INFECTION AND IMMUNITY, May 2005, p. 2728–2735 Vol. 73, No. 5
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.5.2728–2735.2005

Outer Membrane Protein P6 of Nontypeable *Haemophilus influenzae* Is a Potent and Selective Inducer of Human Macrophage Proinflammatory Cytokines

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Received 16 September 2004/Returned for modification 4 November 2004/Accepted 22 January 2005

Interactions of nontypeable *Haemophilus influenzae* (NTHI) with human macrophages contribute to the pathogenesis of NTHI-induced infection in humans. However, the immunologic mechanisms that initiate and perpetuate NTHI-mediated macrophage responses have not been well explored. Outer membrane protein (OMP) P6 is a conserved lipoprotein expressed by NTHI in vivo that possesses a Pam_3Cys terminal motif, characteristic of immunoactive bacterial lipoproteins associated with Toll-like receptor signaling. We theorized that OMP P6 is a potent immunomodulator of human macrophages. To test this hypothesis, we purified OMP P6 as well as OMP P2, the predominant NTHI outer membrane protein, and lipooligosaccharide (LOS), the specific endotoxin of NTHI, from NTHI strain 1479. Human blood monocyte-derived macrophages, purified from healthy donors, were incubated with each outer membrane constituent, and cytokine production of macrophage supernatants interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), IL-10, IL-12, and IL-8 was measured. OMP P6 selectively upregulated IL-10, TNF-α, and IL-8. While OMP P6 (0.1 μg/ml for 8 h) elicited slightly greater concentrations of IL-10, it resulted in over ninefold greater concentrations of TNF-α and over fourfold greater concentrations of IL-8 than did OMP P2. OMP P6 at doses as low as 10 pg/ml was still effective at induction of macrophage IL-8, while OMP P2 and LOS were not. OMP P6 of NTHI is a specific trigger of bacteria-induced human macrophage inflammatory events, with IL-8 and TNF-α as key effectors of P6-induced macrophage responses.

Nontypeable *Haemophilus influenzae* (NTHI) is a major cause of sinopulmonary infections, with a particular propensity for people with chronic obstructive pulmonary disease (COPD) (26, 31). NTHI strains are the most common pathogenic bacteria isolated from the airways of patients with COPD, in both stable and exacerbated states (32). Outer membrane protein (OMP) P6 is a highly conserved 16-kDa lipoprotein of NTHI, expressed in vivo. Of 115 strains of NTHI tested by immunodot or Western blot, all expressed OMP P6 (21). P6 sequences are conserved among strains, making P6 a strong potential vaccine candidate. OMP P6 elicits bactericidal antibody responses and evokes proliferative lymphocyte responses in vitro that correlate with relative protection from COPD exacerbations in humans (1). OMP P6 possesses a tripalmitoyl (Cys-Pam_3) terminus, common to immunoregulatory bacterial lipoproteins (3, 16). As is characteristic of immunoactive bacterial lipoproteins, OMP P6 induces intracellular signaling by activation of NF-κB (34).

Increasing evidence supports a key role for macrophages in the pathogenesis of NTHI infections. Macrophages interact with NTHI to promote binding and phagocytosis (28, 42). In fact, NTHI are capable of surviving macrophage phagocytosis in vivo and have been recovered, fully viable, from macrophages of hypertrophied adenoids and of explanted lungs of patients with COPD (11, 24). The importance of macrophage function to NTHI infection is further illustrated by the increased susceptibility to severe, invasive NTHI infections of adults with AIDS (8).

Chronic inflammatory stimuli of human macrophages are theorized to contribute to the progression of human diseases, including sinopulmonary infections. The key similarities of OMP P6 to immunoactive bacterial lipoproteins suggest that OMP P6 belongs to a family of bacterial lipoproteins associated with Toll-like receptor (TLR) activation and supports experiments directed at investigating OMP P6-macrophage interactions (2, 6). The role of macrophages in NTHI infections and the immunoregulatory phenomena of bacterial lipoproteins support a paradigm of OMP P6 as a key immune regulator of human macrophage responses, contributing to inflammatory states in humans. To test this hypothesis, we selected a panel of cytokines for studies to determine the spectrum of responses of human macrophages in response to OMP P6 in comparison to related outer membrane constituents of NTHI. Interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) are indicative of macrophage activation and modulate T-cell responses. IL-12 is a proinflammatory cytokine of macrophages that can increase proliferation of recruited T-cell subsets and modulate production of gamma interferon (IFN-γ) (38). IL-10 can downregulate T-cell responses, including antigen presentation to Th1 cells (35). IL-8 is a principal chemoattractant for neutrophils in the lung (19). Defining the cytokine milieu stemming from interactions between human macrophages and NTHI provides a critical first step toward determining the regulatory signals by which NTHI induction of

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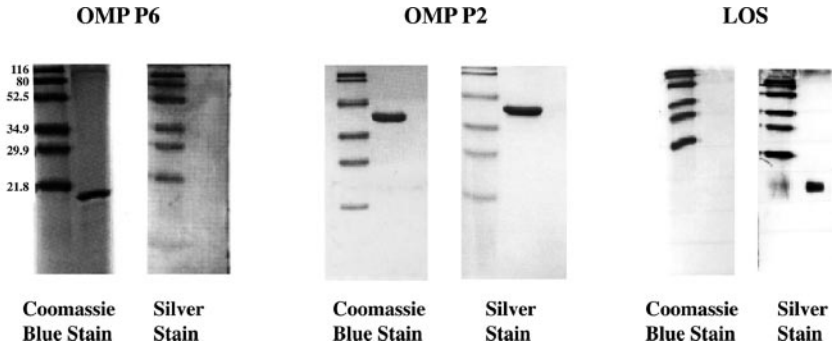

FIG. 1. Purified preparations of NTHI outer membrane constituents. Purified preparations were made of OMP P6, OMP P2, and LOS of NTHI 1479. Purity of each a preparation (5 to 10 μg) was confirmed with Coomassie blue stain and by absence of other bands with silver stain in addition to Limulus assay for endotoxin. Molecular weight standards are shown to the left of each lane and are labeled in the first lane.

macrophages influences the behavior of T cells and neutrophils.

MATERIALS AND METHODS

Reagents. RPMI and fetal bovine serum (FBS) were purchased from BioWhittaker, Walkersville, Md. Endotoxin-free human AB-positive serum was purchased from Nabi, Miami, FL.

Purification of outer membrane components. All outer membrane components were purified from NTHI strain 1479, a clinical respiratory isolate from an adult with COPD. A chocolate agar plate was inoculated with NTHI 1479 from a frozen stock and incubated overnight at 35°C. Brain heart infusion broth supplemented with 10 μg/ml hemin and 10 μg/ml NAD was inoculated with bacteria and incubated for 24 h at 35°C. Outer membrane constituents were purified as follows.

The total OMP preparation of NTHI1479 was purified by suspending bacteria in 10 mM HEPES buffer (pH 7.4) and repeatedly sonicating before centrifuging (1,700 × *g* for 30 min) to remove intact cells. Cell envelopes were recovered by centrifugation (100,000 × *g*, 4°C for 60 min) and were incubated in 10 mM HEPES and 2% sarcosyl in 10 mM HEPES buffer (1:1) at room temperature for 30 min. The detergent-insoluble fraction was obtained by centrifugation (100,000 × *g*, 4°C, 60 min) and suspended in pyrogen-free water (29).

OMP P6 was purified from NTHI 1479 as described previously (21). In brief, bacteria were pelleted by centrifugation (9,000 × *g*, 30 min at 4°C). The bacterial pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged again before resuspending in buffer B (1% sodium dodecyl sulfate [SDS], 0.1 M Tris, 0.5 M NaCl, 0.1% β-mercaptoethanol, pH 8) with repeated sonication. The suspension was incubated in a 37°C shaking water bath for 30 min before centrifugation (13,000 × *g*, 30 min) at room temperature. The supernatant was discarded, and three additional cycles of incubation followed by centrifugation were performed with buffer B with RNase A (10 μg/ml) and twice with buffer B alone. The pellet was resuspended in buffer A (0.01 M Tris, 0.15 M NaCl, pH 7.4) and incubated at 65°C for 30 min to dissociate P6 from peptidoglycan. After centrifugation (100,000 × *g*), P6-containing supernatant was collected and concentrated to a final volume of 10 to 15 ml.

OMP P2 was purified from NTHI 1479 by a modification of previously described methods (44). In brief, bacteria were suspended in 1 M sodium acetate-1 mM β-mercaptoethanol (pH 4.0), to which nine volumes of 5% Zwittergent 3-14 in 0.5 M $CaCl_2$ was added. After stirring, ethanol was added to 20% and the suspension was centrifuged (17000 × *g* for 10 min) to remove nucleic acids. After discarding the pellet, ethanol was added to the supernatant to 80% to precipitate proteins, and the suspension was centrifuged (17,000 × *g*). The pellet was resuspended in buffer Z (0.05% Zwittergent, 0.05 M Tris, 0.01 M EDTA, pH 8.0), stirred at room temperature, and centrifuged (12,000 × *g*). P2 was further purified from supernatant by applying the Zwittergent extract to a DEAE-Sephacel column from which P2 was eluted. Fractions containing P2 and LOS were precipitated with ethanol, dissolved in 0.05 M Tris–0.15 M NaCl–1.5% sodium deoxycholate (pH 9.0), and the pH was adjusted to 11. After centrifugation, the supernatant was applied to a BioGel P100 column and eluted. P2-containing fractions were precipitated with ethanol and solubilized in buffer Z. OMP P2, purified by this method, retains antigenic reactivity with human bactericidal antibodies (27, 28).

LOS was extracted from NTHI 1479 and purified by the phenol-water method of Westfall and Jann (41). In brief, bacteria were lyophilized and suspended in endotoxin-free water, heated to 65°C, placed in 90% phenol for 15 min, and then cooled to 10°C. After centrifugation, the LOS-containing supernatant was removed. Remaining material was extracted in phenol two additional times before the combined supernatants were centrifuged. Supernatants were removed and dialyzed across 7,000-molecular-weight dialysis tubing. Samples were centrifuged (80,000 × *g*, 8 h at 20°C), and supernatants removed. Remaining pellets were again extracted and supernatants removed. Pellets containing LOS were taken up in endotoxin-free water and quantitated by weight (7).

Purity of each outer membrane constituent was confirmed by Coomassie blue stained SDS-polyacrylamide gel electrophoresis (PAGE). Absence of contaminating endotoxin was confirmed by silver stain (Fig. 1). Endotoxin contamination of purified P2 and P6 was <0.1 ng/μg protein by Limulus amebocyte lysate assay (Associates of Cape Cod). Final protein concentrations of OMP P6, OMP P2, and the total OMP preparation were determined by Lowry protein assay (Sigma Chemical Co, St. Louis, Mo.). LOS was quantitated by dry weight and volume adjusted for concentration.

Purification of human macrophages. Human mononuclear phagocytes were purified from buffy coat suspensions obtained from healthy, human immunodeficiency virus (HIV)-seronegative volunteers from the Red Cross of Western New York. Cells for individual experiments were from single donors and were not pooled. Macrophages from three separate donors were used for determination for each individual cytokine. Mononuclear cells were then purified by density centrifugation (Histopaque; Sigma Chemical Co., St. Louis, Mo.) and seeded onto 96-well tissue culture plates (4×10^6 cells/ml) in RPMI 1640 supplemented with 10% heat-inactivated human AB-positive serum. After incubation at 5% CO_2, 95% humidity, 37°C for 7 days, nonadherent cells were removed with serial rinses of warm (37°C) phosphate-buffered saline. Remaining monocyte-derived macrophages were incubated in RPMI 1640 with 10% fetal calf serum and were consistently 98 to 100% esterase positive (5).

Cytokine assays. Adherent human macrophages derived from 2×10^6 cells/well were incubated with 0.1 to 10 μg/ml of each NTHI outer membrane constituent for 8 to 48 h in 96-well Linbro tissue culture plates (Hampton Research, Aliso Viejo, CA). Data is presented for responses to 0.1 μg/ml of each antigen, unless stated otherwise. No loss of cell viability was detected by live-dead cell assay (Molecular Probes, Inc., Eugene, OR) or by trypan blue exclusion. To accommodate donor variability, cells of each donor were treated with each bacterial constituent in the same experiment for assay of each elicited cytokine.

Cellular supernatants were assayed by sandwich-type solid phase immunoassay for IL-1β, TNF-α, IL-10, IL-12, and IL-8 (R&D Systems, Inc., Minneapolis, MN). Supernatants of treated cells were harvested at appropriate time points, centrifuged, and removed from pelleted cells before being frozen. Standards and samples (200 μl) were added to individual wells precoated with monoclonal antibody to each cytokine and incubated. After repeated washing, polyclonal antibody to each cytokine, conjugated to horseradish peroxidase, was incubated in each well. After acidic 'stop' solution was added and followed by developer, the optical density (OD) of each well was measured at 450 nm within 30 min by an enzyme-linked immunosorbent assay (ELISA) reader and the concentration of each cytokine was calculated from a standard curve. All experiments included separate wells treated with *Escherichia coli* K235 lipopolysaccharide (LPS) (1 μg/ml) (Sigma Chemical Co., St. Louis, MO) or with the buffer diluent for each OMP as additional controls. In each experiment, cytokine induction in response to all buffers used for outer membrane constituents was confirmed to be indistinguishable from baseline cytokine values. Experiments were repeated with cells

from three separate donors. All conditions were performed in triplicate, as previously described (4).

Statistical significance between groups was determined using Student's two-tailed *t* test and was confirmed by post-hoc analysis of variants using Scheffe's F test.

RESULTS

Characterization of purified outer membrane components. To confirm that immunologic effects were not influenced by contaminating material, the purity of each outer membrane constituent was assessed by subjecting purified P6, P2, and LOS to SDS-PAGE and staining with Coomassie blue and silver stains (Fig. 1). Purified P6 and P2 separated as single bands, and silver stain showed absence of LOS. Preparations were further assessed by Limulus amebocyte lysate assay (Associates of Cape Cod), which confirmed that endotoxin content was <0.1 ng/μg protein. Subjecting LOS to SDS-PAGE and staining with Coomassie and silver stain further confirmed absence of contaminating proteins or other bacterial components.

OMPs P6 and P2 are weak antigenic stimuli for macrophage IL-1β. To investigate the potential of NTHI outer membrane constituents for IL-1β induction, human macrophages were incubated with OMP P6, OMP P2, LOS, and the total OMP preparation, each at 0.1 μg/ml, and supernatants were assayed over time (8 to 48 h) for IL-1β (Fig. 2). IL-1β induction in response to all buffers used for outer membrane constituents was statistically no different from basal (unstimulated) IL-1β production which, for all donors, was 0.5 ± 0.3 pg/ml at 8 h, 0.4 ± 0.2 pg/ml at 24 h, and 0.4 ± 0.3 pg/ml at 48 h (means ± standard errors of the means [SEM]). In all experiments, macrophages in separate wells also produced IL-1β in response to incubation with *E. coli* K235 LPS (1 μg/ml).

Measurements were taken from triplicate samples of each donor and were reproducible with cells from three separate donors. Single donor results (means ± SEM) are shown in Fig. 2, panel A. Among NTHI constituents, the total OMP preparation was the most potent antigenic constituent for IL-1β induction, while purified constituents P6, P2, and LOS had lesser effects. P6, P2, and LOS, even at doses of 10 μg/ml, were comparatively weak elicitors of IL-1β (not shown). Increasing concentrations of all outer membrane constituents to 1 to 10 μg/ml resulted in greater IL-1β induction from macrophages treated with the total OMP preparation, to 220.5 ± 112.0 and 480.2 ± 47.0 pg/ml, respectively (not shown).

To accurately compare relative responses from several donors and to accommodate relative differences in overall macrophage responsiveness between individual donors, results from three donors are combined and expressed as means ± SEM of IL-1β induced by each antigen (P6, P2, LOS) relative to IL-1β production from the total OMP preparation (Fig. 2, panel B). Composite data from multiple donors for each cytokine is also presented this way, so that results from relatively hyper- or hyporesponsive macrophages are not over- or underrepresented. Although LOS was nearly equivalent to total OMPs at 8 h, IL-1β concentrations were low for all constituents. By 24 h, relative IL-1β concentrations from P6- and LOS-treated macrophages were 0.23 ± 0.04 and 0.21 ± 0.19, respectively, compared with the total OMP preparation. Effects of each continued to diminish, relative to effects of total OMPs, by 48 h.

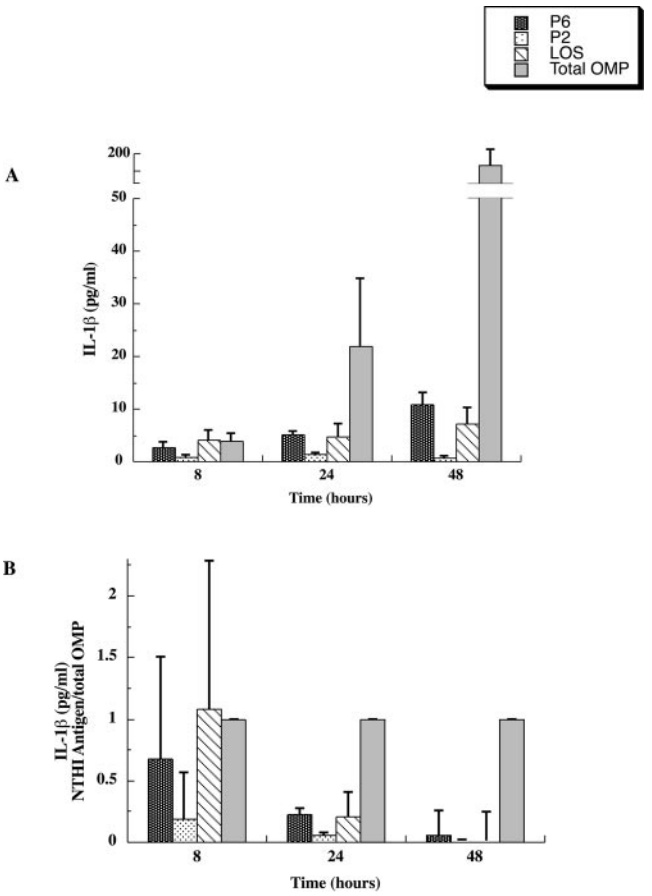

FIG. 2. NTHI outer membrane antigen induction of macrophage IL-1β. Macrophages were incubated with OMP P6, OMP P2, LOS, or the total OMP preparation, each at 0.1 μg/ml, and IL-1β induction was measured for 8, 24, and 48 h. Results shown in panel A are means ± SEM from triplicate samples of one donor and were reproducible with cells from three separate donors. Combined results of all three donors, shown in panel B, are expressed as IL-1β concentration elicited with each antigen relative to IL-1β elicited by total OMPs. Macrophage reactivity was confirmed by treatment with *E. coli* K235LPS (1 μg/ml) for each time point.

OMPs P6 and P2 are weak antigenic stimuli for macrophage IL-12. To determine the relative capabilities of NTHI outer membrane antigens to elicit IL-12 from human macrophages, each antigen was incubated with human macrophages and supernatant was assayed for IL-12 (Fig. 3). IL-12 induction in response to all buffers used for outer membrane constituents was no different from basal (unstimulated) IL-12 production which, for all donors, was 2.9 ± 1.2 pg/ml at 8 h, 2.7 ± 1.1 pg/ml at 24 h, and 2.8 ± 1.1 pg/ml at 48 h (means ± SEM). NTHI outer membrane constituents proved to be relatively weak elicitors of IL-12 in all donors at each time point. Single donor results are shown in Fig. 3, panel A. Higher concentrations (1 to 10 μg/ml) of each outer membrane constituent resulted in the total OMP preparation at 10 μg/ml, inducing 19.5 ± 14.3 pg/ml, 23.3 ± 2.3 pg/ml, and 24.0 ± 12.0 pg/ml at 8, 24, and 48 h, respectively (not shown). OMP P6, OMP P2, and LOS each induced less than 10 pg/ml of IL-12 for all concentrations and time points. IL-12 responses from three donors, relative to

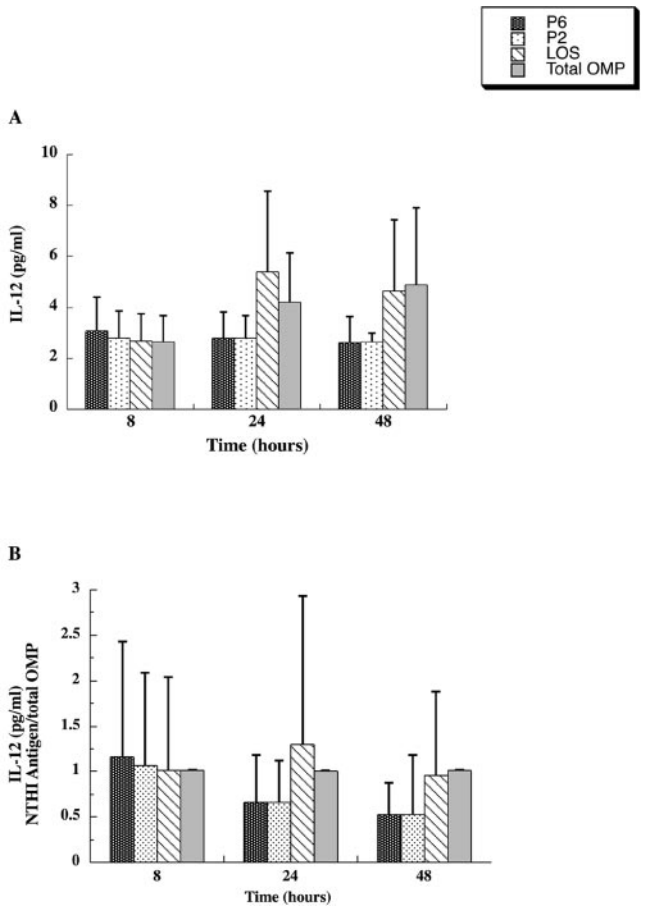

FIG. 3. NTHI outer membrane antigen induction of macrophage IL-12. Macrophages were incubated with each outer membrane constituent as in Fig. 2, and IL-12 induction was measured. Concentrations of each antigen and incubation times are as detailed in Fig. 2. Results shown in panel A are means ± SEM from triplicate samples of one donor. Combined results of all three donors, shown in panel B, are expressed as IL-12 concentration elicited with each antigen relative to IL-12 elicited by total OMPs.

the total OMP response, was not significant for each individual antigen (Fig. 3, panel B).

OMP P6 is a moderate antigenic stimulus for macrophage IL-10. To establish the potential and relative kinetics of NTHI outer membrane constituents to elicit IL-10, human macrophages were incubated with each NTHI outer membrane constituent and assayed for IL-10. As in previous experiments, IL-10 induction in response to all buffers used for outer membrane constituents was statistically no different from basal (unstimulated) IL-10 production which, for all donors, was 4.1 ± 1.3 pg/ml at 8 h, 5.1 ± 1.5 pg/ml at 24 h, and 6.0 ± 2.8 pg/ml at 48 h (means ± SEM). Single donor results are shown in Fig. 4, panel A. OMP P6 (0.1 μg/ml) elicited concentrations of IL-10 comparable to LOS and well in excess of OMP P2. P6-mediated IL-10 production did not increase with higher concentrations (1 to 10 μg/ml).

Comparison of IL-10 production for each antigen (P6, P2, LOS) relative to total OMP induction for three donors is shown in Fig. 4, panel B. OMP P6 produced moderate amounts of IL-10, with a ratio of 0.5 ± 0.1, compared with total OMPs at 24 h. LOS was the most potent antigenic stimulus for IL-10, relative to total OMPs, with ratios of 0.91 ± 0.1, 1.03 ± 0.2, and 0.73 ± 0.23 for 8, 24, and 48 h, respectively.

OMP P6 is a potent stimulus for macrophage TNF-α induction. To further determine the relative capacities of NTHI outer membrane antigens to induce TNF-α, human macrophages were incubated with each constituent and supernatant was assayed for TNF-α. As in previous experiments, TNF-α induction in response to buffers used for outer membrane constituents was no different from basal (unstimulated) TNF-α production which, for all donors, was 3.8 ± 0.4 pg/ml at 8 h, 3.7 ± 1.5 pg/ml at 24 h, and 0.9 ± 0.8 pg/ml at 48 h (means ± SEM). While a wide range of responses were found among individual donors, the relative responsiveness to individual antigens was conserved. For the donor shown in Fig. 5, panel A (means ± SEM of triplicate values), OMP P6 was comparable to LOS and exceeded OMP P2 for all time points.

Comparison of TNF-α production induced by each antigen (P6, P2, LOS) relative to total OMP induction for three donors is shown in Fig. 5, panel B. LOS (0.1 μg/ml) exerted the most potent effect (1.48 ± 0.7). However, OMP P6-induced TNF-α

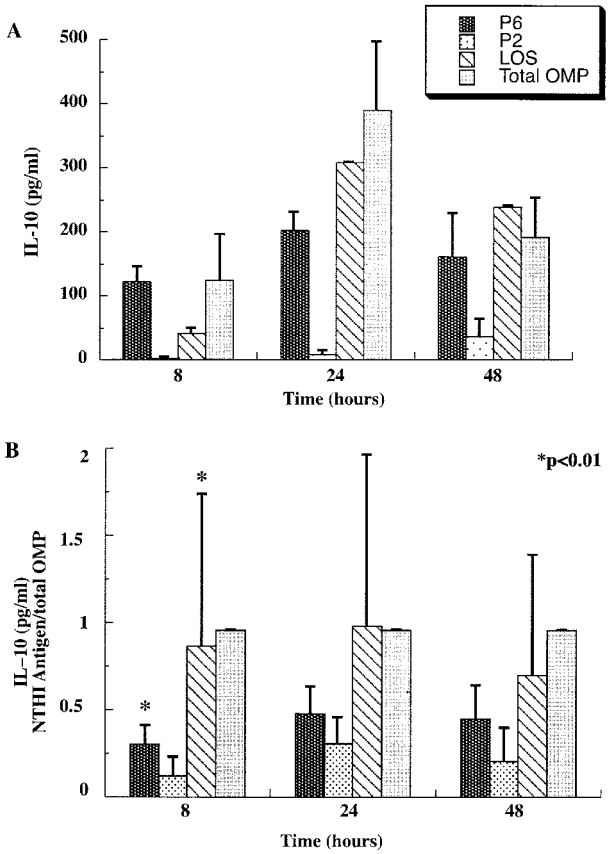

FIG. 4. NTHI outer membrane antigen induction of macrophage IL-10. Macrophages were incubated with each outer membrane constituent as in Fig. 2, and IL-10 induction was measured. Concentrations of each antigen and incubation times are as detailed in Fig. 2. Panel A shows means ± SEM of IL-10 from triplicate samples of one donor and were reproducible with cells from three separate donors. Panel B shows IL-10 concentration elicited with each antigen relative to IL-10 elicited by total OMPs to accommodate donor variability.

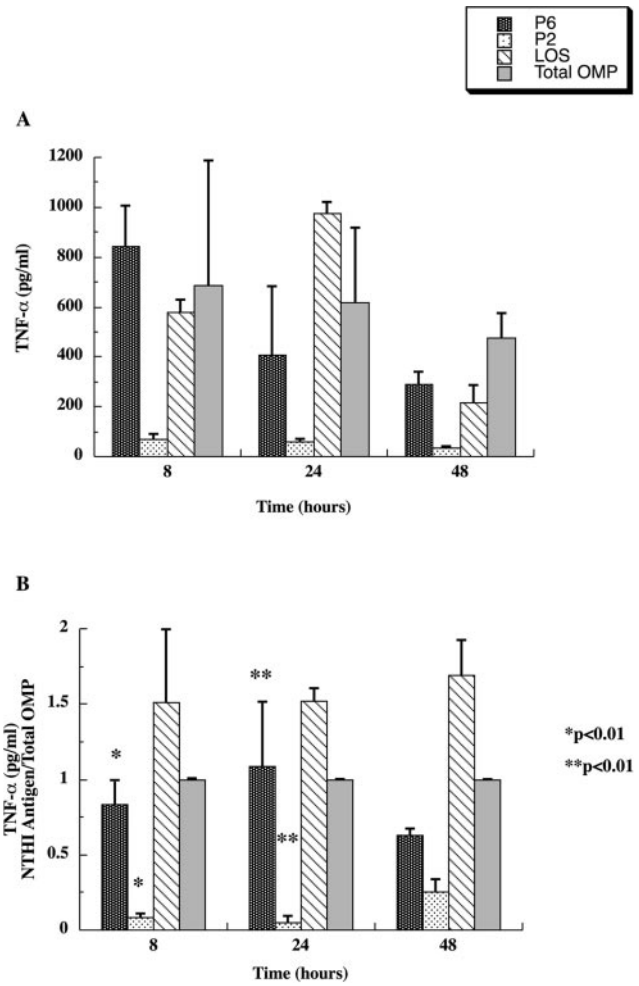

FIG. 5. NTHI outer membrane antigen induction of macrophage TNF-α. Macrophages were incubated with each outer membrane constituents as in Fig. 2, and TNF-α induction was measured. Concentrations of each antigen and incubation times are as detailed in Fig. 2. Panel A shows means ± SEM of TNF-α from triplicate samples of one donor and were reproducible with cells from three separate donors. Results from all three donors are expressed in panel B as TNF-α concentration from each antigen relative to TNF-α concentration from the total OMP preparation.

production at 8 and 24 h (0.9 ± 0.15 and 1.1 ± 0.4) was comparable to the effect of LOS ($P > 0.2$). In comparison, OMP P2-induced TNF-α production (0.1 ± 0.02) was far less effective than that of OMP P6 ($P < 0.01$) at 8 and 24 h. Higher doses of OMP P6 (1 to 10 μg/ml) did not induce greater concentrations of TNF-α (not shown).

OMP P6 is a potent stimulus for macrophage IL-8 induction. To determine if OMP P6 induction of proinflammatory cytokines extends to IL-8, supernatants of human macrophages, incubated with each outer membrane constituent, were assayed for IL-8 (Fig. 6). As in previous experiments, IL-8 induction in response to buffers used for outer membrane constituents was no different from basal (unstimulated) IL-8 production which, for all donors, was 65.0 ± 0.3 pg/ml at 8 h, 63.1 ± 29.7 pg/ml at 24 h, and 79.5 ± 25.2 pg/ml at 48 h (means ± SEM). Some variation of individual values was seen among donors, although relative responsiveness to each antigen was conserved among individual donors. For the donor shown (Fig. 6, panel A), IL-8 induced by OMP P6 was comparable to IL-8 elicited by LOS and total OMPs and far exceeded IL-8 induced by OMP P2.

Proportionate responses to each antigen were maintained with cells of other donors in separate experiments. Therefore, as with earlier cytokine measurements, results of three donors are expressed as IL-8 production from each NTHI antigen relative to IL-8 elicited by total OMP preparation for each donor (means ± SEM) in Fig. 6, panel B. As with TNF-α, OMP P6 at 0.1 μg/ml was an extremely potent antigenic stimulus of IL-8, with a 1.4-fold greater effect than the total OMP preparation, compared with a 0.63-fold effect elicited by LOS at 8 h. In fact, the effect of OMP P6 (0.1 μg/ml) was not statistically distinguishable from that of LOS (0.1 μg/ml) for all time points. In comparison to OMP P6, OMP P2 elicited far

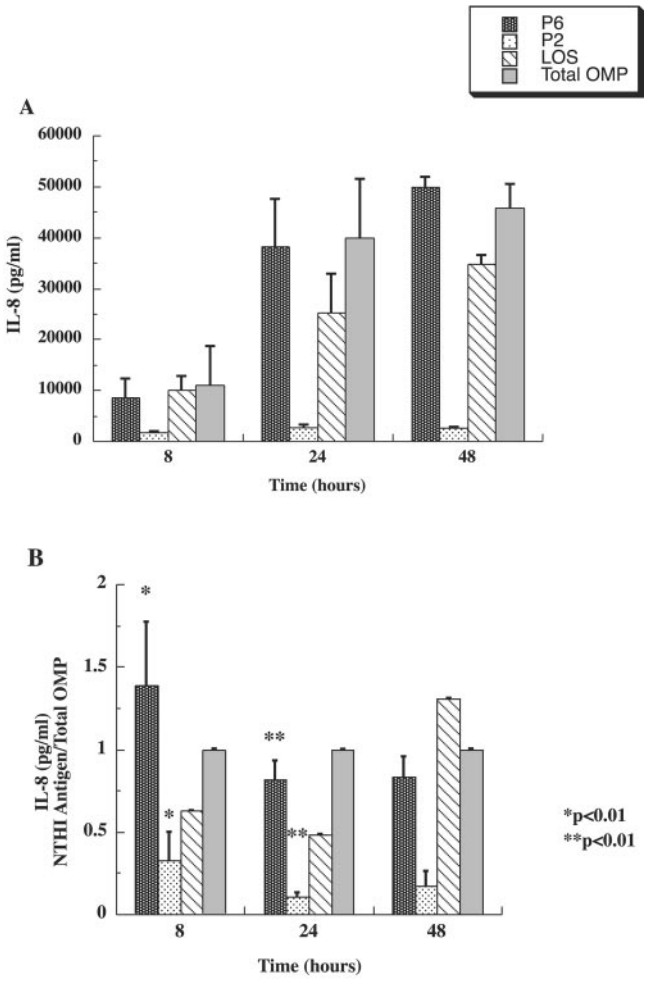

FIG. 6. NTHI outer membrane antigen induction of macrophage IL-8. Macrophages were incubated with each outer membrane constituent as in Fig. 2, and IL-8 induction was measured. Concentrations of each antigen and incubation times are as detailed in Fig. 2. Panel A shows means ± SEM of TNF-α from triplicate samples of one donor and were reproducible with cells from three separate donors. As with TNF-α measurements, relative differences between individual donors are accommodated by expressing values as IL-8 concentration elicited by each antigen relative to IL-8 concentration elicited by total OMPs in panel B.

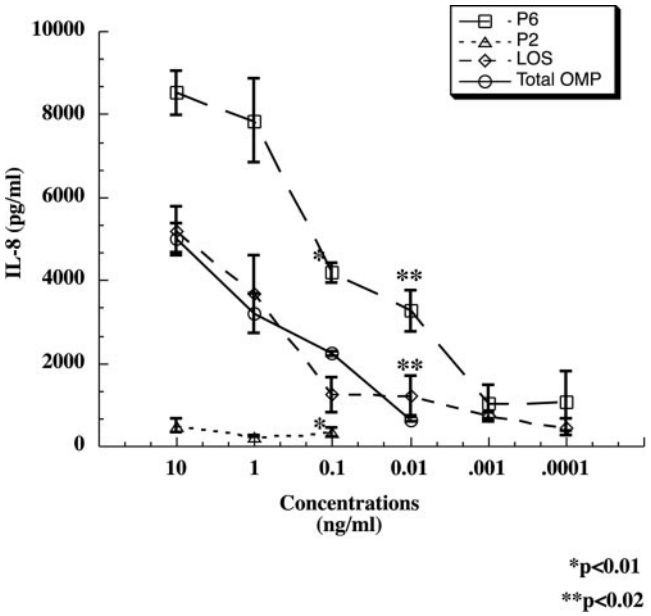

FIG. 7. Macrophage IL-8 induction with diminished concentrations of NTHI outer membrane antigens. Macrophages were incubated with decreasing concentrations of each outer membrane constituent. As in previous experiments, each value represents means ± SEM of three separate measurements.

smaller levels of IL-8 ($P < 0.01$). OMP P6 at higher concentrations (1 to 10 μg/ml) did not elicit higher concentrations of macrophage IL-8 (not shown).

OMP P6 is a potent antigenic stimulus of IL-8 at picogram concentrations. OMP P6 was a potent stimulus of IL-8, relative to other outer membrane constituents, at low (0.1 μg/ml) concentrations. To determine the minimum concentrations of OMP P6 that would retain macrophage IL-8 induction capabilities, cells were treated with serial dilutions of each outer membrane constituent (Fig. 7). At concentrations of 10 ng/ml, the effect of OMP P6 exceeded that of LOS ($P < 0.01$), while OMP P2 did not induce IL-8 above baseline levels ($P < 0.01$). At 10 pg/ml, OMP P6 still elicited 3,261 ± 485 pg/ml of IL-8, while LOS or total OMPs (10 pg/ml) no longer produced IL-8 appreciably above baseline concentrations of untreated cells ($P < 0.02$). Thus, OMP P6 retained IL-8 inductive activity at 10 pg/ml while LOS, OMP P2, and the total OMP preparation did not.

DISCUSSION

These studies are the first to demonstrate a fundamental immunologic role for OMP P6 of NTHI in the regulation of human macrophages. Because NTHI is a strictly human pathogen, human macrophages were used exclusively as the model for these studies. For greater consistency in interpretation of results and to eliminate the potential for variability of individual components among strains, these studies were designed employing purified individual cellular components from the same NTHI strain.

Macrophages are critical to the pathogenesis of NTHI infections in humans. Examination of hypertrophied adenoid tissue, removed at adenoidectomy from 10 children, revealed intracellular NTHI in all 10 adenoids. The reservoirs for NTHI were large subepithelial mononuclear cells which contained up to 200 viable and actively dividing intracellular NTHI organisms per cell (11). Moreover, examination of explanted lungs from patients undergoing lung transplantation for COPD revealed NTHI in 8 of 16 individuals. NTHI was widely distributed and was again found in macrophages (24). Macrophages are also vital to the binding and phagocytosis of NTHI (28, 42). In fact, human macrophages preferentially phagocytosed NTHI in vitro more avidly than type b *H. influenzae* (20, 30). In one study of macrophage-NTHI interactions, 82% of 33 clinical NTHI isolates persisted and remained viable intracellularly after having been ingested by phagocytes of a macrophage cell line (9). *Haemophilus* species also interact with macrophages to modulate phagocytosis and promote disease in nonhuman mammals (14, 15). Collectively, these studies support a paradigm of NTHI interactions with human macrophages as a key source of persistent inflammatory responses.

Once activated, the ability of macrophages to promote inflammation is in part a virtue of their ability to express cytokines (23). Thus, defining the cytokine milieu elicited by bacterial antigens and identifying key immunoregulatory antigens determines the regulatory signals by which macrophages influence the behavior of T cells and neutrophils. Our total NTHI OMP preparation contained not only LOS but also peptidoglycan and numerous immunoactive molecules of the outer membrane. Thus, it was not surprising that the total OMP preparation should globally stimulate macrophage cytokine output. In fact, NTHI possess additional small molecules that induce IL-8, although study has been confined to effects on epithelial cells (39).

Traditionally, lipooligosaccharide (LOS), the endotoxin of NTHI, has been considered a prime immune activator of macrophages. LOS of NTHI is a prime stimulant of inflammatory mediators from human cells, including IL-8 (22). In fact, our studies confirm LOS as a potent stimulus for human macrophage induction of proinflammatory cytokines. Despite the association of porin proteins with bacterial endotoxins, OMP P2, the porin of NTHI 1479, was a less effective stimulus of proinflammatory cytokines in human macrophages (18). The porin protein (Hib porin) of type b *H. influenzae* also activates human and murine cells and induces proinflammatory cytokines, signaling through Toll-like receptors (12, 13). While higher doses of Hib porin had greater effect on THP-1 cells, smaller doses (0.05 μg/ml) elicited concentrations of TNF-α comparable to those elicited with OMP P2 (0.1 μg/ml) in human macrophages in our study (13).

Our findings identify OMP P6 as a potent and specific trigger of proinflammatory macrophage cytokines, specifically IL-10, TNF-α, and IL-8. The induction of macrophage TNF-α by OMP P6 provides the ability to recruit T-cell subpopulations, while IL-10 may further modulate T-cell responses, including antigen presentation to Th1 cells. This provides a means of further modifying macrophage responses, perpetuating a succession of inflammatory events. At early time points, OMP P6 was a less potent stimulus of IL-10 than were either the total OMP preparation or LOS, but it was a more potent stimulus of TNF-α and IL-8. This probably reflects the incorporation in the total OMP preparation of numerous immunoactive molecules, not specifically studied in this analysis, with distinct immunologic properties and of numerous components that do

not activate human macrophages to produce IL-8 and TNF-α. OMP P6 is also an immunomodulator of peripheral blood lymphocyte proliferation (1). The induction of IL-8 by OMP P6 further provides a means for recruiting neutrophils to an inflammatory site. The elaboration of a spectrum of cytokines supports a paradigm of NTHI OMP P6 initiating inflammatory events by several immunologic routes. Perhaps most intriguing, OMP P6 not only possesses potent immunologic properties but, at doses as low as 10 pg/ml, also was more potent than LOS at eliciting macrophage IL-8.

OMP P6 possesses a terminal cysteine-tripalmitoyl (Cys-Pam_3) motif, common to immunoregulatory bacterial lipoproteins (43). *Mycobacterium tuberculosis* expresses a 19-kDa lipoprotein that not only activates human macrophages through Toll-like receptors (TLRs) but that also possesses a Cys-Pam_3 terminus (6). Lipoproteins possessing a cysteine-tripalmitoyl terminus show preferential recognition for TLR-2 (25). A synthetic lipopeptide expressing a Cys-Pam_3 motif activates human mononuclear cells through transcription of NF-κB (2). Although study has thus far been limited to epithelial cell lines, OMP P6 of NTHI also activates NF-κB through TLR-2 (8). *H. influenzae* infection can also initiate immune cell activation in vivo by elaboration of cytokines through Toll-like receptor signaling, as demonstrated in a murine model (40). A Cys-Pam_3 terminus has also been identified on bacterial lipoproteins of numerous additional bacterial genera (10, 17, 36). A synthetic lipopeptide expressing a Cys-Pam_3 motif activates human mononuclear cells through transcription of NF-κB (2). However, deacylation of the lipopeptide abrogated its immune effect, supporting a role for the lipid terminus as a critical component to lipoprotein-TLR interactions. Determination of whether the lipid terminus of OMP P6 bears a comparable role in human macrophage signaling is a focus of our ongoing studies.

In related work of our group, significantly higher IL-8 concentrations in sputum during COPD exacerbations were associated with the presence of NTHI, whereas those associated with *Moraxella catarrhalis* were not (33). Among a variety of chemoattractants in bronchoalveolar lavage fluid from smokers with and without emphysema, only IL-8 correlated with the presence of emphysema, although the cellular source of IL-8 remained to be determined (37). Each study supports IL-8-mediated inflammatory responses as a marker of the destructive changes of emphysema and, taken with our current findings, support OMP P6 as a possible trigger for IL-8 induction. The recognition of OMP P6 as a potent and specific trigger of proinflammatory macrophage cytokines, notably IL-8 and TNF-α, supports a role for OMP P6 induction of human macrophages as a contributor to inflammatory disease states in humans.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of Jane M. Smigiera for these studies.

This work was supported by research grants 1RO1HL6654901 and AI19641 (T.F.M.) from the National Institutes of Health (C.S.B., S.S.) and the Department of Veterans Affairs.

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Editor: J. B. Bliska