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Dendritic Cell Activation and Cytokine Production Induced by Group B *Neisseria meningitidis*: Interleukin-12 Production Depends on Lipopolysaccharide Expression in Intact Bacteria

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Interactions between dendritic cells (DCs) and microbial pathogens are fundamental to the generation of innate and adaptive immune responses. Upon stimulation with bacteria or bacterial components such as lipopolysaccharide (LPS), immature DCs undergo a maturation process that involves expression of costimulatory molecules, HLA molecules, and cytokines and chemokines, thus providing critical signals for lymphocyte development and differentiation. In this study, we investigated the response of in vitro-generated human DCs to a serogroup B strain of *Neisseria meningitidis* compared to an isogenic mutant *lpxA* strain totally deficient in LPS and purified LPS from the same strain. We show that the parent strain, *lpxA* mutant, and meningococcal LPS all induce DC maturation as measured by increased surface expression of costimulatory molecules and HLA class I and II molecules. Both the parent and *lpxA* strains induced production of tumor necrosis factor alpha (TNF-α), interleukin-1α (IL-1α), and IL-6 in DCs, although the parent was the more potent stimulus. In contrast, high-level IL-12 production was only seen with the parent strain. Compared to intact bacteria, purified LPS was a very poor inducer of IL-1α, IL-6, and TNF-α production and induced no detectable IL-12. Addition of exogenous LPS to the *lpxA* strain only partially restored cytokine production and did not restore IL-12 production. These data show that non-LPS components of *N. meningitidis* induce DC maturation, but that LPS in the context of the intact bacterium is required for high-level cytokine production, especially that of IL-12. These findings may be useful in assessing components of *N. meningitidis* as potential vaccine candidates.

Dendritic cells (DCs) are highly specialized antigen-presenting cells that form a gateway between the innate and adaptive immune system. Exposure of DCs to invading pathogens triggers a series of activation events involving antigen uptake and processing as well as migration to specialized lymphoid tissue for antigen presentation to T cells (3). In addition, activated DCs generate signals that alert the immune system to potentially dangerous foreign material and modulate subsequent lymphocyte activation and differentiation (14, 27). Some of these signals are mediated by direct contact through the costimulatory molecules CD40, CD80 (B7.1), and CD86 (B7.2), which are increased upon DC maturation, and others are mediated by cytokines and chemokines (28, 30, 31)

Cytokines generated by DCs are critically important for subsequent T-cell differentiation. For example, interleukin-12 (IL-12) produced by DCs is pivotal for the development of Th1 responses (8, 17, 38). This in turn can be modulated by ligation of CD40 (8) and production of gamma interferon (IFN-γ) by activated T lymphocytes, which is most likely to occur after DCs have migrated to T-cell areas of lymphoid tissues and thus have encountered the signals necessary for activation and maturation. In addition, exposure to stimuli such as tumor necrosis factor alpha (TNF-α) and IL-1 or other inflammatory mediators at sites of local inflammation can influence the capacity of DCs to mature, migrate to T-cell areas in lymphoid tissue, and present antigen (21). Thus, the nature of both stimuli from invading pathogens and the local microenvironmental milieu is important for DC behavior and the subsequent immune response (13). Whole bacteria, protozoa, and microbial products such as lipopolysaccharide (LPS) can induce DC maturation in vitro and in vivo, resulting in increased expression of costimulatory molecules and production of proinflammatory cytokines that influence the subsequent immune response (21, 27, 29, 43). The gram-negative bacterium *Neisseria meningitidis* is an important cause of mortality and morbidity worldwide (11). Effective subunit vaccines using capsular polysaccharide have been developed against *N. meningitidis* serogroups A and C (10), but a safe and effective vaccine has not yet been developed against serogroup B. In this study, we investigated DC responses to a clinical isolate of *N. meningitidis* B and the isogenic *lpxA* mutant, which is totally deficient in LPS (35). We show that both purified LPS and the *lpxA* strain can activate DCs, but neither was able to induce production of IL-12. Only

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intact parent bacteria induced IL-12 production, showing that LPS in the context of the intact bacterium is required for this response.

MATERIALS AND METHODS

Bacteria and LPS. The serogroup B *N. meningitidis* strain H44/76, isolated from a case of fatal septicemia (2), and a viable LPS-deficient isogenic mutant constructed by insertional inactivation of the *lpxA* gene with a kanamycin resistance cassette (35) were used in this study. The enzyme *lpxA* is required for the first committed step in lipid A biosynthesis. The absence of endotoxin activity in the mutant was established by *Limulus* amoebocyte lysate assay, by whole-cell enzyme-linked immunosorbent assay (ELISA) with LPS-specific monoclonal antibody, and by gas chromatography-mass spectrometry analysis (35). The purity of the *lpxA* mutant was maintained by culturing on agar plates containing kanamycin (100 μg/ml; Sigma, Poole, United Kingdom). Both strains were grown on gonococcal agar (Difco, Basingstoke, United Kingdom), supplemented with Vitox (Oxoid, Basingstoke, United Kingdom) in 6% CO_2 in air at 36°C. The bacteria were used in the stationary phase after culture for 18 h. Suspensions of bacteria were prepared in RPMI 1640 medium with no phenol red (Gibco, Paisley, United Kingdom), and their optical density at 540 nm (OD_{540}) was measured. Viability counts demonstrated that an OD of 1.0 was equivalent to 10^9CFU/ml. Bacteria were fixed in 0.5% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and washed thoroughly in RPMI medium. This treatment rendered the bacteria nonviable, as judged by viability counts and propidium iodide staining. Meningococcal LPS from strain H44/76 was prepared by hot aqueous phenol extraction, ultracentrifugation, gel filtration, and cold ethanol-NaCl precipitation, as described previously (1, 39).

Antibodies. The following monoclonal antibodies were used: CD25 (MO731) (Dako, Ely, United Kingdom); CD40 (LOB7/6), CD83 (HB15A), mouse anti-rat immunoglobulin G2c (IgG2c) (MARG 2c-3), and HLA-ABC (W6/32) (all from Serotec, Oxford, United Kingdom); CD14 (HB246) and CD3 (UCH-T1) (kind gift from P. C. L. Beverley, Jenner Institute, Compton, United Kingdom); CD1a (NA1/34) (kind gift from A. McMicheal, Oxford University, Oxford, United Kingdom); CD19 (BU12) and CD86 (BU63) (kind gifts from D. Hardie, Birmingham, United Kingdom); phycoerythrin (PE)-conjugated antihuman IL-1α (364-3B3-14) and IL-12 (p70/p40)-specific (C11.5) (both from Pharmingen/BD, Oxford, United Kingdom); PE-conjugated anti-human IL-6 (AS12) (Becton Dickinson, Oxford, United Kingdom); and TNF-α (6402.31) and IgG1 (11711.11) control (both R and D Systems, Abingdon, United Kingdom). Fluorescein isothiocyanate (FITC) conjugated anti-mouse rabbit polyclonal antibody was purchased from Dako.

DC culture and activation. DCs were generated from human peripheral blood mononuclear cells (PBMCs) as described previously (45). In brief, PBMCs were prepared from venous blood anticoagulated with 4.2 mM EDTA by centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway) at 400 × *g* for 30 min at room temperature. Mononuclear cells recovered from the interface were washed and resuspended to a concentration of 3×10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum (PAA Laboratories, Kingston-upon-Thames, United Kingdom), 0.05 M 2-mercaptoethanol, 100 U of penicillin-streptomycin per ml, and 2.4 mM L-glutamine (all GIBCO). The cells were then cultured in six-well tissue culture plates at 10^7 cells per well for 3 h at 37°C in an atmosphere of 5% CO_2 in air. Nonadherent cells were removed by gentle aspiration, and the adherent cells were incubated for 7 days in fresh culture medium supplemented with 100 ng of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) per ml (Schering-Plough, Welwyn Garden City, Hertsfordshire, United Kingdom) and 50 ng of recombinant human IL-4 per ml (Schering-Plough). The cells were then washed gently and cultured for 24 h with fixed parent or *lpxA* bacteria or with purified meningococcal LPS. Brefeldin A (10 μg/ml) (Sigma) was added to the cultures when intracellular cytokines were to be measured. In our hands, inclusion of brefeldin A for 18 to 24 h gave the best results, particularly for IL-12, and was not toxic to the DCs. At the end of the culture period, the cells were examined by light microscopy for characteristic features of dendritic cells. They were then collected and centrifuged over Lymphoprep at 400 × *g* for 30 min to remove dead cells and cell debris. All reagents used for the preparation and culture of DCs were shown to be endotoxin free. For surface staining, the cells were incubated with unconjugated monoclonal antibody followed by FITC-conjugated rabbit anti-mouse antibody (IgG1). Unstimulated DCs at day 8 were $CD14^{low}$ $CD83^-$ $CD86^{low}$ $CD25^-$ expressed HLA-DR, HLA-DQ, HLA class I, and CD40 and CD1a; and were negative for both CD19 and CD3.

To measure intracellular cytokine production, cultured DCs were fixed with 4% paraformaldehyde in PBS at 4°C for 15 min and then washed in PBS containing 0.1% sodium azide (Sigma) and again in Hanks buffered saline solution (with calcium and magnesium) (GIBCO) containing 0.1% saponin (Sigma), 2 mM HEPES (GIBCO), and 0.05% sodium azide. The cells were then resuspended in 200 μl of the saponin buffer and incubated with cytokine-specific monoclonal antibody or the appropriate isotype-matched controls as indicated, for 45 min at room temperature in the dark. The cells were then washed twice in saponin buffer and resuspended in PBS containing 0.1% azide for analysis by flow cytometry on a FACScalibur with Cell Quest software (Becton Dickinson). DCs were contained in a distinct population of cells identified by forward and right angle scattering. At least 95% of cells in this population were DCs defined by expression of major histocompatibility complex class II (MHC II), CD1a, CD25, CD80, CD83, and CD86. At least 5,000 events within the gates corresponding to dendritic cells were collected for analysis.

RESULTS

DC maturation induced by *N. meningitidis* H44/76 parent and *lpxA* strains compared with LPS. Expression of surface activation markers on DCs was determined after 24 h of culture with the *N. meningitidis* H44/76 parent strain, the *lpxA* strain, purified LPS, or a combination of the *lpxA* strain and LPS (Fig. 1). Similar results were obtained from five independent experiments. Maturation of the DCs, as indicated by loss of CD14 (data not shown), and increases in expression of CD25, CD40, CD83, and CD86 were observed with each of the stimuli used, although higher levels of CD40 were obtained with LPS than with either the parent or *lpxA* strain (Fig. 1). LPS also stimulated higher expression of HLA-DQ (Fig. 1). Interestingly, the reverse was seen with CD83 and CD25, which were higher on activation with the parent bacteria than either LPS or the *lpxA* strain (Fig. 1). Moreover, addition of the *lpxA* bacteria and LPS did not restore the response to the level obtained with the parent bacteria. Further variation was seen with MHC class I expression, which was consistently higher in response to the parent and *lpxA* bacteria than to LPS. Two important conclusions can be drawn from these results. First, DC activation and maturation induced by *N. meningitidis* can occur in the absence of LPS. Second, the surface markers measured are not all expressed concordantly on DC activation. Rather the level of expression of each depends on the particular stimulus used.

Cytokine production by DCs activated with *N. meningitidis* H44/76 parent and *lpxA* strains compared with LPS. DCs were cultured for 24 h in the presence of brefeldin A to block cytokine secretion with a range of concentrations of the parent H44/76 bacteria, the *lpxA* bacteria, or purified LPS. Intracellular cytokine levels in the gated DC population were then measured by flow cytometry. The results from a typical experiment are shown in Fig. 2. High levels of TNF-α and IL-1α were detected in DCs stimulated with parent H44/76 bacteria at concentrations ranging from 10^5 to 10^7 bacteria per ml. Equivalent levels were obtained with the higher concentration (10^7/ml) of *lpxA* bacteria, but at 10^6 bacteria per ml, levels of TNF-α and IL-1α were lower than those obtained with the parent H44/76 strain, and at 10^5 bacteria per ml, there was little or no cytokine production. Notably, purified LPS induced very low levels of TNF-α and IL-1α. A different pattern was observed with IL-6. In this case, similar levels were obtained on DC activation with both parent H44/76 and the *lpxA* strains at 10^7 and 10^6 bacteria per ml. In addition, higher levels of IL-6 were obtained in response to LPS compared to other cytokines measured (Fig. 2 and Table 1). The most dramatic difference

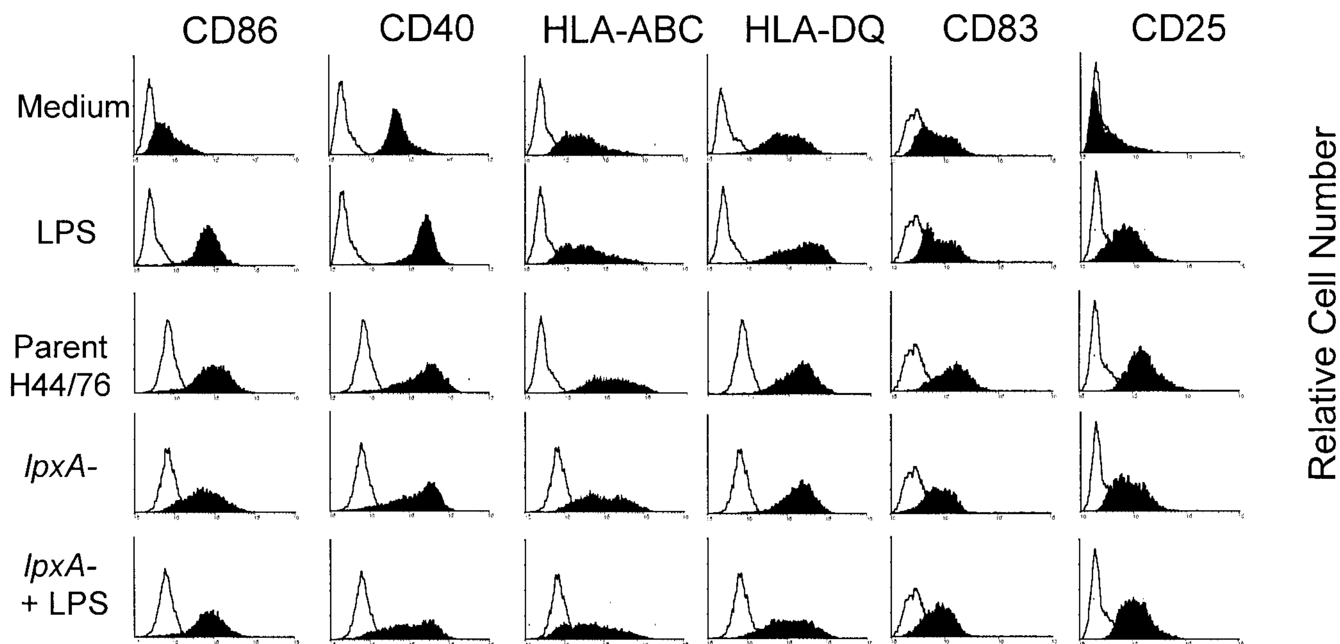

FIG. 1. Representative flow cytometric profiles of surface phenotypic markers on day 8 DCs stimulated with medium, LPS, or the *N. meningitidis* H44/76 parent or *lpxA* strain. Day 7 DC cultures were incubated for 24 h with medium, 100 ng of meningococcal LPS, or 10^7CFU of parent or *lpxA* organisms per ml. Open histograms show staining of appropriate isotype-matched controls. Solid histograms show staining of the antibody raised against the indicated surface marker. The data are representative of five separate experiments.

between the different stimuli was observed with IL-12. Although high levels were detected in DCs stimulated with the parent H44/76 bacteria at concentrations ranging from 10^5 to 10^7 per ml, little or no IL-12 was detected in response to the *lpxA* mutant at any concentration used. Furthermore, no IL-12 was detected in response to LPS. These results were repeated over many experiments and reveal major differences in DC responses to *N. meningitidis*, depending on the presence of LPS. To examine this further, cytokine production by DCs was compared after culture with parent H44/76 bacteria, the *lpxA* strain, LPS, and a combination of *lpxA* bacteria and LPS. As shown, the addition of purified LPS together with the *lpxA* strain did not reconstitute IL-1α, IL-6, or TNF-α production to the levels obtained with the parent H44/76 bacteria (Fig. 3). Essentially the same result was obtained with IL-12 production (Fig. 4). These results show that optimal cytokine production, particularly of IL-12, depends on the presence of LPS and other bacterial components in the context of the intact bacteria.

DISCUSSION

Our results show that serogroup B *N. meningitidis* is an extremely potent activator of human DCs in vitro. Culture of DCs with 10^5 to 10^7 bacteria/ml resulted in a marked increase in expression of costimulatory molecules CD40 and CD86, markers of maturation CD83 and CD25, and HLA class I and II molecules. In addition, high-level production of TNF-α, IL-1α, IL-6, and IL-12 in response to the parent strain was observed. All of our experiments were carried out with fixed bacteria, because live bacteria killed the DCs within the time required for DC activation. In previous work, however, we have shown that live and fixed bacteria elicit essentially the same response by endothelial cells, which require much shorter incubation times (9). Our initial experiments suggested that meningococcal LPS was a key bacterial component responsible for the DC response, as shown by its ability to induce expression of surface markers. LPS is an extremely potent activator of host inflammatory responses (40) and was considered to be the primary stimulus for the proinflammatory cytokine production, disseminated intravascular coagulation, and endothelial damage characteristically seen in gram-negative sepsis, including meningococcal disease (5, 22). It was therefore interesting that purified LPS induced only low levels of TNF-α and IL-1α compared to the parent bacteria and invariably failed to induce measurable levels of IL-12.

There are a number of potential explanations for the observed differences in DC activation by intact bacteria and LPS. First, the effective dose of LPS provided by the parent bacteria may have been greater than that of purified LPS. Quantitation of the LPS content of *N. meningitidis* based on spectrophotometric analysis of the LPS-specific sugar 2-keto-3-deoxyoctonic acid has demonstrated that there are approximately 1.5×10^5 molecules of LPS per bacterium. This makes 100 ng of purified LPS per ml, equivalent to about 10^8 bacteria/ml (P. van der Ley, personal communication), and yet good DC responses were obtained with as few as 10^5 organisms of the parent strain per ml. Low-level expression of CD14 on DCs cannot explain

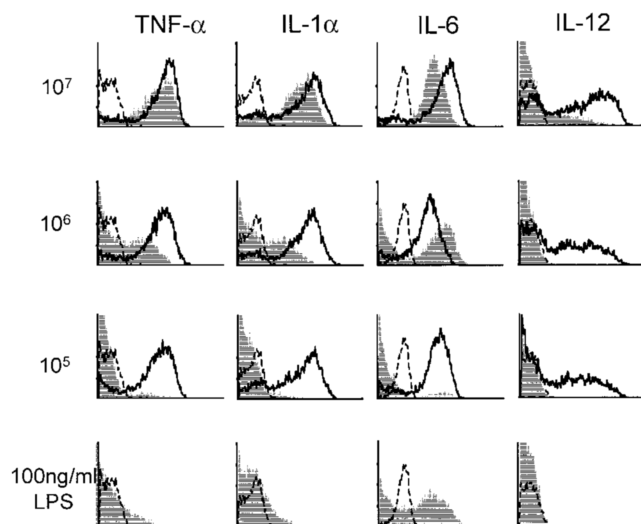

FIG. 2. Dose-dependent cytokine production in DCs in response to the parent H44/76 strain, the *lpxA* strain, and meningococcal LPS. DCs were stimulated with either medium, 100 ng of LPS, or 10^5 to 10^7 CFU of H44/76 parent or *lpxA* strain per ml in the presence of 10 μg of brefeldin A per ml. Intracellular cytokine production was assessed after 24 h. Open histograms represent staining in response to the parent strain. Solid histograms show responses to the *lpxA* mutant or LPS as indicated. Dashed lines show staining in unstimulated DCs. The data are representative of six experiments yielding comparable results.

the difference in response either for three reasons. First, although LPS alone did not induce IL-12 production, it did increase expression of surface activation markers (Fig. 1) and IL-6 (Fig. 2). Second, the LPS antagonist rBPI (bactericidal permeability increasing factor) completely inhibited the response to LPS; Third, the serum concentrations used were able to provide sufficient soluble CD14 and LPS-binding protein (LBP) for LPS activation of endothelial cells, which do not express CD14 (9).

The most likely explanation for our results is that bacterial components other than LPS are playing an important role in DC responses to meningococci. This was investigated by using the LPS-deficient *lpxA* mutant of *N. meningitidis*, which was found to induce similar changes in surface markers to those of the parent, indicative of activation and maturation of DCs. These results show that both LPS and non-LPS components of meningococci activate DCs. The response to the *lpxA* strain and its parent did differ, however, in two significant ways. With the exception of IL-6, the *lpxA* strain was a less potent inducer of cytokines than the parent, which was particularly marked at lower bacterial concentrations (Fig. 2). Most importantly, the *lpxA* mutant induced little or no IL-12, even at the highest concentration used.

A number of meningococcal components other than LPS have been shown to activate various cells of the immune system. Porins from *N. meningitidis* are activators of B cells and antigen-presenting cells, and porin-specific T-cell responses have been described (18, 26, 44). T-cell responses to porins from the related organism *Neisseria gonorrhoea* have also been described (32). *N. gonorrhoea* components have been shown to activate transcription factors Nf-κB and AP-1/c-jun in epithelial cells (24, 25). We have also shown that the *lpxA* mutant can induce activation of Nf-κB and ATF2 and AP-1/c-jun in endothelial cells (G. Dixon, unpublished data). The outer membrane components of H44/76 are well characterized, and the *lpxA* strain has an outer membrane protein composition similar to that of the parent organism (35). In addition, both the parent and *lpxA* strains contain lipoproteins and peptidoglycans in the cell wall as well as bacterial DNA, all of which are known inflammatory mediators in human cells (6, 34, 46). Nevertheless, we cannot exclude altogether the possibility that some of these components are expressed differently in the parent and *lpxA* strains. Recent evidence suggests that human Toll-like receptors (TLRs) play a fundamental role in innate immune recognition of and signaling induced by these microbial products, including LPS (4, 6, 46). DCs express TLRs (23), but it remains to be determined which combination of these components and which TLRs and/or other receptors, such as the mannose receptor, are responsible for LPS-independent activation of DCs by meningococci.

Our finding that the parent *N. meningitidis* H44/76 strain is a potent inducer of IL-12 production in DCs whereas meningococcal LPS is not deserves further comment. A number of studies have described IL-12 production by DCs stimulated with LPS (7, 12, 43). In each of these studies, ELISA of culture supernatants was used to assay IL-12 production. In contrast, others have found that LPS does not induce significant IL-12 production by DCs (8, 15). It has also been reported that high IL-12 production by human DCs requires two signals, such as CD40L and IFN-γ, and that LPS can replace either one of these signals, but not both (33). Our results described here were unequivocal. In at least 20 experiments, purified LPS from either *N. meningitidis* or *Escherichia coli* (data not shown) was unable to induce significant TNF-α or IL-1α production by

TABLE 1. Differences in cytokine production by DCs in response to the *N. meningitidis* H44/76 parent and LPS-deficient *lpxA* strains and purified meningococcal LPS[a]

Stimulus	MFI (% positive)		*P*
	Median	Interquartile range	
IL-1α			0.0067
Medium	3.7 (3)	3.5–4.2 (2–3)	
LPS	3.5 (6)	3.2–3.5 (5–16)	
H44/76 parent	15.1 (59)	12.9–33.1 (56–64)	
lpxA	6.4 (27)	4.9–8.5 (23–33)	
TNF-α			0.015
Medium	2.1 (1)	2.0–2.6 (1–2)	
LPS	3.2 (18)	2.9–9.7 (15–32)	
H44/76 parent	62.1 (75)	26.7–66.7 (62–86)	
lpxA	7.9 (48)	2.8–21.8 (16–51)	
IL-6			0.015
Medium	3.7 (3)	3.5–5.2 (2–4)	
LPS	5 (36)	3.2–14.2 (32–62)	
H44/76 parent	33.1 (66)	15.1–66.1 (62–85)	
lpxA	6.4 (66)	5.0–8.5 (47–77)	

[a] DCs were stimulated with 10^7organisms/ml, 100 ng of purified meningococcal LPS per ml, or medium in the presence of brefeldin A for 24 h Cytokine production was assessed by the intracellular fluorescence flow cytometry protocol described in Materials and Methods. Results are expressed as both MFI and percentage of positive events. The ranking order of cytokine production as measured by MFI was compared by using a Friedman two-way analysis of variance (H44/76 parent > H44/76 *lpxA* > purified LPS).

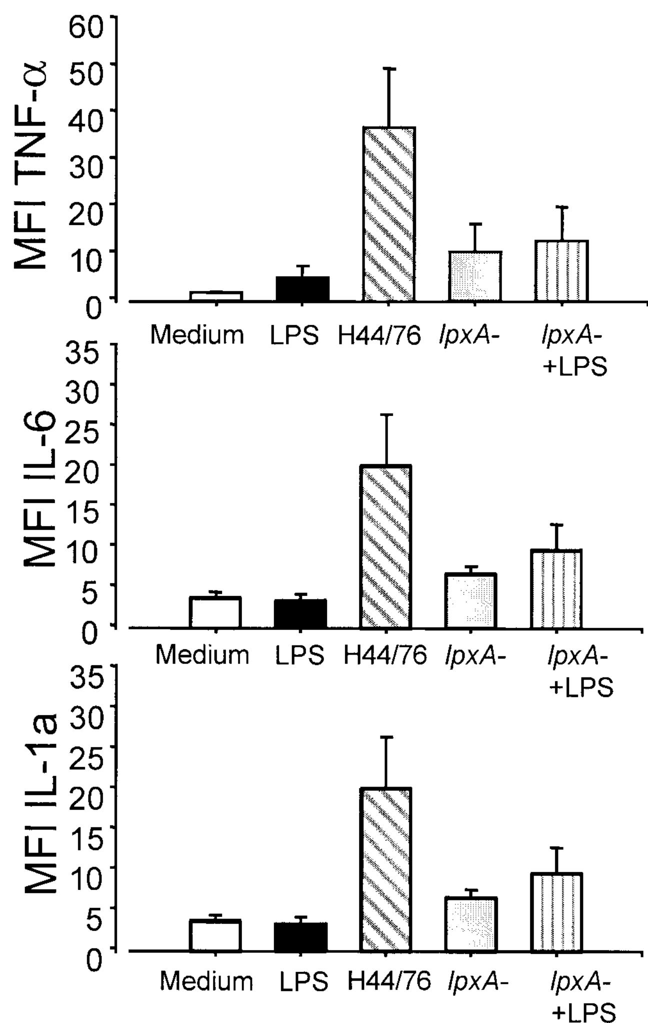

FIG. 3. Addition of exogenous LPS only partially restores IL-1α, TNF-α, and IL-6 induction in DCs in response to the *lpxA* mutant. DCs were stimulated with 100 ng of LPS per ml, 10^7CFU of parent or *lpxA* bacteria per ml, or 10^7 CFU of *lpxA* strain plus 100 per ml ng of LPS per ml in the presence of 10 μg of brefeldin A per ml. Intracellular IL-6, TNF-α, and IL-1α production was assessed after 24 h. Results are presented as mean MFI ± standard error from three separate experiments.

DCs and invariably failed to induce detectable IL-12. The same LPS preparations, however, did induce cytokine production by monocytes (42) and did activate DCs, as shown by the increased expression of surface activation antigens (Fig. 1) and production of IL-6 (Fig. 2). The reason for these apparently conflicting findings is not known, but the different methods used to measure cytokine production may be important. In our experiments, intracellular cytokines were measured in gated cells with the phenotypic characteristics of DCs. DCs identified by immunohistochemical staining have also been found not to make IL-12 in response to LPS (15). On the other hand, ELISAs of culture supernatants will detect IL-12 made by other cells, including monocytes and macrophages that exist in the cultures after 7 days of incubation with GM-CSF and IL-4. These would not be included in the gated population of DCs identified by flow cytometry used in our study or by methods using immunohistochemical stains.

Even more striking was the inability of the *lpxA* strain to induce significant levels of IL-12. Surprisingly, addition of exogenous LPS to the *lpxA* strain did not reconstitute IL-12 production or, for that matter, IL-1α, IL-6, and TNF-α production. This suggests that high-level IL-12 production by DCs in response to *N. meningitidis* requires LPS expressed in the membrane of the intact bacteria. The integrity of pathogen molecular motifs like LPS within bacteria may therefore determine the biological response by human DCs. A recent study showed that recognition of microbial products by TLRs might occur within phagosomes, perhaps in concert with receptors involved in particulate uptake of microorganisms (41). This would be a reasonable explanation for why the molecular orientation and context of pathogen-associated motifs may be such a critical determinant of DC response to microorganisms. It is interesting that outer membrane complexes from the *lpxA* strain or heat-inactivated bacteria elicit poor immune responses in mice (36). Protective responses and bactericidal antibody production, including class switching to IgG2a and IgG2b, could be obtained by addition of LPS to outer membrane complexes, but LPS did not restore the antibody response to intact *lpxA* bacteria. IL-12 production was not mea-

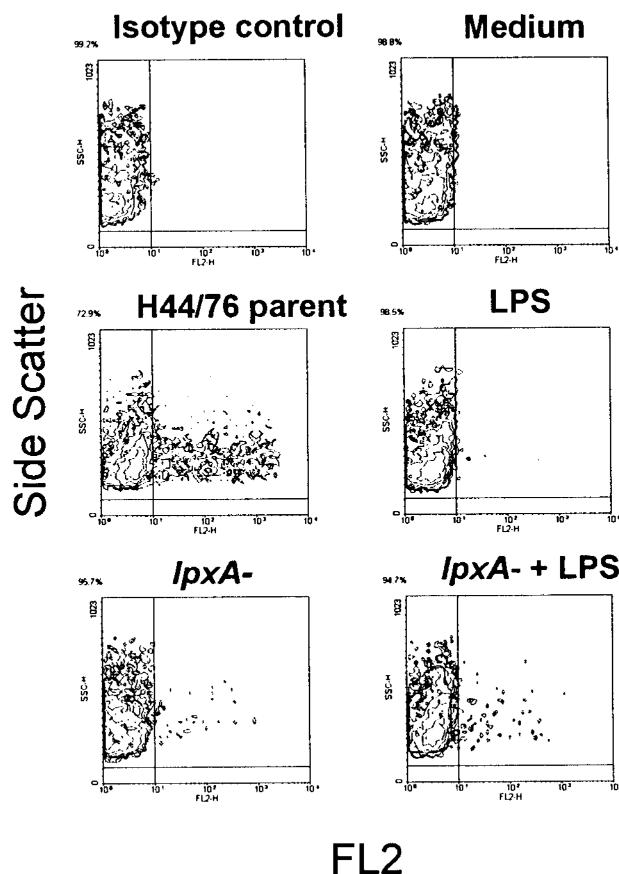

FIG. 4. Addition of exogenous LPS does not reconstitute the ability of the *lpxA* strain to induce IL-12 production. DCs were stimulated with 100 ng of LPS per ml, 10^7 CFU of parent or *lpxA* strain per ml, or 10^7 CFU of the *lpxA* strain plus 100 ng of LPS per ml in the presence of 10 μg of brefeldin A per ml. Intracellular IL-12 production was assessed after culture for 24 h. The data are representative of three independent experiments.

sured, however, and it would be of interest to know whether production of IL-12 by antigen-presenting cells, especially DCs, in response to these outer membrane components plays a role in this process. This may be important in view of the effects IL-12 has on T helper cell-dependent immune response and antibody class switching in vivo (20).

Because of their capacity to process and present antigen efficiently and provide necessary costimulatory signals to both naive and activated lymphocytes, DCs are obvious targets for evaluating responses to candidate vaccines. Strategies that have used transfer of DCs pulsed with various pathogens to nonimmune animals have successfully induced protective immune responses (16, 19, 37). It is clear that the context in which adjuvant-like molecules such as LPS are presented to the immune system is also important and should be taken into account in future vaccine design.

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