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Toll-Like Receptor 2 Ligands as Adjuvants for Human Th1 Responses

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Bacterial lipopeptides (bLPs) are increasingly used as adjuvants to activate cell-mediated immune responses to foreign Ags. To explore mechanisms whereby bLPs adjuvant T cell responses, we stimulated human PBMCs with bLPs. We found that bLPs stimulate T cells to proliferate and produce IFN-γ in an accessory cell-dependent manner and in the absence of exogenous protein Ags. The ability of bLPs to stimulate T cell proliferation was Toll-like receptor 2 dependent and required IL-12, interaction with costimulatory molecules, and MHC proteins. Our data suggest that bLPs adjuvant adaptive Th1 responses by enhancing Ag presentation of endogenous peptides. The Journal of Immunology, 2003, 170: 194–200.

Primitive organisms rely exclusively on preprogrammed host defense receptors to recognize and respond to microbial pathogens. Mammals have conserved these pattern recognition receptors but in addition use receptors whose genes rearrange to adapt to the unique structures of diverse pathogens. This increased level of complexity allows mammals to elicit a three-phase host immune response upon exposure to an infectious microorganism. Innate immunity provides an immediate and direct response in which pattern recognition receptors, such as the Toll-like receptors (TLRs), recognize and respond to various pathogen-associated molecular patterns (1) by eliciting direct antimicrobial pathways (2, 3) and by inducing NO (4) and phagocytosis (5). Activation of TLRs during the innate response also triggers the release of cytokines and chemokines (6–8) that mark the beginning of the early induced response. Early induced responses follow within hours of the innate response, allowing for additional inflammatory cells to be activated and recruited to the site of infection. Lastly, the adaptive immune response relies primarily on T and B cells and is distinct from the first two phases in its degree of specificity and its ability to generate immunological memory, two processes that combine to arm the host against future challenges by the same pathogen.

Bacterial lipoproteins activate cells of the innate immune system (9, 10) through TLR2 in combination with TLR1 (11), eliciting a signaling cascade resulting in NF-kB activation (4) and inflammatory cytokine production (10). The active portion of bacterial lipoproteins is an amino-terminal triacylated cysteine (12), a common microbial pattern. Synthetic lipopeptides comprising the active portion of bacterial lipoproteins as well as other TLR ligands are increasingly being used as adjuvants in animal vaccine models (13, 14), have been shown to be safe in human vaccine trials for HIV (15) and stimulate immunity against malaria (16) and hepatitis B virus (17) in human volunteers. However, the mechanism of TLR-mediated adjuvant function is not fully characterized. We sought to develop an in vitro system to investigate the adjuvant effect of natural microbial pattern molecules using bacterial lipopeptides (bLPs). The adjuvant activity of bLPs is mediated by TLR2, requires the expression of costimulatory proteins and inflammatory cytokines from APCs, and results in the activation of adaptive Th1 responses.

Materials and Methods

Reagents and Abs

Synthetic bLPs were obtained from commercial sources (N-palmitoyl-(s)\[2,3-bis(palmitoyloxy)-(2S)-propyl]-Cys synthetic bLPs obtained from Roche Applied Science, Indianapolis, IN; Mycobacterium tuberculosis 19-kDa lipopeptide obtained from Bachem, King of Prussia, PA). Treponema pallidum lipopeptide (TP47) was a gift from Dr. M. Norgard (9) (University of Texas Southwestern, Dallas, TX). Abs used were obtained from the following sources: anti-TLR2 (18); C8.6 (mouse anti-human IL-12 (BD PharMingen), L243 (mouse anti-human MHC class II; American Type Culture Collection, Manassas, VA), isotype controls (BD PharMingen), and cyclosporine (Novartis Pharmaceuticals, East Hanover, NJ).

Isolation of PBMCs and culture system

PBMCs were isolated by density gradient centrifugation (Ficoll-Paque; Amersham Pharmacia, Piscataway, NJ) from healthy human subjects after obtaining informed consent. PBMCs (2 × 10^7/200 μl) were cultured in the presence of bLPs for 3–7 days. For proliferation assays, [3H]thymidine was added for the last 4 h of culture. Cells were harvested and counted in a liquid scintillation counter. To evaluate the frequency of reactive cells, PBMCs were labeled with CPSE (15 min) before culture with bLPs. For cytokine ELISAs, culture supernatants were removed (24–48 h) from microwell plates prepared as for thymidine incorporation assays. Cytokine ELISAs were performed using matched Ab pairs for IFN-γ, IL-4, and GM-CSF (BD PharMingen) for PBMC cultures and IL-12 (BD PharMingen) for adherent cell cultures.

Isolation of cell populations

T cell, T cell subset, and monocyte populations were enriched using Rosette Sep (StemCell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer’s instructions. Cell lineage depletions were

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3 Abbreviations used in this paper: TLR, Toll-like receptor; bLP, bacterial lipopeptide.
performed using Dynabeads (Dynal, Lake Success, NY) as previously described (20). T cells were enriched by depleting B, T, NK, dendritic cells, monocytes and granulocytes; conversely, monocytes were enriched by depleting B, T, NK, T cells, and granulocytes. When cell populations were combined, T cells (2 × 10^5) were added to monocytes (2 × 10^5) in 200 µl. To identify the TLR2 cell population required for T cell proliferation to bLPs, whole blood was divided into two portions. One portion was enriched for monocytes using Monoocyte Rosette Sep (StemCell Technologies), whereas the other portion was enriched for T cells using T cell Rosette Sep (StemCell Technologies). Monocytes were cultured with anti-TLR2 Ab or isotype control (30 min) followed by bLPs (30 min). Cells were washed and added to T cells. T cell proliferation was measured by [3H]thymidine incorporation.

Preparation of monocytes and monocytederived dendritic cells
PBMCs were isolated by density gradient centrifugation and monocytes were enriched by adherence (2 h, 37°C in medium containing 10% FBS) to plastic. To evaluate cytokine production and costimulatory protein expression, plastic-adhered monocytes were stimulated with lipopeptides in culture medium. To measure IL-12 production from monocytes, supernatants were harvested (24 h) and cytokine was quantitated by ELISA (BD Pharmingen). To measure costimulatory protein expression, cells were harvested (48 h) and CD80 and CD86 expression was determined by flow cytometry. Samples were acquired at the University of California, Los Angeles Flow Cytometry Core Laboratory.

Monocyte-derived macrophages and dendritic cells were prepared as described previously (21). Briefly, macrophages were derived by culturing adherent cells with medium and FBS, whereas dendritic cells were derived using the same conditions, but adding rGM-CSF (800 U/ml Leukine; SmithKlineBeecham) and rIL-4 (1000 U/ml; PeproTech, Rocky Hill, NJ). Macrophages and dendritic cells were harvested after 6 days and cultured with autologous T cells in the presence or absence of T cell growth factors, rIL-2 (1 nM; Chiron, Emeryville, CA) or rIL-12 (1 nM; BD Pharmingen).

Results
PBMC proliferation and cytokine production to bLPs
bLPs and other TLR ligands are increasingly used as adjuvants for adaptive immune responses. Therefore, we attempted to devise an in vitro system to dissect the mechanism of their adjuvant activity. We cultured human PBMCs with bLPs (six amino acids in length) rather than lipoproteins since peptides of this small size do not stimulate T cells directly through MHC Ag presentation. The synthetic bLP N-palmitoyl-(s)-[2,3-bis(palmitoyloxy)-(2R,3R)-propyl]-Cys stimulated PBMCs in a dose-dependent manner (Fig. 1A). When monocytes were added, we found that PBMC proliferation was readily detectable, although the extent of the response was quite variable (Fig. 1B). Proliferation was detected at 5 days of culture with peak proliferation at 7 days, suggestive of an adaptive response rather than innate or early induced. PBMC proliferation was not due to adjuvant activity toward xenogenic or allogeneic peptides in bovine or human serum since lipopeptides stimulated proliferation in serum-free medium (data not shown).

Lipoproteins are found in numerous bacteria and proinflammatory activity has been described for many of these bacterial lipoproteins (9, 22, 23); therefore, we wished to ascertain whether activation of PBMC proliferation was representative of lipoproteins of microbial origin. We tested synthetic lipopeptides representing lipoproteins from M. tuberculosis and Treponema pallidum, the causative agents of tuberculosis and syphilis, respectively. Both 19-kDa (M. tuberculosis) and Tp47 (T. pallidum) lipoproteins stimulated proliferation of human PBMCs (Fig. 1C). In addition, all three lipopeptides examined stimulated IFN-γ (Fig. 1, D and E) and GM-CSF (data not shown), but no detectable IL-4 production (Fig. 1E), indicating that these lipopeptides induce a cytokine pattern characteristic of Th1-like responses. These findings contrast with those of Pulendran et al. (24) who found that a TLR2 ligand stimulated TH2 responses in mice. These disparate findings can be explained by 1) contribution of other cells in vivo in mice not present in our in vitro system, 2) species differences in TLR responsiveness, or 3) use of distinct TLR2 ligands.

The inflammatory properties of bacterial lipoproteins are dependent on the acylated portion of the protein (9). To determine whether the PBMC proliferative response to bLPs required the lipid portion of the peptides, we used Tp47 in both the acylated and nonacylated form. Proliferation was measured by [3H]thymidine incorporation after 7 days in culture at several concentrations of lipoprotein with a single donor (A) or at one concentration using several donors (B). bLPs from three sources stimulated proliferation (C) and IFN-γ, but not IL-4 production (D and E). F. Bacterial peptide devoid of lipid (Lipid−) had no ability to stimulate PBMC proliferation. Values expressed are the mean cpm ± SEM of triplicate samples.

FIGURE 1. bLPs stimulate PBMC proliferation and cytokine production. Human PBMCs (2 × 10^5) were stimulated with bLPs. Proliferation was measured by [3H]thymidine incorporation after 7 days in culture at several concentrations of lipoprotein with a single donor (A) or at one concentration using several donors (B). bLPs from three sources stimulated proliferation (C) and IFN-γ, but not IL-4 production (D and E). F. Bacterial peptide devoid of lipid (Lipid−) had no ability to stimulate PBMC proliferation. Values expressed are the mean cpm ± SEM of triplicate samples.

T cells and monocytes are necessary and sufficient for PBMC proliferation to bLPs
To examine the mechanism whereby bLPs activate cellular proliferation, we first evaluated the cell lineages required. PBMCs were depleted of distinct cell lineages by immunomagnetic selection. Depletion of T cells (CD3) or monocytes (CD14) abrogated proliferation to bLPs (Fig. 2A), in contrast to depletion of B cells (CD19), which did not diminish the proliferation of PBMCs to bLPs.
To determine whether T cells and monocytes were sufficient for a proliferative response to bLPs, we independently enriched for T cells and monocytes by depleting other lineages. Neither T cells nor monocytes cultured alone responded to bLPs (Fig. 2B), but when cultured together proliferation to bLPs was restored. Flow cytometric analysis demonstrated that only CD3+ cells remained after the 7 days of culture, indicating that T cells were proliferating to the bLP stimulus (data not shown). Our data show that T cells and monocytes are necessary and sufficient for the proliferative responses of T cells to bLPs.

We next wished to determine the phenotype of T cells activated with bLP, thus we enriched for either CD4 or CD8 T cells and added them to monocytes in the presence of bLPs. Both CD4+ and CD8+ T cells proliferated in the presence of bLPs (Fig. 2C), indicating that both major T cell subsets are activated by bLPs.

**bLPs adjuvant T cells responses by activating monocytes through TLR2**

We and others have previously shown that bacterial lipoproteins activate innate immune responses through TLRs on monocytes (4, 25). To determine whether TLRs are involved in the PBMC response to bLPs, we used neutralizing Abs to TLR2 (18) and TLR4 (19). Anti-TLR2 Abs inhibited T cell proliferation to bLP in contrast to anti-TLR4 (Fig. 3, A and B); in contrast, TLR4 Abs did inhibit PBMC proliferation to LPS (26) (data not shown). These data demonstrate that T cell proliferation to bLPs is mediated by TLR2 activation.

Immunological adjuvants act to accelerate, prolong, or enhance an adaptive immune response. We next wanted to determine whether bLP activation of monocytes acted to adjuvant adaptive immune cells. We therefore enriched for both T cells and monocytes, cultured the monocytes separately with bLPs and then combined with CD3-enriched cells. The T cell populations were then combined with CD14-enriched cells and proliferation was measured by [3H]thymidine incorporation. Values expressed are the mean cpm ± SEM of triplicate samples.

**FIGURE 2.** T cells and monocytes are necessary and sufficient for PBMC proliferation to bLPs. A, PBMCs were depleted (depl.) of discrete cell populations using immunomagnetic selection and cultured in the presence of bLPs. B, Cell populations (T cells, CD3 and monocytes, CD14) were enriched using Rosette Sep and cultured in the presence of bLPs. Proliferation was measured as described in the legend of Fig. 1. C, T cell populations that proliferate to bLPs. T cell lineages were enriched using Rosette Sep and then recombined with CD14-enriched cells. The T cell populations were then combined with CD14-enriched cells and proliferation was measured by [3H]thymidine incorporation. Values expressed are the mean cpm ± SEM of triplicate samples.

**FIGURE 3.** bLPs stimulate monocytes through TLR2 to adjuvant T cells. PBMCs from an individual donor (A) or multiple donors (B) were stimulated with bLP in the presence or absence of neutralizing TLR2 or TLR4 Abs or isotype control Abs. Proliferation was determined by [3H]thymidine incorporation. C, Monocytes mediate the T cell response to bLPs via TLR2. PBMCs were divided in two, half were enriched for CD14 cells and the other half was enriched for CD3 cells. CD14 cells were cultured in the presence of Ab before the addition of bLPs and then combined with CD3 cells. Proliferation was measured by [3H]thymidine incorporation. Values expressed are the mean cpm ± SEM of triplicate samples.
of neutralizing anti-TLR2 Abs, then added T cells. Under these conditions, anti-TLR2 blocked bLP-induced T cell proliferation (Fig. 3C), in contrast to an isotype control Ab. The data indicate that bLPs stimulate monocytes directly through TLR2 to adjuvant adaptive T cell responses.

bLP stimulation of T cells is dependent on IL-12 and B7.2 (CD86)

To understand the mechanism whereby monocytes transduce the signal from bLPs to T cells, we used neutralizing Abs against T cell growth factors and costimulatory molecules. Since the T cell response to bLPs was Th1 in nature, we evaluated the role of IL-12, a growth and differentiation factor for Th1 cells (27). T cell proliferation to bLPs was inhibited in the presence of anti-IL-12, but not an isotype control Ab (Fig. 4), indicating a requirement for IL-12. Neutralizing Abs to other T cell growth factors including IL-15 and IL-18 did not diminish bLP-stimulated T cell proliferation.

To determine the role of costimulation, we used Abs against the two best-characterized costimulatory proteins, B7.1 (CD80) and B7.2 (CD86). Using specific Abs against the discrete forms of B7 would allow us to distinguish whether one or both of these B7 isoforms were required. As demonstrated in Fig. 5, CD86 Abs profoundly inhibited T cell proliferation to bLPs. In contrast, CD80 Abs did not show this inhibitory activity. Together, the data indicate that bLP-stimulated T cell proliferation requires IL-12 and CD86 ligation.

bLPs induce IL-12 production and CD86 expression on monocytes

Our data indicated a role for IL-12 and CD86 in the T cell response to bLPs, thus we wanted to determine whether bLPs stimulate monocytes to produce IL-12 and express CD86. We cultured monocytes with bLPs and measured IL-12 production by ELISA and CD86 expression by flow cytometry. As anticipated from our previous studies (4), Tp47 stimulated IL-12 production from peripheral blood monocytes (Fig. 6A). In addition, flow cytometric analysis demonstrated that Tp47 induced CD86 but not CD80 on monocytes in a dose-titratable manner (Fig. 6B). Thus, as predicted from our studies showing a requirement for IL-12 and CD86 increases in response to bLPs, we detected an up-regulation of IL-12 production and CD86 expression in response to bLPs. Up-regulation of costimulatory proteins is a hallmark of dendritic cell differentiation (28, 29); thus for comparison to lipopeptide activated monocytes, we derived dendritic cells using GM-CSF and IL-4. We found that monocytes cultured with GM-CSF and IL-4 expressed higher levels of CD86 in comparison to monocytes cultured with medium alone (Fig. 6D) and more like lipopeptide-activated monocytes.

FIGURE 4. PBMC proliferation to bLP requires IL-12. PBMCs of an individual donor (A) or three donors (B) were stimulated with bLPs in the presence or absence of IL-12 or an isotype control Ab. Proliferation was determined using [3H]thymidine incorporation. Values expressed are the mean cpm ± SEM of triplicate samples.

FIGURE 5. PBMC proliferation to bLPs requires the costimulatory molecule CD86. PBMCs of an individual donor (A and B) or four donors (C) were stimulated with bLPs in the presence or absence of CD80, CD86, or isotype control Abs. Proliferation was determined using [3H]thymidine incorporation. Values expressed are the mean cpm ± SEM of triplicate samples.
**T cell activation by bLP requires MHC proteins**

Our studies demonstrated that lipopeptide-activated T cells required a costimulatory pathway, signal 2 required for T cell activation, but a role for MHC-TCR, signal 1 had not been addressed. Therefore, we used Abs that block MHC class II-TCR interactions to assess the role of MHC in lipopeptide activation of T cells. We depleted CD8\(^+\) T cells from human PBMCs to concentrate on lipopeptide activation of CD4\(^+\) T cells, reasoning that CD8\(^+\) T cells would not require MHC class II. Neutralizing MHC class II Abs inhibited the activation of CD4\(^+\) T cells cultured with bLPs (Fig. 7, A and B). In contrast, the Abs did not prevent CD1b-restricted T cells from responding to bacterial glycolipids (data not shown), indicating the specificity for MHC-restricted T cells. To determine whether activation of T cells occurred through the TCR, we used cyclosporin A, which disrupts signaling through the TCR (30). Cyclosporin A inhibited T cell proliferation to bLPs (Fig. 7, C and D), suggesting that the TCR was involved in responding to lipopeptides. To gain additional insight into the nature of the T cell response, PBMCs were labeled with CFSE, then stimulated with a mitogen, PHA or bLPs. More than 80% of cells underwent cell division in response to PHA, in contrast to bLPs where the frequency of dividing cells was only 7%, indicating that the T cell response was not polyclonal, but resembles more that of peptide Ags. Together the data indicate that MHC class II-TCR interactions are required for lipopeptide activation of T cells, presumably by reducing the activation threshold to endogenous peptides.

**Discussion**

The influence of TLR ligands on host defense is exerted most directly on early phases of the immune response. However, the widespread use of TLR ligands as vaccine adjuvants indicates that they promote adaptive responses in vivo as well. We wished to ascertain the mechanism of the adjuvant properties of bLPs on adaptive immunity in an in vitro system; therefore, we cultured human PBMCs with bLPs. We found that bLPs stimulate T cells to proliferate and produce Th1 cytokines in an accessory cell-dependent manner. The ability of bLPs to stimulate T cell proliferation was TLR2 dependent and required IL-12, expression of costimulatory molecules, and MHC
NF- and Environment for microbial patterns and transmitting a signal through BE (53) were cultured or multiple donors (53). Human PBMCs from one donor (A/H9260) or multiple donors (A/H11006) were used for stimulating APC function.

Figure 7. T cell proliferation to bLP requires MHC class II. A and B, Human PBMCs from one donor (A) or multiple donors (B) were cultured in the presence of anti-MHC class II Abs or an isotype control before the addition of bLPs. Proliferation was measured using [3H]thymidine incorporation. Values expressed are the mean cpm ± SEM of triplicate samples.

proteins. We interpret these findings to indicate that bLPs adjuvant adaptive Th1 responses by enhancing APC function.

Activation of T cells by bLPs required a TLR2-mediated signal on monocytes, which elicits a signaling cascade that includes NF-κB activation (4). We found that bLP-stimulated T cells required IL-12 production and CD86 up-regulation from monocytes, two NF-κB-dependent processes (31, 32). By surveying the environment for microbial patterns and transmitting a signal through NF-κB to cause the up-regulation of inflammatory cytokines and costimulatory proteins, TLR2 allows cells of the innate immune system to influence the adaptive immune response in concert with their ability to elicit more rapid responses.

Ag-specific T cell responses require TCR engagement from MHC and peptide (signal 1) as well as costimulation (signal 2), which is thought to reduce the threshold of signal required for T cell activation (33). Although T cells recognize peptide epitopes within bacterial lipoproteins through their TCRs (34, 35), there is presently no evidence for recognition of the N-terminal acylated portion. The small size (six amino acids) of the lipopeptides precludes their binding to MHC for TCR stimulation, thus it is unlikely that T cells responded to bLPs through the TCR. However, our data also revealed that MHC and costimulation were required for T cell responses to bLPs. Taken together, the data suggest to us that lipopeptides enhance the Ag-presenting function of monocytes. One potential mechanism for enhanced Ag presentation in response to TLR ligation would be the rapid differentiation of monocytes into dendritic cells, a subject of ongoing studies. We speculate that TLR-activated monocytes differentiate into dendritic cells and form synapses with T cells, reducing the threshold to activate T cells recognizing endogenous peptides (36, 37).

Alternatively or coincidentally, TLR ligand activation of T cells may sustain memory T cells via activation of accessory cells, as has been shown for TLR ligands poly(I:C) and LPS (26, 38, 39). Our study revealed that IL-12 and costimulation were required for T cell proliferation to a TLR2 ligand. Studies in mice indicate that costimulation combined with IL-12 activates Th1 clones whereas naive T cells require costimulation but no IL-12 (40), further implying that bLPs activate memory T cells. Our future studies are designed to further identify the phenotype of T cells responding to bLPs.

What advantage to the host is provided by bLP-mediated T cell activation? First, TLR2 signaling promotes dendritic cell maturation, resulting in enhanced Ag presentation to peptide-specific T cells (41, 42). We found that both CD4+ and CD8+ T cells were activated by bLPs, indicating that helper and CTL activity could be enhanced by bLPs. Second, bLPs skewed the T cell cytokine pattern toward Th1, suggesting that cell-mediated immune responses are promoted by TLR2 activation. A burst of inflammatory lymphocytes are recruited into the site of infection, yet only a fraction of the inflammatory cells (~1 in 500) are Ag specific (43). A third function of TLR activation may be to recruit Ag-nonspecific T cells providing a critical mass in situ of inflammatory lymphocytes that activate macrophages for a short time but undergo activation-induced cell death if not rescued by Ag stimulation (44).

An unwanted consequence of the adjuvant activity of TLR ligands may be immunopathology. Costimulation and IL-12 mediate reactivity against self as well as non-self-Ags (45). By increasing costimulation and lowering the threshold for T cell activation, bLPs and other TLR ligands could promote T cell responses to endogenous peptides as our data suggests. In chronic infections where TLR ligands persist such as Lyme disease, Borrelia burgdorferi lipoproteins stimulate inflammatory events through TLR2 (46). TLR activation also can take place in the absence of infection via degradation of the extracellular matrix (47) induced by UV irradiation (48), a common cause of flare in systemic lupus erythematosus. Increased adjuvant activity from TLR ligation combined with an insufficient regulatory T cell response (49) could increase susceptibility to autoimmune disease. In considering the clinical utility of lipopeptides and other TLR agonists as adjuvants, it will be important to determine ways to prevent these deleterious side effects. With greater understanding of the mechanism of TLR adjuvant activity, it should be possible to target TLR responses in vaccines and other immunotherapies to prepare for the ever-increasing threat of infectious predators while diminishing the likelihood of anti-self-responses.

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References

TLR2 ACTIVATION BY bLPs STIMULATES Th1 RESPONSES