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Lipoteichoic Acid Preparations of Gram-Positive Bacteria Induce Interleukin-12 through a CD14-Dependent Pathway

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Interleukin 12 (IL-12) strongly augments gamma interferon production by natural killer (NK) and T cells. IL-12 also promotes effective cell-mediated immune responses, which are particularly important against intracellular bacteria such as *Listeria monocytogenes*. While the lipopolysaccharide (LPS) of gram-negative bacteria induces monocyte production of IL-12, the relevant gram-positive components which induce IL-12 production are uncharacterized. We used the human monocytic cell line THP-1 to study IL-12 induction by gram-positive bacteria. Muramyl dipeptides as well as the major muramyl tetrapeptide component of *Streptococcus pneumoniae* were inactive for inducing IL-12. In contrast, lipoteichoic acid (LTA), a predominant surface glycolipid of gram-positive bacteria, potently induced IL-12 p40 gene expression. A competitive LPS antagonist, *Rhodobacter sphaeroides* LPS, inhibited LTA-induced IL-12 production, suggesting a common pathway for LPS and LTA in IL-12 activation. Pretreatment of cells with anti-CD14 monoclonal antibody blocked both LPS and LTA induction of IL-12 p40 expression. LTA also induced Th1 development in naive CD4 T cells by an IL-12-dependent mechanism, indicating direct induction of physiologic levels of IL-12. Together, these results show that LTA is a potent surface structure of gram-positive bacteria which induces IL-12 in monocytes through a CD14-mediated pathway.

Interleukin-12 (IL-12) is a heterodimeric cytokine composed of a constitutively expressed 35-kDa (p35) subunit and an inducible 40-kDa (p40) subunit, which enhances activity of natural killer (NK) cells, promotes development of cytolytic T cells (CTL) and T helper 1 (Th1) cells, and enhances gamma interferon (IFN-γ) production (17, 40, 44). IL-12 is primarily produced by macrophages in response to gram-positive and -negative bacteria as well as other pathogens such as *Mycobacterium tuberculosis* (4, 5, 36, 37). While lipopolysaccharide (LPS) from gram-negative bacteria induces IL-12 production (7, 14, 41), the microbial structures by which intracellular gram-positive pathogens such as *Listeria monocytogenes* induce IL-12 is unknown. Furthermore, it is unknown to what extent pathogenic extracellular gram-positive bacteria such as *Streptococcus pneumoniae* and *Staphylococcus aureus* induce IL-12. The receptor through which gram-positive bacteria deliver this stimulus is uncharacterized. The purpose of the current study was to identify the active components of gram-positive organisms for induction of IL-12 and define the pathway for its induction. We find that lipoteichoic acid (LTA), a class of surface glycolipid similar to LPS, is a potent IL-12-inducing agent on gram-positive bacteria and have characterized the receptor mechanisms through which macrophages respond to this material.

LTAs are a highly diverse class of sugar phosphate polymers which bear a single lipid side chain anchored to a ribitol or glycerol backbone. A nonacylated form of LTA, teichoic acid (TA), is covalently attached to gram-positive peptidoglycan. Many of the clinical features observed during gram-positive sepsis have been related to the potent inflammatory properties of LTA and TA (1, 11, 19, 20, 30, 34, 38). Like LPS, LTA induces activation of macrophages, including stimulation of several cytokines (IL-1β, tumor necrosis factor alpha [TNF-α], IL-6, and IL-8) and inducible nitric oxide synthetase (1, 26, 33). The cell surface receptors through which LTA exerts these effects are unknown, although a recent report has described LTA binding to type I macrophage scavenger receptors (9).

The macrophage appears to be the most significant source of IL-12, although some B cell lines produce IL-12 at low levels constitutively or with phorbol ester induction (40). Expression of the p35 subunit is constitutive in many cell types, although recent studies have noted p35 induction in IFN-γ-primed macrophages (2, 13). Exposure of macrophages to bacteria or bacterial products causes rapid induction of the p40 subunit of IL-12 (2). mRNA expression of the p40 subunit correlates with secreted levels of IL-12 in vitro (44). In fact, soluble forms of free p40 are secreted along with biologically active heterodimers (2, 44).

Since the first clear demonstration of pathogen-induced development of Th1 cells by IL-12 involved gram-positive organisms (17), we wished to identify the mechanism of induction of IL-12. Thus, we set up an in vitro system to characterize IL-12 induction by bacterial products. THP-1 is a human monocytic leukemia cell line which is responsive to bacterial stimulation of p40 and IL-12 (22). In THP-1 cells, we find that LTA was a potent stimulant of not only the p40 subunit of IL-12 but also multiple other early response cytokines (IL-1β, TNF-α, IL-6, and IL-10). Other major structural components, including the major GlcNAc-MurNAc-tetrapeptide of pneumococcus, were inactive. THP-1 responsiveness to LTA was inhibitable by an LPS antagonist, *Rhodobacter sphaeroides* LPS (RS-LPS). Furthermore, blocking antibody to the LPS binding receptor, CD14, abrogated the IL-12 induction by LTA. LTA and LPS

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TABLE 1. Oligonucleotide primer pairs and probes for human gene products measured by RT-PCR (all 5′-3′)

Gene product	Sense oligonucleotide	Antisense oligonucleotide	Probe
p35	TTTATGAAGACTTGAAGATGTACCAG	TCAAAGTTTTATAAAAATGACAACGG	CATGCTTTCAGAATTCGGGC
p40	GAGTCTGCCCATTGAGGTCAT	AATTTTCATCCTGGATCAGAACC	CATTAGCGTGCGGGCCCAGGA
IL-1	ATGAAGTGCTCCTTCCAGGACCTG	CCTGGAGGTGGAGAGCTTTCAGTT	GAACCTATCTTCTTCGACACATGGG
IL-6	GTGTTGCCTGCTGCCTTCCCTG	CTCTAGGTATACCTCAAACTCCAA	CAAAGATGTAGCCGCCCCACACAG
IL-10	GCTGGAGGACTTTAAGGGTTACCTGGG	TCTTGGAGCTTATTAAAGGCATTCTTCACC	AGGCTACGGCGCTGTCATCGATTTCTTCCC
TNF-α	GGACGTGGAGCTGGCCGAGG	TGGGAGTAGATGAGGTACAGGCCC	CTCTAATCAGCCCTCTGGCCCAG
HPRT	TGGGAGGCCATCACATTGT	GCTTTTCCAGTTTCACTAATGACA	GGTGGAGATGATCTCTCAACTTT

behaved similarly in an in vitro IL-12-dependent Th1 cell development assay. Our results provide evidence that LTA is a potent IL-12-inducing compound and suggest that LTA signaling is mediated through interaction with CD14 on the surface of macrophages.

MATERIALS AND METHODS

Cell lines and culture conditions. The promonocytic cell line THP-1 was obtained from D. Chaplin (Washington University, St. Louis, Mo.) and maintained in RPMI medium supplemented with 10% fetal calf serum, essential amino acids, 2-mercaptoethanol, and antibiotics. For activation, 10^6 cells were placed in 1-ml cultures in 24-well plates and stimulated with the following bacteria or subcomponents: LPS (*E. coli* serotype O55:B5; Sigma Chemical Co., St. Louis, Mo.), LTA derived from *Staphylococcus aureus* (ribitol phosphate backbone) or *Streptococcus pyogenes* (glycerol phosphate backbone; Sigma), *Streptococcus pneumoniae* R6 or its purified cell wall or major peptidoglycan subcomponents (11), or muramyl dipeptide (Sigma). The endotoxin activities of these commercial LTA products were found to be undetectable in the *Limulus* amebocyte lysate assay at a sensitivity of 3 pg/ml (BioWhittaker, Walkersville, Md.). RNA was harvested from cultures for use in reverse transcriptase (RT) PCR measurement of mRNA of various cytokines. In some experiments, THP-1 cells were pretreated for 1 h at room temperature with RS-LPS (List Biological Laboratories, Campbell, Calif.) or anti-CD14 (MY4; Coulter Immunology, Hialeah, Fla.) at the indicated concentrations before stimulation with LPS or LTA. Purified monoclonal immunoglobulin G2b (IgG2b; Coulter Immunology) was used as an isotype-matched control. Other controls included nonblocking antibodies to CD14 (LeuM3; Becton Dickinson, San Jose, Calif.) and anti-CD11b (anti-Mac-1; Pharmingen, San Diego, Calif.).

RT-PCR. The RT-PCR assay used in this work has been detailed extensively elsewhere (27). PCR primer pairs for all cytokines and hypoxanthine phosphoribosyltransferase (HPRT) were designed to span intron-exon splice sites to avoid amplification of any genomic DNA. Sense and antisense oligonucleotides for all gene products are listed in Table 1. Total cellular RNA was extracted from culture wells as listed above with a Stratagene (La Jolla, Calif.) RNA isolation kit. THP-1 cells (10^6) yielded approximately 1 μg of RNA, from which 0.1 μg was used for reverse transcription with Moloney murine leukemia virus enzyme (Gibco, Gaithersburg, Md.). Approximately 15% of this RT product was used in subsequent PCRs with a 30-cycle amplification with Amplitaq (Perkin-Elmer, Branchburg, N.J.). Temperatures and times for the PCR were as follows: 94°C for 30 s, 50°C for 30 s, and 74°C for 30 s. Single PCR products of appropriate size were identified on agarose gels. For quantitation of PCR product, the PCR product was then blotted onto nitrocellulose and hybridized to ^{32}P-end-labeled oligonucleotide probes specific for sequences internal to the PCR primers. End-labeling was achieved with T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.). All probe sequences are listed in Table 1. Following washing, specific hybridization was quantified by phosphorimaging (Kodak, Rochester, N.Y.), and relative mRNA levels for each cytokine product were normalized to the signal of HPRT-hybridized samples generated from the same experiment. Under these conditions, PCR products were demonstrated to be in the linear range of the assay, and input RNA was demonstrated to be nonsaturating.

Luciferase assay for p40 promoter constructs. Details concerning the development of the murine DM-4300 p40 promoter construct in a luciferase expression vector were described by Murphy et al. (28a). J774 cells were stably transfected with DM-4300 containing 4.3 kb of proximal 5′ sequence and the untranslated first exon of the p40 promoter. Cells were simultaneously transfected with the neomycin resistance plasmid pSRαneo72.3 and maintained under selection. Cells were stimulated for 4 h with various stimuli prior to lysis in Promega lysis buffer and subsequent measurement of luciferase activity.

T-cell development assay. T cells were obtained and purified as described previously (16) from peripheral lymph nodes of α/β T-cell-receptor transgenic mice with antigen specificity for ovalbumin peptide 323-339 (OVA) (17, 28). Briefly, purification involved centrifugation over a Ficoll-Hypaque density gradient (Histopaque-1119; Sigma), depletion of CD8 or class II-positive cells with monoclonal antibodies 3.155 (31) and CA4 with rabbit complement (Cedarlane, Hornby, Canada). B cells were removed by sheep anti-mouse IgG magnetic beads (Dynal, Lake Success, N.Y.). Fluorescence-activated cell sorter analysis of the resulting population routinely achieved a purity of 92 to 95% for CD4 staining. Cells were plated at 1.25×10^5 in 1.0-ml cultures in 48-well plates in the presence of 0.3 μM OVA with 2.5×10^6 irradiated (2,600 rads) BALB/c splenocytes. LPS and LTA were added in various concentrations at the initiation of culture. Positive controls for Th1 development included the addition of 10^6 heat-killed *L. monocytogenes* (obtained from E. Unanue, Washington University, St. Louis, Mo.) or recombinant IL-12 (5 U/ml; generous gift of S. F. Wolf, Genetic Institute, Cambridge, Mass.). Monoclonal anti-IL-12 (TOSH; 3 μg/ml; generous gift of K. S. Tripp, Washington University School of Medicine, St. Louis, Mo.) was used to neutralize in vitro-produced IL-12. After 1 week of stimulation and expansion, the cells were washed and placed in culture at 1.25×10^5 with fresh peptide and antigen-presenting cells with no other stimulus. After 2 days, supernatants were harvested, and IL-4 and IFN-γ content was measured by enzyme-linked immunosorbent assay (ELISA). Controls for the T-cell development assay included conditions in which antigen-presenting cells or OVA was excluded from both the primary and secondary stimulation or in which LTA was added in the absence of antigen-presenting cells or OVA. In these instances, no cytokine production was discernible.

RESULTS

LTA is a potent inducer of the p40 component of IL-12. Several gram-positive bacteria are strong activators of IL-12 production by macrophages. For example, *L. monocytogenes* (16), *Staphylococcus aureus* (37), and the R6 strain of *Streptococcus pneumoniae* (Fig. 1) are all very potent for IL-12 induction and activation of p40 mRNA in THP-1 cells, a human monocytic cell line responsive to multiple stimuli for p40 (22). In the case of pneumococcus, cell surface molecules have been

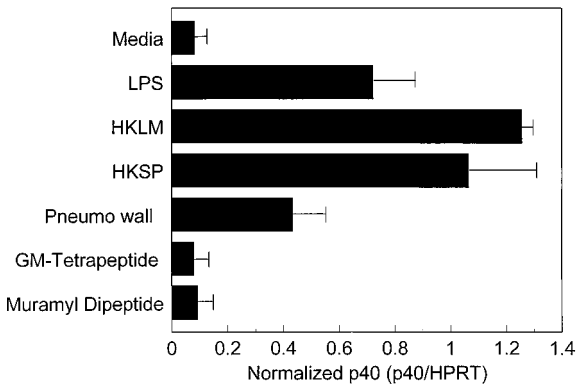

FIG. 1. LTA is the predominant structure of gram-positive bacteria which induces IL-12 p40. THP-1 cells were placed in culture for 4 h with LPS (0.1 μg/ml), heat-killed *L. monocytogenes* (HKLM) or *Streptococcus pneumoniae* (HKSP; 10^8), purified pneumococcus cell wall (Pneumo wall) or its predominant peptidoglycan tetrapeptide (GM-Tetrapeptide; at the indicated bacterial equivalents), and muramyl dipeptide (0.1 mg/ml). RNA was then harvested, and expression of p40 and HPRT was determined by RT-PCR. Dot blotted PCR products were hybridized to end-labeled internal oligonucleotide probes, and the resulting signal was quantified by phosphoimagery. p40 values were normalized by division of the HPRT signal from the same sample. Data are representative of four separate experiments.

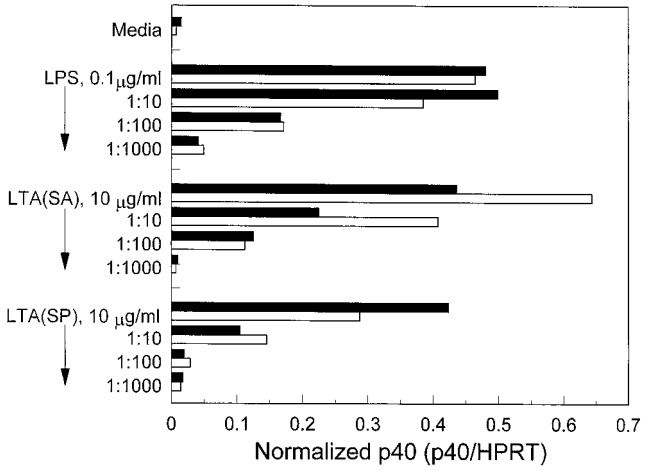

FIG. 2. LTA and LPS induce IL-12 p40 gene expression as measured by RT-PCR. THP-1 cells were placed in culture with various concentrations of LPS or LTA derived from *Staphylococcus aureus* (SA) or *Streptococcus pyogenes* (SP) for 4 h. p40 and HPRT were measured by RT-PCR as described in the legend to Fig. 1. Pairs of bars are duplicates. The data are representative of four separate experiments.

sufficiently well characterized to allow testing of individual components for this bioactivity (11, 35). Purified pneumococcal cell wall-bearing TA was highly active for induction of p40 (Fig. 1). Cell wall is a heteropolymer of TA and peptidoglycan. The lack of activity of purified peptidoglycan monomers (*N*-acetylglucosamine-*N*-acetyl muramic acid tetrapeptide or GM-tetrapeptide) suggested that the TA or its acylated analog, LTA, was instrumental in IL-12 induction (Fig. 1). Even at very high doses, GM-tetrapeptide failed to activate p40 mRNA transcription. Furthermore, muramyl dipeptide was inactive (Fig. 1). Thus, we suspected that TA and LTA of gram-positive organisms were responsible for IL-12 induction.

While LPS is presumably the predominant gram-negative component responsible for induction of IL-12 (2, 3), no studies to date have examined gram-positive lipid constituents for IL-12 induction. As a prominent surface structure of gram-positive organisms, LTA remained a possible candidate (1, 26, 33). We tested the two classes of LTA (ribitol- and glycerol-containing) preparations for induction of p40 by RT-PCR in THP-1 cells. Figure 2 shows dose-response curves for p40 mRNA induction by LTA derived from *Staphylococcus aureus* or *Streptococcus pyogenes*. Both preparations of LTA were potent stimulants of p40 expression in THP-1 cells. Near-maximal induction was achieved with 10 μg of *Staphylococcus aureus*-derived LTA per ml. LTA was found also to be active in similar concentrations when added to other cell sources, including elutriated human peripheral blood monocytes, J774 (a murine macrophage cell line), and mouse peritoneal exudate cells, spleen cells, and bone-marrow-derived macrophages (data not shown). Endotoxin activity by *Limulus* amebocyte lysate assay of these LTA preparations was undetectable. These LTA preparations have been used by others and have been found to be LPS free (8, 19, 20, 33). This range of bioactive concentrations is in agreement with previous reports of LTA induction of other macrophage cytokines and activities (19, 33). These results indicate that LTAs are a relatively potent IL-12-inducing structural component of gram-positive bacteria.

LTA induces multiple other macrophage cytokines simultaneously with p40. IL-12 is a rapidly induced cytokine when macrophages are exposed to bacteria (7). We investigated the kinetic differences between mRNA signals generated from THP-1 cells treated with LPS or LTA for times between 1 and 24 h. RNA from activated THP-1 cells was harvested, and expression of p40 and multiple other cytokines was assessed by RT-PCR (Fig. 3). p40 mRNA expression was rapidly induced by both LPS and LTA, with maximal induction occurring at 4 to 6 h. p35 mRNA demonstrated constitutive expression at these same time points (data not shown). A similar time course of induction for multiple other cytokines was observed for both LPS and LTA treatment of THP-1 cells. Consistent with previous reports, TNF-α, IL-1, and IL-6 undergo induction in macrophages exposed to LTA (1, 25, 33). Figure 3 confirms these observations and also shows the novel finding of LTA induction of IL-10 expression. TNF-α expression was the most rapid to rise and to fall, while IL-10 showed delayed induction. These data indicate that IL-12 p40 is rapidly induced with either LPS or LTA treatment. Thus, no differences between LPS and LTA were found in the induction of p40 and multiple other cytokine mRNAs.

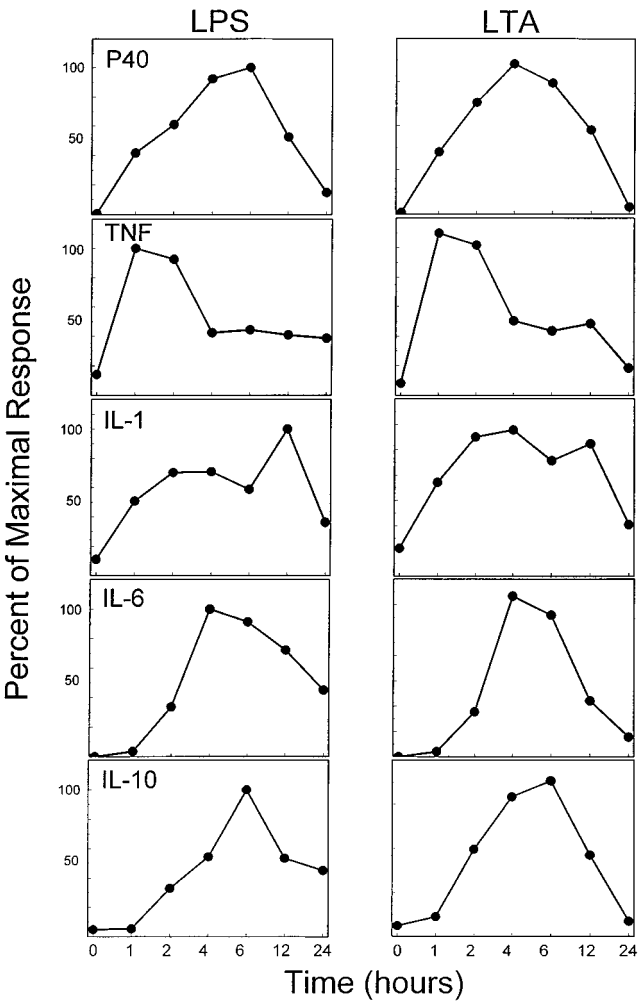

FIG. 3. LPS and LTA demonstrate similar time course induction of p40, TNF-α, IL-1β, IL-6, and IL-10. THP-1 cells were placed in culture and stimulated for various lengths of time with LPS (0.1 μg/ml) or LTA (1.0 μg/ml; from *S. aureus*). Cytokine and HPRT mRNA levels were measured by RT-PCR as described in the legend to Fig. 1. Normalized values are shown as a percentage of maximal inductive response for this particular experiment. Datum points are averages of duplicates, with less than 20% variance between duplicates. The results are representative of two separate experiments.

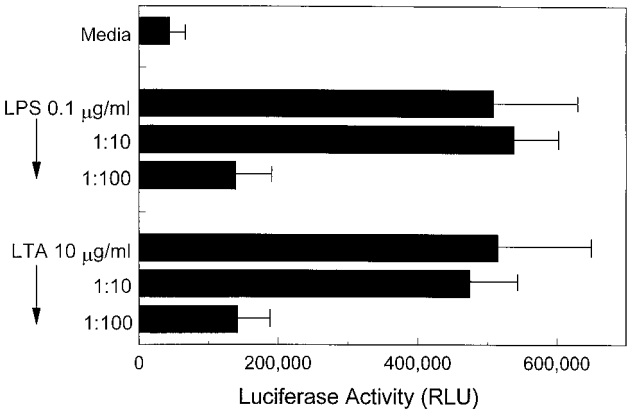

FIG. 4. LTA and LPS both induce p40 promoter elements. J774 cells, stably transfected with a murine p40 promoter luciferase reporter, were treated with LPS or LTA at various doses for 4 h prior to measurement of luciferase. Data presented are averages of triplicate wells, with standard deviations indicated by error bars. Results are representative of three separate experiments. RLU, relative light units.

LTA activates transcription via p40 promoter elements. To confirm the results described above in a functional system, we used LPS and LTA in a luciferase-based reporter assay to study induction of the p40 promoter. LPS induction of multiple cytokines occurs through induction of NF-κB (18). Recent data also suggest that gram-positive cell walls are also potent stimulators of NF-κB (32). We have shown recently that the LPS activation of the IL-12 p40 promoter similarly occurs through induction of NF-κB binding to a unique NF-κB consensus sequence (28a). We next addressed whether LTA could induce p40 promoter elements previously found to be inducible by LPS. Figure 4 shows that LTA induces luciferase activity in a dose-dependent fashion from a luciferase reporter containing a 190-bp fragment of the p40 promoter (DM-4300) transfected stably into J774, a murine monocytic cell line. LTA and LPS induced similar levels of luciferase activity at the same concentrations used for mRNA induction. The results indicate that LPS and LTA are similar in at least two different p40 induction systems, suggesting similar modes of activation.

LPS antagonist also inhibits LTA induction of p40. RS-LPS has been used as an antagonist for LPS-mediated responses and cytokine production in macrophages (12, 15). The inhibitory activity of RS-LPS resides within the lipid A portion of the molecule and is thought to act as a competitive inhibitor of LPS-binding receptors on the macrophage cell surface (21, 25). To determine whether LTA and LPS act through the same pathway, we next addressed whether RS-LPS could inhibit both LPS and LTA induction of p40. THP-1 cells were pretreated with various concentrations of RS-LPS for 1 h before activation with LPS or LTA (Fig. 5). RS-LPS completely inhibited LPS induction of p40. This inhibition was overcome by dilution of RS-LPS. Interestingly, LTA induction of p40 was also inhibited by prior treatment of cells with RS-LPS (Fig. 5), with nearly complete inhibition when 0.1 μg of RS-LPS per ml was used to inhibit 1.0 μg of LTA per ml. Again, RS-LPS inhibition of LTA was lost with dilution of the inhibitor. These results indicate that LTA-mediated induction of p40 was susceptible to inhibition by a well-characterized LPS antagonist, RS-LPS.

Monoclonal antibody to CD14 inhibits LTA and LPS induction of p40. The similarity of kinetics for p40 induction by LTA and LPS and the similar sensitivities to RS-LPS inhibition suggested that LTA and LPS may use the same macrophage signaling pathway. In serum-containing in vitro systems, CD14 is the primary receptor through which LPS effects are mediated in macrophages (43). LPS interacts with soluble LPS-binding protein present in media and subsequently binds to CD14. MY4 is a monoclonal anti-human CD14 antibody used extensively to block LPS-mediated effects in macrophages (24). We used MY4 to examine LTA induction of p40 (Fig. 6). THP-1 cells were subjected to pretreatment for 1 h with MY4, followed by a 4-h stimulation with either LPS or LTA. MY4 blocked p40 induction by LPS in a dose-dependent manner (Fig. 6A). In contrast, 10 μg of monoclonal isotype control IgG2b antibody per ml had no effect on LPS- or LTA-induced p40 expression. Anti-CD14 similarly inhibited LTA induction of p40 (Fig. 6A). This inhibition was lost with antibody dilution. In addition, a nonblocking monoclonal antibody to CD14, LeuM3, and an antibody to a distinct cell surface marker of macrophages, anti-Mac-1 (CD11b), had no effect on the induction of IL-12 p40 by LTA (Fig. 6B). These data indicate that CD14 is the primary receptor through which LTA induces p40, suggesting that LTA may be a second, previously unrecognized ligand for CD14.

LTA induces Th1 phenotype development. The data presented above suggested that LTA is the primary component of gram-positive bacteria responsible for stimulating IL-12 production from macrophages. In addition, LTA and LPS induced similar patterns of cytokine expression. However, the physiologic relevance of IL-12 induction by LTA was not shown directly. We used an in vitro T-helper-cell development assay to examine the effects of LTA (16, 17). Purified lymph node T cells from DO11.10 T-cell-receptor-transgenic mice, specific for OVA, were activated in vitro with various concentrations of LTA or LPS, OVA, and irradiated spleen cells. After 1 week, T cells were examined for Th1 or Th2 development as described previously (17). Conditions known to induce Th1 development, such as the addition of heat-killed *L. monocytogenes* or LPS, resulted in high IFN-γ levels, while Th2-producing conditions resulted in high IL-4 production. Transgenic T cells stimulated with OVA under neutral conditions develop into a predominantly Th2 population with significant production of IL-4 and very low levels of IFN-γ. The addition of heat-killed *L. monocytogenes* led to the predicted Th1 skewing

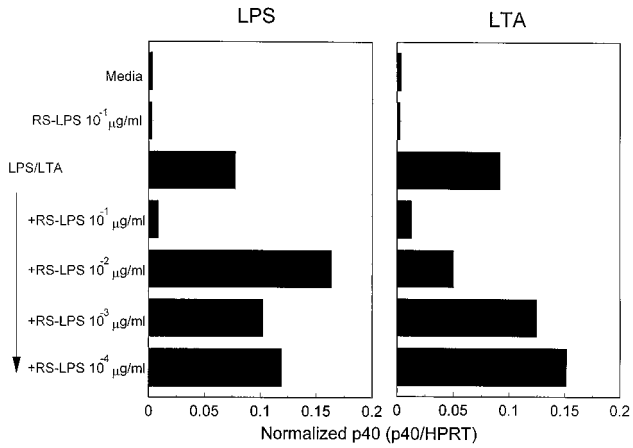

FIG. 5. RS-LPS inhibits p40 induction by both LPS and LTA. THP-1 cells were pretreated for 1 h at room temperature with or without RS-LPS at the indicated doses. Cells then were stimulated with LPS (0.01 μg/ml) or LTA (1.0 μg/ml) for 4 h prior to RT-PCR. Bars represent the averages of duplicates, which showed less than 25% variance (as in Fig. 2), and are expressed as a normalized ratio of p40 to HPRT signal. Results are representative of three separate experiments.

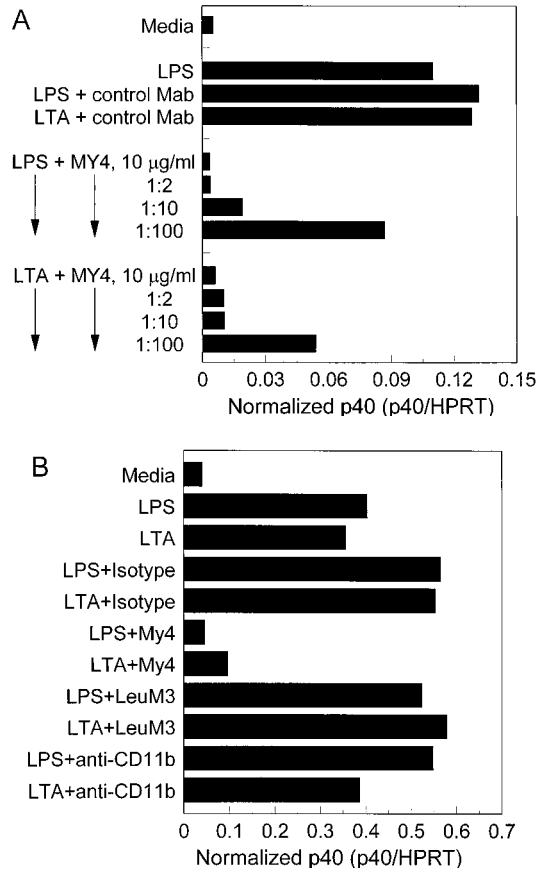

FIG. 6. Anti-CD14 blocks p40 induction by both LPS and LTA. THP-1 cells were pretreated for 1 h at room temperature with or without anti-CD14 (MY4) at the indicated doses. Purified monoclonal IgG2b antibody served as an isotype control (10 μg/ml). Cells were stimulated with either LPS (0.01 μg/ml) or LTA (1.0 μg/ml) for 4 h, and data are expressed as described in the legend to Fig. 4. Data are representative of three separate experiments. In panel B, LeuM3 (5 μg/ml) and anti-CD11b (5 μg/ml) were used as controls. Mab, monoclonal antibody.

of the population, with high levels of IFN-γ produced (Fig. 7). The addition of LPS also led to a dose-dependent shift in T-cell cytokine production toward a Th1 population (Fig. 7A). Similarly, the addition of LTA at various concentrations led to the production of high levels of IFN-γ and low levels of IL-4. The Th1-inducing effect of both LPS and LTA was due to the production of IL-12, since addition of anti-IL-12 monoclonal antibody specifically reversed the effect on Th1 development (Fig. 7B), as described previously (16, 17). In summary, these data indicate that LTA induces Th1 development in a manner dependent on IL-12 production.

DISCUSSION

The results of the present study suggest that the polyol phosphate class of compounds, including TA and LTA, is very likely an active component of gram-positive bacteria responsible for induction of IL-12. Activation of IL-12 was shared by LTAs of both the glycerol and ribitol backbone variety. Teichoicated cell wall and LTA were both active, suggesting that acylation was also not critical to activity. Very recent evidence has suggested that reverse-phase high-performance liquid chromatography fractionation of phenol-extract-prepared LTA, which most investigators have used in the past, results in the isolation of high-molecular-weight lipids distinct from LTA which stimulate the release of monocyte cytokines IL-1 and IL-6 (23, 34). These heterogeneous compounds do not contain protein and are distinct from peptidoglycan. Both these components and LTA bind to CD14; however, fractionated LTA has little to no stimulatory activity. These lipid components very likely may be responsible for our observations that LTA preparations stimulate IL-12 production in monocytes through CD14.

Our findings that the signaling of the LTA preparation was sensitive to a competitive inhibitor of LPS, RS-LPS, and that a monoclonal antibody to CD14 blocked stimulation are strong evidence that the induction by LTA of IL-12 can occur through CD14. In addition to LPS, lipoarabinomannan, the major LPS of mycobacteria, has been shown to induce IL-12 (42). Lipoarabinomannan also binds to and stimulates cytokine production through CD14 similarly to whole gram-positive bacteria (29). Apparently, the potency of both LTA and LAM with regards to cytokine production is less than that of gram-nega-

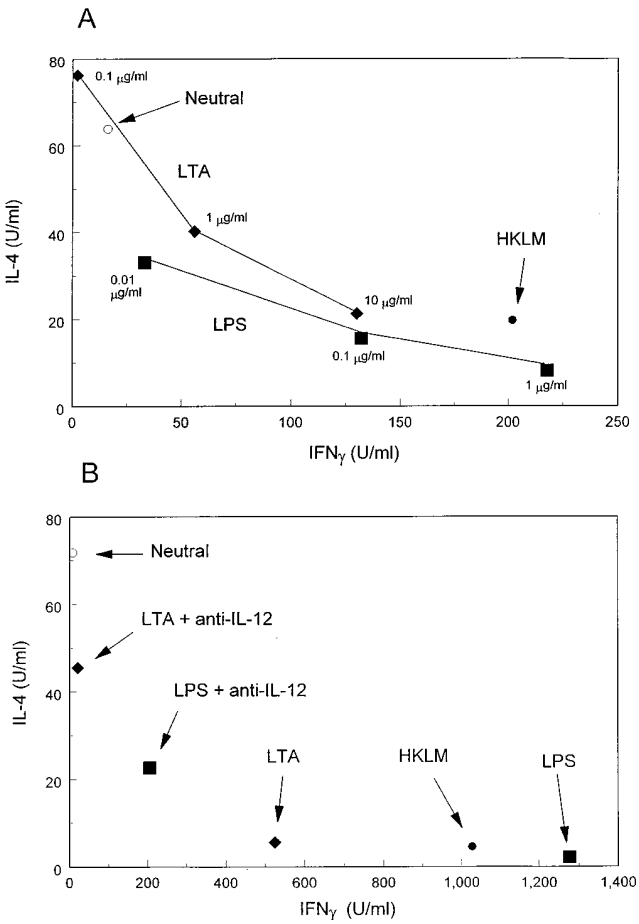

FIG. 7. LTA and LPS induce Th1 cells in a T-cell developmental assay. (A) Purified T cells from an α/β T-cell-receptor-transgenic BALB/c mouse were placed in culture with stimulatory antigen (OVA) and irradiated spleen cells to serve as antigen-presenting cells. LTA (at 0.1, 1.0, and 10 μg/ml), LPS (at 0.001, 0.01, and 0.1 μg/ml), and heat-killed *L. monocytogenes* (HKLM; at 10^6/ml) were added at the initiation of culture. After 1 week, the cells were washed and given fresh antigen-presenting cells and antigen with no other stimulus. Two days later, supernatants were harvested and IFN-γ and IL-4 were measured by ELISA. (B) T cells were placed in culture as described for panel A with or without LPS (0.1 μg/ml) or LTA (10 μg/ml). In other wells, anti-IL-12 (TOSH; 3.0 μg/ml) was added along with LPS or LTA. Results shown are representative of two separate experiments for each set.

tive bacterium-derived LPS. In addition, soluble peptidoglycan induces IL-1 and IL-6 in human monocytes in a CD14-dependent manner (39). Furthermore, polymers of uronic acid also induce monocyte cytokine production through CD14 (10). These results suggest that LTA may not be the only compound of gram-positive bacteria responsible for IL-12 induction. They also suggest that CD14 can bind several biologically active glycolipids from multiple organisms, constituting a family of CD14 ligands. LTA could also, perhaps, interact with other LPS receptors to induce IL-12. Scavenger receptors and platelet-activating factor receptor are reported to bind LTA and LPS (6, 9).

LTAs bear many similarities to LPS with regards to macrophage stimulation. The results of this study indicate that LTA is a relatively potent and rapid IL-12-inducing agent and that this cytokine is induced concomitantly with multiple other macrophage activation cytokines (TNF-α, IL-1β, IL-6, and IL-10). The kinetics of LTA induction of cytokines is similar to that of LPS, suggesting common pathways of stimulation.

LTA-induced Th1 development in a T-cell system is known to be dependent on antigen-presenting cell production of IL-12 (16, 17). These findings indicate that LTA, even at relatively low concentrations (1 to 10 μg/ml), induced physiologically relevant levels of IL-12 in a biologic assay system. Thus, the data suggest that the means by which gram-positive bacterial products stimulate IL-12 production in macrophages is through interaction with CD14. In addition, the apparent ligand for this stimulatory response is the predominant surface glycolipid of both gram-positive and -negative organisms, thus providing a rationale for many of the similarities in pathogenesis for these distinct organisms.

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