells, and LPS can have a positive effect on Th17 differentiation *in vitro* due to its ability to induce inflammatory cytokines.[66–68] Culturing naïve CD4 T cells with medium from LPS-stimulated DCs, anti-CD3- and anti-CD28-coated beads, and TGF-β resulted in Th17 differentiation.[68] Although IL-6 was the most important product of LPS stimulation, IL-1β and TNF accentuated the effect of IL-6 (Fig. 2). LPS can also increase TGF-β production in multiple cell types, however, exogenous TGF-β must be added to the LPS-conditioned medium in order for Th17 differentiation to occur *in vitro*.[68–72] In the future it will be interesting to examine effects of LPS on Th17 differentiation *in vivo* as well as a possible role for Treg-derived TGF-β.

LPS can also negatively regulate Th function. Infecting mice with *Salmonella* followed by injecting peptide 3 days later resulted in IL-2 production by Ag-specific CD4 T cells.[73] However, injecting LPS along with peptide reduced IL-2 levels and secondary clonal expansion, suggesting that LPS induced factors that inhibited the T cells. With human cells, pre-treating PBMCs with LPS decreased TNF and IFN-γ production as well as T cell proliferation in response to TCR stimulation.[74] This effect involved the COX-2-PGE$_2$ axis and correlated with an ability of LPS-stimulated monocytes to induce Foxp3 expression in CD4$^+$CD25$^-$ cells (Fig. 2). Also, directly treating human Tregs with LPS enhances their ability to suppress neutrophil function and viability.[75] Interestingly, LPS decreased Foxp3 expression in Tregs, indicating that levels of this transcription factor do not always correlate with suppressor function. Murine splenic Tregs were found to express TLR4 at higher levels than non-Tregs, and incubating them with LPS caused their upregulation of activation markers, proliferation, and greater suppressor function.[76] In addition, a recent study found that transferring LPS-activated, IL-10 producing DCs into mice increases the number Foxp3-positive cells.[77] These studies suggest that LPS might promote Treg differentiation or function *in vivo*, a relatively new area of LPS research. Injecting mice with Ag alone generates a CD4 T cell population that produces IL-10 following recall, and including LPS during immunization increases IL-10 production as well as IFN-γ (Fig. 2).[78] These IL-10 producing Tregs appear to be functional, as they can limit a parallel CD8 T cell response.[79] Although it is well known that the quality of immune responses are determined by interactions between multiple cell types, it is underappreciated that these cell types may have opposing responses to the same adjuvant.

In summary, although many examples have shown enhanced Th1 responses by LPS, this adjuvant is also capable of supporting differentiation into Th2, Th17, and Treg lineages (Fig. 2). The ability of LPS to support differentiation into multiple lineages may be attributed to experimental conditions such as genetics, route of immunization, dosage, and timing. Understanding how these variables impact T cell responses can be useful for designing efficacious human vaccines. Following their activation, effector CD4 T cells traffic to non-lymphoid tissues but little is known about how LPS impacts effector function at those sites. Perhaps the APC subtype or tissue microenvironment plays an overlooked role in Th differentiation, and more studies should examine the impact of LPS on already committed effector T cell populations. In the next section we will touch on this subject by reviewing the ability of LPS to support CD4 T cell activation.

V. A MOLECULAR UNDERSTANDING OF LPS ADJUVANTICITY

LPS adjuvanticity is a multi-step process, and the mechanisms have been investigated at multiple levels including events that are extrinsic and intrinsic to Ag-specific CD4 T cell populations. T cell-extrinsic processes include the involvement of other cell types and their intracellular signaling pathways, cytokines, surface phenotype, and the influence of regulatory populations. T cell-intrinsic processes include their surface receptors, signaling pathways, and survival molecules. In addition, studying the extent to which LPS cooperates with other adjuvants can provide insight into its own biological functions. This section discusses how LPS detection leads to Ag-specific CD4 T cell proliferation, survival, and effector differentiation with an emphasis on *in vivo* findings.

A. Effects of LPS on APCs

The cell types required for LPS responsiveness is an interesting question, and studies in the late 1970s reported that while LPS detection by T cells may contribute to antibody production *in vitro*, macrophages elicit the best production in those systems.[80, 81] This indicated a specialized role for APCs in LPS-responsiveness. Another issue is the extent to which certain cytokines are capable of substituting for LPS. Interleukin-1 is produced

following LPS injection and can inhibit tolerance induction in Th cells similar to LPS.[82] However, whereas LPS generated both IgG1 and IgG2a antibodies, IL-1β only generated IgG1 which is indicative of a Th2-biased response.[83] Therefore, IL-1 can only partially substitute for LPS. This is not surprising considering the complexity of LPS signaling (Fig. 1), which leads to a more robust immune response compared to the effects from individual cytokines. In addition to cytokines, the ability of LPS to upregulate costimulatory molecules on APCs contributes to its ability to drive CD4 T cell proliferation *in vitro*.[84, 85] In a human study, LPS stimulated proliferation of T cells from LPS-non-responders as long as the cultures contained monocytes from responders, indicating the LPS-derived signals were received by T cells indirectly from APCs.[85] Overall, LPS influences CD4 T cell activation in part by stimulating the release of inflammatory cytokines and upregulating co-stimulatory molecules on APCs (Fig. 3A).

B. LPS Promotes T Cell Survival Through Accessory Cells

Perhaps the most dramatic effect of LPS on CD4 T cells is the increased accumulation observed following immunization.[52] This ability of LPS to enhance T cell survival requires MyD88, probably due to its role in the induction of inflammatory cytokines (Fig. 3A).[86] The observation that MyD88-deficient CD4 T cells undergo normal survival indicates that non-Ag-specific T cells, possibly APCs, are responsible for detecting LPS in mice and that CD4 T cells do not require IL-1 or IL-18 which also signal through MyD88.[86, 87] This mechanism of survival via bystander stimulation is supported by *in vitro* data showing that LPS does not directly enhance the survival of activated murine CD4 T cells.[88] TNF is one of the MyD88-dependent factors required for T cell survival and is quickly produced following LPS stimulation (Fig. 3A). *In vivo* neutralization of TNF significantly impairs the ability of LPS to generate T cell survival, and IFN-γ neutralization reduces survival by a lesser degree.[52] It is unknown if the T cells themselves must detect TNF or if this cytokine functions through accessory cells. Although pro-inflammatory cytokines certainly are involved in the LPS adjuvant effect, they do not fully explain the observations. Injecting TNF or IL-1 along with Ag increases T cell accumulation, but not to the same level as LPS.[53, 54] One study found that IL-1 did enhance CD4 T cell accumulation to a similar degree as LPS, however, multiple injections were given.[54] Since TNF and IL-1 do not have an additive effect on T cell survival, it suggests other factors besides inflammatory cytokines are important for the full adjuvant effect.[53] One possibility was IL-18 which profoundly enhances the differentiation of Ag-stimulated T cells, however, IL-18 only had a small effect on long-term T cell survival.[89]

Costimulatory molecules are candidates to complement the function of inflammatory cytokines during LPS-induced immune responses, including CD28 on T cells that binds to CD80 and CD86 on APCs. Blocking the ligands for CD28 by using a CTLA4-Ig fusion protein reduces clonal expansion in response to superantigen.[53] However, CTLA4-Ig did not affect the ability of LPS to enhance T cell survival, suggesting that LPS upregulates compensatory costimulatory ligands or bypasses the need for costimulation.[52, 53] This data complements that from adoptive transfer models in which CD28 is required for LPS to enhance CD4 T cell clonal expansion.[90] The greater dependency of peptide-stimulated CD4 T cells on CD28 signaling may result from an ability of superantigens to activate APCs better than peptides,[91, 92] or to deliver stronger TCR signals to both CD4 and CD8 T cells. Overall, LPS appears to enhance CD4 T cell survival by stimulating innate cells to produce MyD88-dependent factors that fully activate APCs, resulting in T cells receiving an appropriate combination of signals from Ag, costimulatory molecules, and cytokines (Fig. 3A). Many important questions remain such as the cell types required for LPS responsiveness *in vivo*, MyD88-dependent factors in addition to TNF, the activation phenotype of APCs, and molecules that directly stimulate T cells.

C. Effects of LPS on T Cell-Intrinsic Survival Factors

T cells primed in an LPS-stimulated environment are qualitatively different than those primed without adjuvant. One aspect is that LPS can make Ag-stimulated CD4 T cells capable of surviving independently of growth factors despite having increased metabolic activity.[93] Progress has been made in the identification of molecules that either positively or negatively affect T cell viability. Firstly, certain Bcl family members positively affect T cell survival (Fig. 3B). Mitchell et. al., demonstrated that immunization with LPS and anti-CD40 increases Bcl-3 levels in superantigen-stimulated T cells, and enforced Bcl-3 expression increases T cell recovery *in vitro* and *in vivo*.[94] No effects on cell division were observed, indicating that Bcl-3 is a bona fide survival factor. Bcl-6 has also been investigated, and immunizing Bcl-6-deficient DO11.10 T cells *in vivo* with peptide plus LPS resulted in their normal accumulation for the first three weeks, but then a dramatic decline at week four.[95] In contrast, high numbers of WT DO11.10 T cells were maintained in tissues for at least twelve weeks. Bcl-6 was not involved in effector differentiation, indicating it was specifically required for long-term T cell survival.[95] These reports suggest that studying signaling pathways leading to the upregulation of Bcl-3 and Bcl-6 will be important to discern how extracellular signals can directly improve T cell survival. On the other side of the coin, GSK-3β expressed in T cells inhibits their survival following activation.[96] GSK-3β was transiently inactivated by phosphorylation after antigen injection, and the inclusion of LPS during immunization resulted in prolonged phosphorylation that correlated with increased T cell survival. Therefore, these interesting results showed that LPS blocked an intrinsic inhibitor of T cell survival. In the future, studying how Bcl family members and GSK-3β affect activated CD4 T cell survival may yield important insight into the generation and maintenance of immunological memory (Fig. 3B).

D. Pathways from LPS to Th differentiation

Mechanisms leading to Th1 differentiation have been fairly well documented with many studies examining IL-12. Using IL-12 as an adjuvant mimicked the effect of LPS on generating Ova-specific IgG2a antibodies *in vivo*, correlating with an increased percent of CD4 T cells capable of producing IFN-γ (Fig. 2).[54] Interestingly, IL-12 did not generate IgG1 antibodies, in contrast to TNF, suggesting that *in vivo* effects of LPS on Th1 and Th2 differentiation can be mediated through IL-12 and TNF, respectively.[54] Dendritic cells are major IL-12 producers following LPS injection, and depleting them in mice that have diphtheria toxin-sensitive CD11c-positive cells reduced the percent of Ag-specific CD4 T cells capable of producing IFN-γ.[86, 97] Production of TNF and IL-2 by T cells from these mice was normal, indicating a specific defect in Th1 differentiation. It is tempting to speculate that CD11c-positive cells are required to provide IL-12 to activated CD4 T cells, although this has not been formally proven *in vivo*. Interestingly, mice with IL-2-deficient DO11.10 T cells immunized with Ova plus LPS were also defective in IFN-γ production, suggesting that studying events downstream of the IL-2 receptor may yield insights into how adjuvants influence differentiation.[90]

Direct effects of LPS on DCs was assessed from mixed lymphocyte reactions in which bone marrow-derived DCs were either pre-treated with LPS or left untreated prior to incubation with allogeneic CD4 T cells.[98] LPS increased IFN-γ production and decreased IL-4, indicating Th1 polarization. This effect was reversed if the DCs were derived from MyD88-deficient mice, resulting in less IFN-γ and more IL-4.[98] The addition of IL-12 to cultures containing MyD88-deficient DCs increased IFN-γ production without affecting IL-4, suggesting that another MyD88-dependent factor inhibited IL-4 production. The Notch-like ligand Delta 4 is induced on CD8-negative DCs by LPS and can drive Th1 differentiation.[99] To compare the roles of IL-12 and Delta 4 *in vivo*, OTII cells were transferred into mice that were immunized with Ova plus LPS. Two weeks later the cells were restimulated *in vitro*,

and the percent of OTII cells capable of producing IFN-γ was approximately 3-fold lower when they were primed in IL-12p40-deficient mice.[99] Blocking Delta 4 interactions in the IL-12p40-deficient mice further reduced the number of IFN-γ producers by another 50 percent. Therefore, Th1 differentiation in this system was almost entirely explained by the combined actions of IL-12 and Delta 4. Since both of these factors depend on MyD88 for their expression in response to LPS, MyD88 uses at least two mechanisms to support Th1 differentiation. Th1 differentiation can also be MyD88-independent, indicating the TRIF pathway is sufficient (Fig. 2).[86] Taken together, LPS uses redundant mechanisms to drive Th1 differentiation.

E. LPS-primed CD4 T cells are Resistant to Suppression

Tregs participate in preventing autoimmunity and function in part by suppressing effector CD4 T cell activity, as reviewed elsewhere.[100] However, pathogen-specific CD4 T cells must become properly activated in order to deal with dangerous infections. Pasare and Medzhitov examined how Tregs influence CD4 T cell priming, and found that conditioned medium from LPS-stimulated DCs caused effector CD4 T cells to overcome Treg-mediated proliferative suppression *in vitro*.[101] MyD88 was required for this effect, in part by inducing IL-6. Both MyD88 and IL-6 are required for optimal T cell priming *in vivo* when LPS is the adjuvant, and depletion of Tregs using the CD25-specific PC61 antibody enhanced the primary CD4 T cell response in MyD88- and IL-6-deficient mice to levels comparable with normal WT mice.[101, 102] Treg depletion in WT mice further enhanced the response. CD28 was required for T cell priming in the presence and absence of Treg, suggesting that APCs must deliver costimulatory signals even when the influence of Treg is negated.[102] Interestingly, Treg depletion only enhanced the primary, not memory, CD4 T cell response in MyD88-deficient mice, indicating that MyD88-dependent signals present at the time of initial priming are required for the maintenance of a functional CD4 T cell population.[102]

F. LPS Conditions T cell Responses During an Early Phase

Ultimately, many effects of LPS may be explained by its ability to enhance T cell/APC interactions. Injecting LPS after Ag causes CD4 T cells to become trapped in lymphoid tissues for about 48 hours, during which the T cells are detectable by histology but not very well by flow cytometry unless the tissues are first digested with collagenase.[103] During this period the T cells are tightly associated with APCs, forming doublets or larger order interactions. This indicates the effects of LPS are programmed into CD4 T cells early in the response, and this process has been termed "clonal conditioning."[103] Perhaps clonal conditioning offers an explanation for why the timing of LPS injection is important: The T cells must be in a receptive state to respond to survival and differentiation signals during the period they are tightly associated with APCs. Evidence for this is supported by the observation that LPS increased the percent of Ag-stimulated CD4 T cells expressing the growth factor receptor CD25 during the clonal conditioning phase.[103] Secondly, this may also apply to a functioning memory response.[104] Therefore, long-term effects of LPS may become imprinted on CD4 T cells during the first 48 h, although the identities of the signals are unknown.

G. Combining LPS Treatment with Costimulatory Agonists

Since different types of adjuvants may have non-overlapping functions, combination approaches can be useful for obtaining desired responses. Costimulatory molecules have fundamental roles in T cell clonal expansion and effector differentiation, and agonistic monoclonal antibodies (mAb) directed against them are effectively used as adjuvants. The combination of an OX40 agonist mAb plus LPS had a massive synergistic effect on T cell accumulation, possibly due to LPS indirectly increasing expression of OX40 on Ag-stimulated T cells.[105] Mice that received both adjuvants generated much better clonal

expansion compared to single-treated mice, and the T cell population underwent very little contraction afterwards. MyD88 was found to be dispensable for the clonal expansion phase but, importantly, required for long-term T cell survival.[86] A similar synergy was observed between a CD40 agonist mAb and LPS, however, the inclusion of both anti-OX40 and anti-CD40 with LPS did not further increase T cell survival.[106] This suggests that anti-OX40 and anti-CD40 may work through similar mechanisms and anti-CD40 has been shown to increase expression of OX40-ligand on DCs.[107] Since OX40 stimulation potently drives Th1 differentiation just as well as LPS,[86] our data indicate that synergy between costimulatory agonists and LPS may be explained by the ability of costimulatory molecules to drive CD4 T cell clonal expansion and effector differentiation while LPS mainly provides survival signals through the MyD88 pathway (Fig. 3A). This conclusion may be simplistic, however, based on the following data. The inflammatory cytokine TNF is essential for optimal T cell survival when mice are immunized with Ag and LPS, but not when mice are immunized with Ag, LPS, and a CD40 agonist mAb.[52, 106] When analyzed in total, our data have generated two major points on this issue. First, the LPS effect cannot merely be explained by an increase in the costimulatory function of APCs, and second, costimulatory agonists can bypass a requirement for any single pro-inflammatory cytokine. It is apparent that multiple roads may lead to full T cell activation *in vivo*.

VI. NON-TOXIC LPS DERIVATIVES: PROGRESS WITH MONOPHOSPHORYL LIPID A

Although LPS is a powerful adjuvant, its toxicity prevents human use. Several investigators have searched for methods to chemically modify LPS so that it loses toxicity while retaining adjuvanticity. Early studies found that treating LPS with sodium hydroxide increases the lethal and pyrogenic dose but does not impair B cell mitogenicity.[108, 109] Furthermore, periodate reduced LPS toxicity without affecting pyrogenicity, indicating these properties are not necessarily linked.[108] Therefore, various physiological responses to LPS are separately impacted by chemical treatments. Studies have shown the less-toxic sodium succinyl-LPS and sodium pthalyl-LPS derivatives can promote antibody responses *in vivo*, formally demonstrating that toxicity is not a requirement for adjuvanticity.[110, 111]

In the early 1980s, Ribi's group studied effects of acid hydrolysis on LPS and found that it significantly lowered pyrogenicity and toxicity while simultaneously promoting tumor rejection.[112] Structurally, the lipid A species contained half the normal amount of phosphate and the product became known as monophosphoryl lipid A (MPL).[112, 113] When given to human volunteers, MPL increases specific antibody levels while only causing mild side effects.[114–116] The relatively mild side effects in comparison to LPS may result from a reduced capacity of MPL to induce inflammatory cytokines such as IL-12, possibly due to an inability of CD14 to amplify MPL signals.[117] It would be interesting to test whether or not CD14 plays a role in MPL adjuvanticity. MPL seems to preferentially drive Th1 differentiation, as both human and murine DCs treated with this adjuvant significantly increased IFN-γ production by T cells.[117, 118] With human cells, it is likely that LPS and its derivatives directly stimulate T cells in addition to APCs. Treating CD3-stimulated human CD4 T cells with MPL increases their CD40-ligand expression, indicating that MPL can contribute indirectly to APC activation through the T cell.[117] Taken together, MPL is a non-toxic LPS derivative with important adjuvant activity.

Mitchell's group has compared the adjuvant effects of MPL versus LPS on murine CD4 T cell responses *in vivo*. They found that MPL can generate similar or even better levels of clonal expansion even though several innate factors were induced more highly by LPS.[119, 120] Serum levels of serum amyloid A, IFN-γ, IL-1β, IL-6, and MIP-1α were much lower in MPL-treated mice, correlating with its lower toxicity. However, some factors such

as IP-10, MCP-1, G-CSF, and IL-10 were similarly induced by both adjuvants.[119, 120] This was attributed to preferential stimulation of the TRIF pathway by MPL. To illustrate this concept, MPL and LPS were shown to induce similar levels of IRF3 phosphorylation in macrophages while the MyD88-dependent cytokine IL-6 was produced at much higher levels by LPS.[120] When analyzing T cell clonal expansion *in vivo*, they found a critical role for TRIF expression by the host. Secondary immunization revealed that on a per cell basis, MPL and LPS had a similar effect on Th1 differentiation; however, the total number of IFN-γ-producing CD4 T cells was much higher in the LPS-treated mice indicating the effect of MPL on effector CD4 T cell accumulation is more transient than LPS.[119] This finding is consistent with a study that found a specialized role for MyD88 in long-term T cell survival.[86] These murine studies have provided valuable information regarding MPL adjuvanticity, including a mechanism for why it may be non-toxic relative to its counterpart LPS. They also highlight how the pleiotropic nature of LPS can be harnessed by utilizing partial TLR4 agonists.

VII. CONCLUSIONS

Although initially studied for its pathogenicity, LPS research has significantly contributed to our understanding of vaccine adjuvants. Exciting new findings have shown that under appropriate conditions LPS supports the differentiation or function of Th1, Th2, Th17, or Treg subsets. Understanding how the adjuvant activity of LPS affects the balance between these subsets *in vivo* will provide insight into the physiological control of T cell differentiation and expansion, and how infectious agents can subvert host immune responses. Natural selection has generated many LPS varieties that differentially stimulate the MyD88 and TRIF pathways,[121] and species differences may have a significant impact on the ensuing T cell response. *In vitro* evidence for this has recently emerged, as LPS derived from two related species differentially impacted the Th1-Th17 cytokine balance.[122] These types of studies are important for learning how to use adjuvants to control the quality as well as quantity of T cell responses, which may ultimately be helpful for a variety of clinical situations.

Acknowledgments

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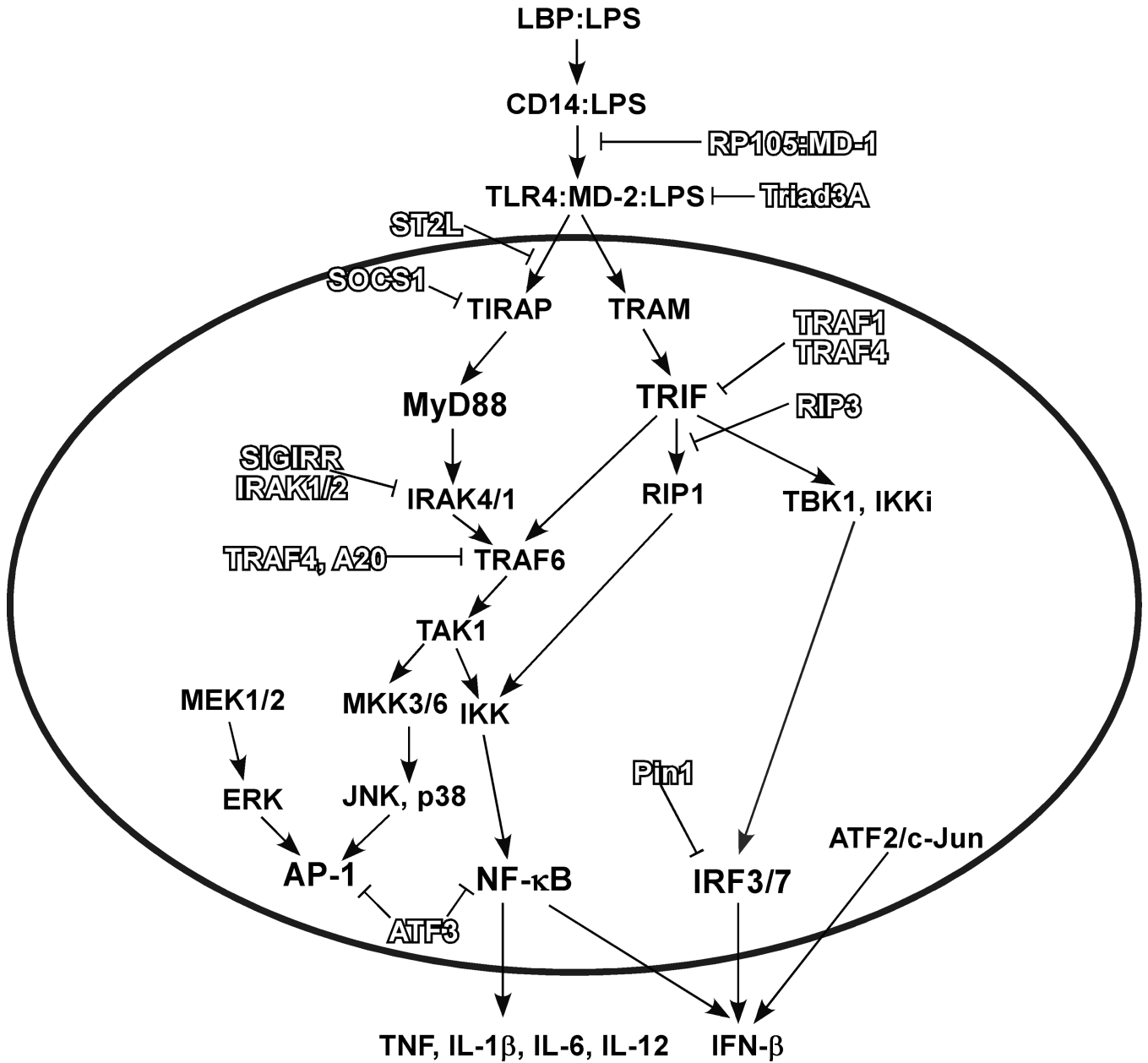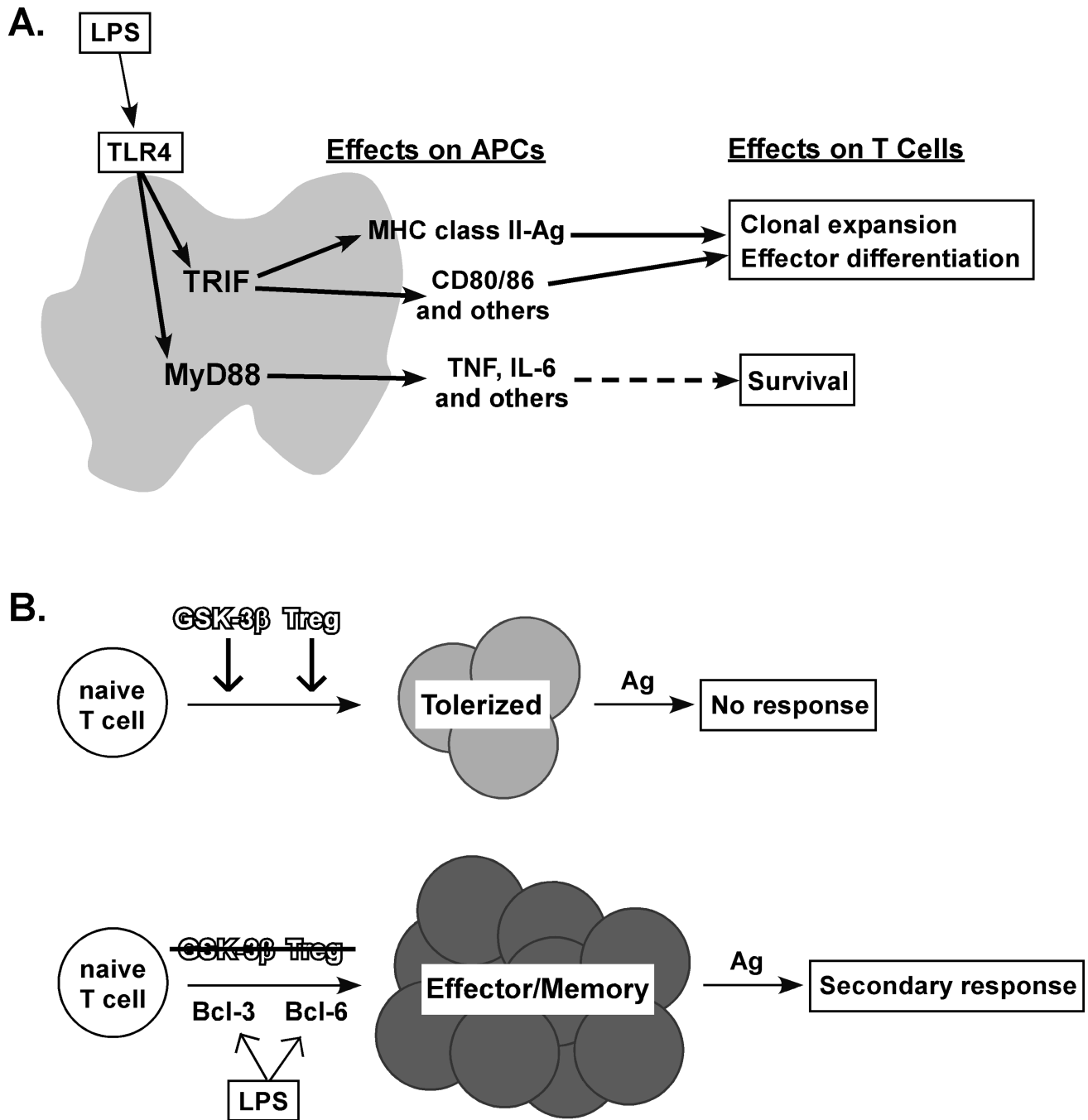
FIGURE 1.
Free LPS molecules are recognized by LPS binding protein (LBP), shuttled to CD14 receptors, and delivered to TLR4:MD-2 complexes. Signaling pathways are propagated through MyD88 and TRIF, resulting in activation of the transcription factors AP-1, NF-κB, IRF3, and IRF7. MyD88 and TRIF can both activate MAP kinases and NF-κB via TRAF6, while only TRIF activates IRF3 and IRF7. Among the plethora of LPS-induced genes are inflammatory cytokines and type I IFNs, which can act in an autocrine or paracrine manner, contributing to bystander activation. LPS responsiveness is negatively regulated at every level by proteins that disrupt specific components of signaling pathways, and some examples are provided (open letters).
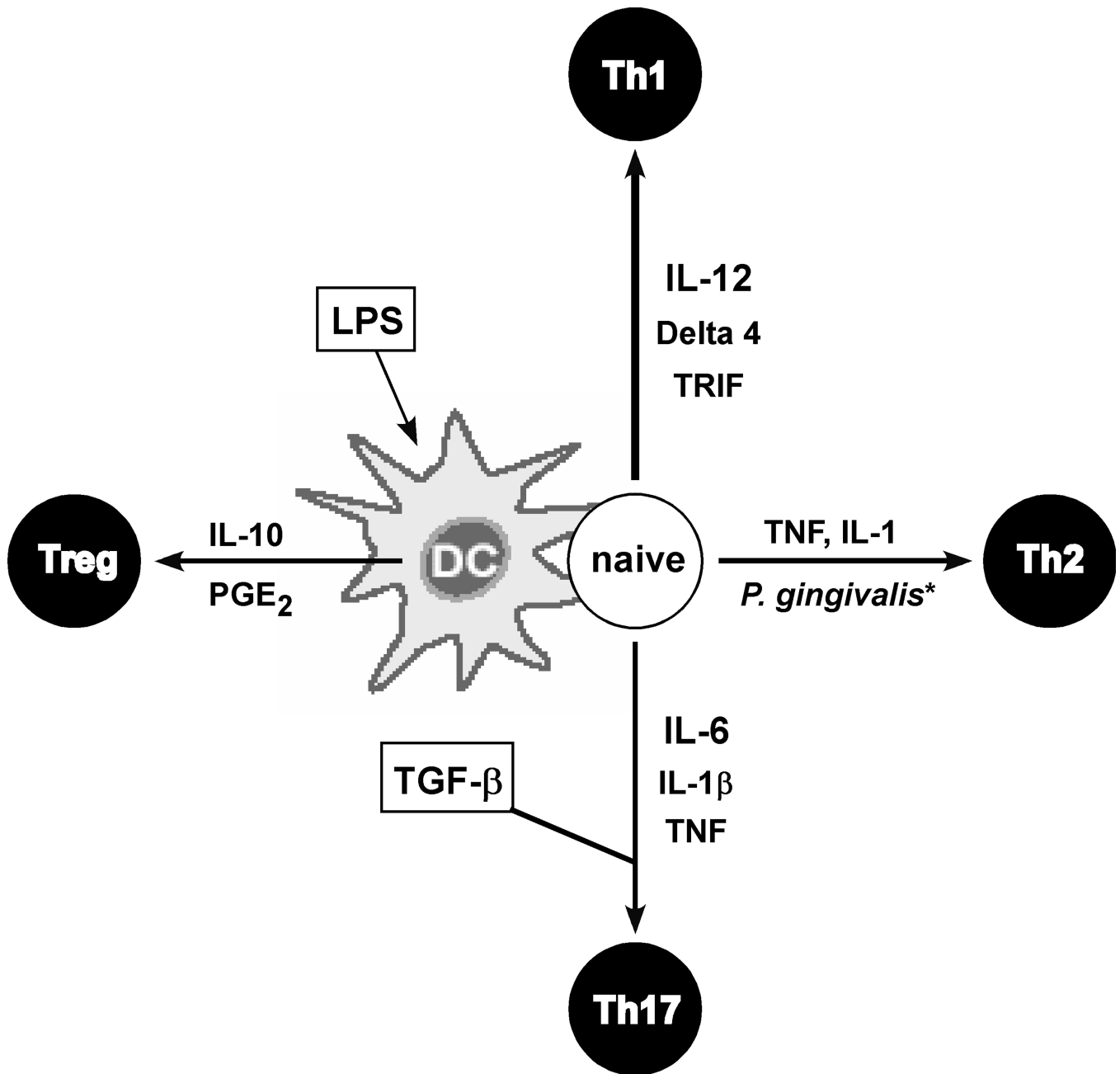
FIGURE 2.
LPS elicits Th1 responses in most models (thick arrow) through MyD88-dependent (IL-12, Delta 4) or –independent (TRIF) pathways. Alternatively, TNF and IL-1 may promote Th2 antibody responses. *P. gingivalis* LPS preparations also generate Th2 responses, possibly due to lipopeptide activity (asterisk).[65] The cytokines IL-6, TNF, and IL-1β can promote Th17 differentiation in a TGF-β-rich microenvironment, while LPS-derived IL-10 or PGE$_2$ supports Treg differentiation.

A.

B.

FIGURE 3.
Innate immune stimulation by LPS positively impacts T cell priming. (A) LPS stimulation through TLR4 causes APCs to upregulate Ag-bearing MHC class II chains[17] and costimulatory molecules, both of which push T cell clonal expansion and effector differentiation. In addition, activated APCs secrete cytokines that enhance T cell survival. The link between TNF and T cell survival is not known (dashed arrow), while IL-6 is thought to render effector CD4 T cells resistant to suppression by Tregs. Since only the TRIF pathway, not MyD88, participates in upregulation of the costimulatory molecules CD40, CD80, and CD86, this suggests LPS can use independent signaling pathways to drive T cell effector differentiation versus survival. In this context, TRIF-dependent responses

may require direct contact between APCs and T cells while MyD88-dependent responses may not. The identity of the LPS-responsive cell types *in vivo* is unclear. (B) In order for naïve T cells to become effector or memory cells, they must overcome suppressive barriers that are intrinsic (GSK-3β) and extrinsic (Treg) to the starting population. LPS-derived signals render T cells resistant to suppression, allowing them to undergo full clonal expansion, effector differentiation, and survival. LPS-induced T cell survival is associated with their expression of Bcl-3 and Bcl-6.