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Pneumococcal Polysaccharides Interact with Human Dendritic Cells

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Dendritic cells (DCs) are critical antigen presentation cells whose influence on murine immune responses to polysaccharide antigens has only recently been elucidated. Little is known about human DC-polysaccharide interactions. We set out to study the interaction between human monocyte-derived DCs and pneumococcal capsular polysaccharides (PPS) in vitro. Immature DCs were generated from peripheral blood monocytes and incubated with fluorescein isothiocyanate-labeled PPS type 9N or 14 for assessment of uptake. DCs were exposed to PPS type 1, 6B, 9N, 14, 19F, or 23F in the absence or presence of *Escherichia coli* lipopolysaccharide (LPS) for assessment of phenotypic DC maturation and cytokine production. PPS were taken up by immature DCs and proceeded to HLA-DR$^+$ and lysosome-associated membrane protein-1$^+$ late endosomal compartments. Uptake was reduced in the presence of cytochalasin D and wortmannin, suggesting that both cytoskeletal rearrangements and phosphatidylinositol 3-kinase activation may be required for internalization. None of the PPS tested induced DC phenotype changes, maturation, or interleukin-12 (IL-12)/IL-10 production. However, PPS were capable of modulating the response of the DCs to a second signal such as LPS. Exposure of DCs to PPS in the presence of LPS resulted in an altered cytokine balance with significantly increased IL-10 production and reduced IL-12 production compared to LPS alone. This effect was not seen using the control antigen tetanus toxoid. DC-pneumococcus interaction may affect subsequent immune responses to pneumococci, as an altered cytokine balance may have a profound effect on DC-driven T-cell priming.

Responses to purified polysaccharide antigens (also called T-independent antigens type 2 [TI-2]) were, until recently, assumed to be completely independent of antigen-presenting cells (APCs), based on the observation that TI-2 antigens generally do not associate with major histocompatibility complex (MHC) class II molecules (10). However, a growing body of evidence suggests a role for APCs in TI-2 responses. Dendritic cells (DCs) are the most potent professional APCs, and a role for them in TI-2 responses was first suggested by the finding that a subset of myeloid DCs, termed plasmablast-associated DCs, supports the differentiation of plasmablasts into antibody-producing plasma cells in response to a soluble TI-2 antigen (6). In murine studies of pneumococcal infection, DCs were necessary and sufficient to promote survival of pneumococcal polysaccharide-specific B cells and their differentiation into immunoglobulin M (IgM)-secreting plasmablasts (2). The critical signals are provided by DCs via the release of B-lymphocyte stimulator protein and a proliferation-inducing ligand (APRIL), which bind with high affinity to specific receptors on the B cells and induce CD40-independent class switching in the presence of appropriate cytokines, i.e., interleukin-10 (IL-10), transforming growth factor β, and/or IL-4 (15).

In addition to supporting polysaccharide-specific B cells, DCs have recently been shown to present at least certain polysaccharides derived from bacterial capsules, including pneumococcal polysaccharide type 1, in the context of MHC class II to CD4$^+$ T cells. Activation of CD4$^+$ cells in this manner causes abscess formation in a rodent model of intra-abdominal abscess formation (12). All the polysaccharides found so far that are able to activate T cells share a zwitterionic charge motif. They are processed to low-molecular-weight carbohydrates inside DCs by a nitric oxide-mediated mechanism, loaded onto MHC, and shuttled to the surface for presentation to T cells. Intriguingly, zwitterionic polysaccharides induced abscess formation only when administered with a sterile cecal contents adjuvant, and subcutaneous administration of zwitterionic polysaccharides alone protected animals from abscess formation after infection with abscess-inducing bacterial pathogens (19).

Immunity against *Streptococcus pneumoniae* and other encapsulated bacteria such as *Haemophilus influenzae* type b and *Neisseria meningitidis* relies on the generation of antibodies against their capsular polysaccharides. Purified capsular polysaccharides, when incorporated into vaccines, are, however, poorly immunogenic TI-2 antigens. They induce a poor antibody response with mainly low-affinity IgM and do not elicit conventional T-cell help, and thus responses fail to demonstrate antibody affinity maturation, isotype switching, or memory formation (13). In addition, some studies report immunological hyporesponsiveness to a second dose of the same vaccine months to years after the original polysaccharide vaccination (8, 20). The limited response to purified polysaccharides has, however, been overcome by coupling the polysaccharides to a highly immunogenic protein carrier. Such glycoconjugate vaccines induce T-dependent responses with good affinity maturation, isotype switching, and memory formation and, most importantly, induce antibodies in the very young, who are particularly susceptible to infection with encapsulated bacteria (9).

Little is known about the role of DCs in the immune re-

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sponse to either purified bacterium-derived polysaccharides or glycoconjugate vaccines in humans. In this study we have thus explored the interaction between purified capsular pneumococcal polysaccharides or protein-polysaccharide conjugate vaccines and human myeloid (monocyte-derived) DCs. Uptake and trafficking, as well as the effect of polysaccharides on DC maturation, have been evaluated. DC cytokine secretion is critical for influencing T-cell immune function in response to antigen presentation, and thus these studies have focused on production of cytokines after incubation of DCs with polysaccharides directly and after lipopolysaccharide (LPS) stimulation of polysaccharide-exposed DCs.

MATERIALS AND METHODS

Antigens and antibodies. Purified capsular polysaccharides (PPS) derived from *S. pneumoniae* serotypes 1, 6B, 9N, 14, 19F, and 23F were obtained from the American Type Culture Collection (Rockville, MD) and diluted in distilled water to a concentration of 1 mg/ml. Concentrations used in cell cultures were between 4.4 and 6.4 μg/ml unless stated otherwise, adjusted for each serotype to match the concentration of the polysaccharide component in 10 μg/ml conjugate. Conjugates of pneumococcal capsular polysaccharides (serotypes 1, 6B, 9N, 14, 19F, and 23F) linked to a tetanus toxoid carrier were obtained from Sanofi Aventis (Lyon, France) and used at a concentration of 10 μg/ml for the carrier component. Tetanus toxoid was used as a control antigen. It was obtained from Aventis Pasteur and used at 10 μg/ml. Fluorescein isothiocyanate (FITC)-labeled pneumococcal polysaccharides were kindly provided by Chris Jones, National Institute for Biological Standards and Control, Potters Bar, United Kingdom.

Fluorescence-activated cell sorter (FACS) analysis was performed using the following antibodies: CD11c (S-HCL-3); CD25 (M-A251); CD45 (2D1); CD83 (HB15e); CD86 (2331); CD40 (5C3); CD14 (M5E2); a Lin-1 cocktail containing antibodies against CD3, CD14, CD16, CD19, CD20, and CD56; and appropriate isotype control antibodies; all were obtained from Becton Dickinson (San Jose, CA).

Generation of DCs. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy adult volunteers by the standard Ficoll-Paque method using Lymphoprep (Nycomed, Roskilde, Denmark). For DC preparations, $CD14^+$ monocytes were positively selected from PBMCs using magnetic anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured for 6 days in RPMI 1640 with 25 mM HEPES and L-glutamine (Invitrogen/Life Technologies, Carlsbad, CA) supplemented with 1% penicillin-streptomycin (10,000 IU/ml; Invitrogen/Life Technologies, Carlsbad, CA) and 10% fetal bovine serum in the presence of 100 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (Leucomax; Sandoz Pharmaceuticals, Camberley, United Kingdom) and 25 ng/ml recombinant human interleukin-4 (gift from D. Katz, University College London, United Kingdom). A specialized batch of fetal bovine serum with no/low endotoxin contamination (Myoclone Superplus; Invitrogen/Life Technologies) was used to minimize unwanted maturation of the generated DCs. On day 6, nonadherent, immature DCs were harvested. This procedure gave pure preparations of immature DCs as assessed routinely by FACS analysis of a panel of antibodies including CD11c, Lin-1, CD14, HLA-DR, CD83, CD86, CD40, CD1a, -b, -c, -d, and appropriate isotype controls.

DCs were activated and matured in some experiments by addition of 0.5 μg/ml LPS (*Escherichia coli* 026:B6; Sigma, St. Louis, MO) on day 6 of culture for 24 h.

Confocal microscopy. DCs in quantities of 1×10^4 to 2×10^4 were fed antigen for indicated periods of time. They were plated onto 18- by 18-mm coverslips (BDH) coated with 10 μg/ml human fibronectin (Sigma, St. Louis, MO) and allowed to adhere and polarize at 37°C for 30 min to 2 h. They were fixed in 4% paraformaldehyde-3% glucose in phosphate-buffered saline (PBS), permeabilized in 0.5% Triton X-100, and blocked with 1% bovine serum albumin in PBS. Cells were stained with 0.1 μg/ml rhodamine phalloidin for localization of filamentous actin and with 2 μM TO-PRO 3-iodide (both from Molecular Probes, Eugene, OR) for visualization of the nuclei. For colocalization with markers of endocytic organelles, cells were incubated with unconjugated, monoclonal mouse anti-human antibodies against CD71 (transferrin receptor, TfnR, specific for early endosomes), CD107a (lysosome-associated membrane protein-1 [LAMP-1], specific for late endosomes and lysosomes), or HLA-DR. HLA-DR localizes to late endosomes and lysosomes in immature DCs and to the cell surface in mature DCs. Alexa 488- or Alexa 546-conjugated antimouse antibodies were used as the second layer. All antibodies were obtained from Molecular Probes (Eugene, OR) and diluted in PBS to 1:40 (primary antibodies) or 1:100 (secondary antibodies). Cells were washed in PBS and distilled H_2O, mounted on glass microscope slides with a drop of Citifluor, and sealed with nail varnish.

Confocal images were obtained using a confocal laser scanning microscope system (TCS NT; Leica, Switzerland) fitted with appropriate filter sets. To combine a three-dimensional *z* series into a two-dimensional image, 10 to 20 optical sections (0.5 μm) spanning the entire cell were projected and superimposed. In double-labeling experiments, bleedthrough corrections were done according to the manufacturer's instructions. To visualize colocalization of two fluorochromes, the images in the two channels were merged. A yellow signal was interpreted as colocalization. Images were processed with the TCS Leica Start and Adobe Photoshop 5.0 software.

In endocytosis experiments, adhered, polarized cells were fed fluorescently labeled antigen for the indicated period of time before fixation, permeabilization, and staining as described above.

Flow-cytometric endocytosis assay. Immature DCs were incubated for the indicated times with FITC-labeled antigen in the dark. Samples were set up in duplicate and incubated either at 37°C, to allow uptake, or on ice, to quantify background staining. In some experiments, cells were preincubated with 10 nM cytochalasin D or 100 nM wortmannin (both from Sigma, St. Louis, MO). Surface-bound antigen was quenched with 75 μl quenching solution (ORPEGEN Pharma, Heidelberg, Germany); cells were washed in PBS and analyzed immediately on a Beckman Coulter XL flow cytometer with Expo2 software.

Cytokine measurements. Cytokine production was analyzed from lymphocyte culture supernatants sampled after 22 h of stimulation. Commercial kits for IL-12 (BD Biosciences Pharmingen, San Diego, CA) and IL-10 (eBioscience, San Diego, CA) were used according to instructions supplied by the manufacturers. Results are expressed as the concentration of cytokine (pg/ml) in the culture supernatant of 4×10^6 cells/ml.

RESULTS

Internalization of pneumococcal polysaccharides by DCs. Uptake of FITC-labeled pneumococcal capsule-derived polysaccharides of serotypes 9N and 14 (PPS9N and PPS14) by human monocyte-derived DCs was examined by both confocal microscopy and FACS analysis. FITC-labeled PPS9N and PPS14 were taken up by immature DCs. They localized to small endocytic vesicles distributed in the perinuclear area (Fig. 1, top panel). Uptake slowed after 4 h but was still ongoing after 24 h (Fig. 1, bottom panel).

Receptor-mediated endocytosis and macropinocytosis are endocytic mechanisms thought to be the most efficient mechanisms for delivery and to play the largest role in internalization by DCs. To distinguish between the mechanisms directly involved in the uptake of PPS, we performed a series of experiments using inhibitors of known specificities. Cytochalasin D, which inhibits actin polymerization and thus, endocytosis, and wortmannin, which is a specific inhibitor of macropinocytosis acting via the phosphatidylinositol 3-kinase, were used to selectively inhibit the different mechanisms of uptake and investigate their relative contribution to uptake of pneumococcal polysaccharides. As seen in Fig. 2, 23.4% of cells stained positive for intracellular polysaccharide after 2 h incubation. When they were preincubated with inhibitory concentrations of cytochalasin D or wortmannin (11), uptake was reduced dramatically in both cases (cytochalasin, 3.5% positive cells; wortmannin, 6% positive cells) (Fig. 2C and D). This suggests that both receptor-mediated endocytosis and macropinocytosis are used by DCs to internalize pneumococcal polysaccharides.

Intracellular processing of pneumococcal polysaccharides. In order to follow the intracellular fate of pneumococcal polysaccharides, immature DCs were incubated with FITC-labeled PPS14, and the various endocytic compartments were stained with their specific markers. Monoclonal antibodies against transferrin receptor were used to label early endosomes,

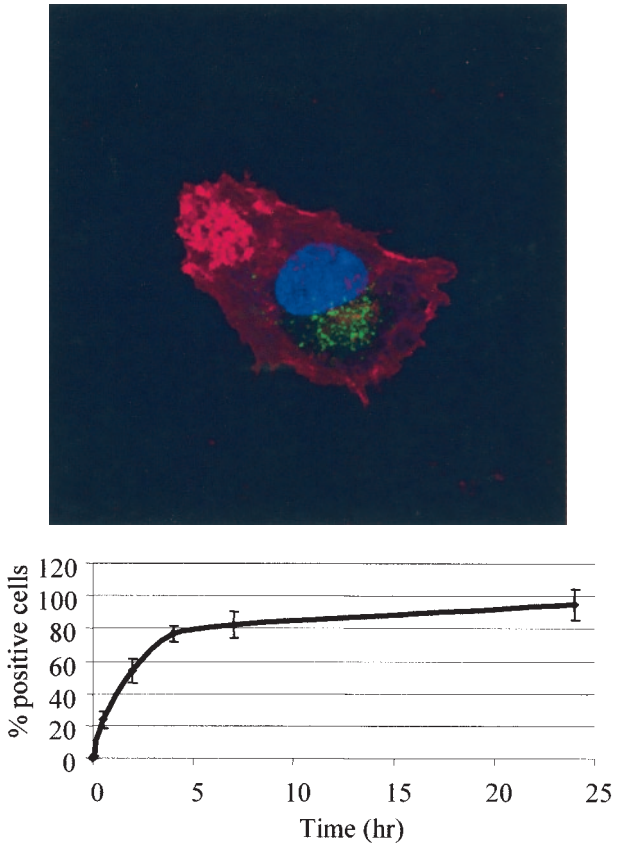

FIG. 1. Internalization of pneumococcal polysaccharides. (Top) Immature DCs were incubated for 1 h with 60 μg/ml PPS14, fixed, stained with rhodamine-phalloidin (green) and TO-PRO (blue) to visualize filamentous actin and the nucleus, respectively, and examined using a confocal microscope. (Bottom) DCs were incubated with 60 μg/ml PPS14 for the periods of time indicated. Surface-bound antigen was quenched and cells were immediately analyzed by flow cytometry. Background uptake on ice was negligible. Results are representative of three independent experiments. Similar results were obtained for PPS9N.

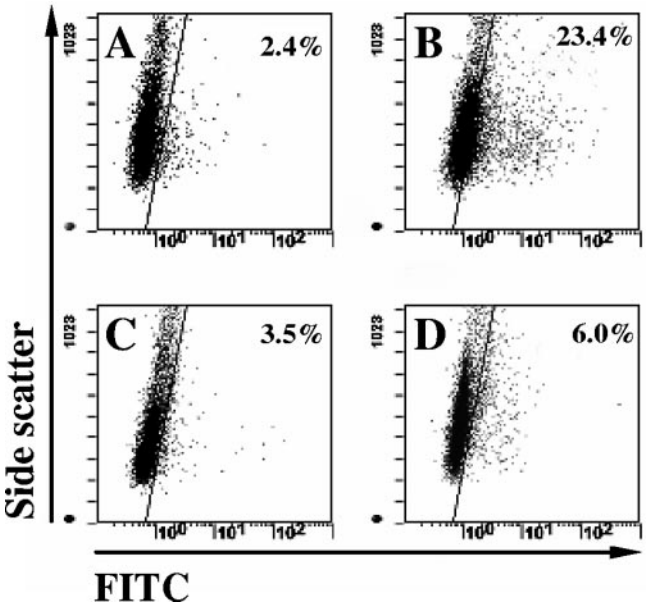

FIG. 2. Inhibition of uptake of pneumococcal polysaccharide type 14. Immature dendritic cells were incubated with 60 μg/ml PPS14 for 2 h on ice (A) or at 37°C (B) to determine background staining and internalization, respectively. Cultures run in parallel were preincubated for 30 min with 10 nM cytochalasin D (C) or 100 nM wortmannin (D). After incubation, surface-bound antigen was quenched and cells were immediately analyzed by flow cytometry. Similar results were obtained in two further experiments and for PPS9N.

LAMP to stain late endosomes and lysosomes, and MHC class II to visualize late endosomal compartments in immature DCs and surface expression in mature DCs. After 24 h of incubation, PPS14 colocalized with LAMP and MHC class II in immature DCs, as shown by a yellow signal, but not with the transferrin receptor, indicating that this polysaccharide reached late endosomal compartments (Fig. 3).

DC maturation in response to pneumococcal polysaccharides and conjugates. To investigate the effect of polysaccharides and conjugates on DC maturation, immature DCs were incubated overnight with pneumococcal polysaccharide type 1, 6B, 9N, 14, 19F, and 23F, and the corresponding conjugates and surface marker expression were analyzed by flow cytometry. FACS analysis showed that, in contrast to LPS, neither PPS nor PPS-tetanus toxoid (TT) (Fig. 4) nor the control antigen TT (data not shown) induced upregulation of critical DC surface markers including HLA-DR, CD83, CD86, and CD40. Analysis of cytokine production showed that immature DCs incubated overnight with PPS (all serotypes), PPS-TT (all serotypes), and TT produced no or very small amounts of IL-12 or IL-10 (Fig. 5). In contrast, DCs produced both cytokines after stimulation with LPS.

Influence of pneumococcal polysaccharides and conjugates on LPS-induced DC maturation. We next determined whether uptake of polysaccharides or conjugates had any effect on subsequent DC maturation induced by LPS. FACS analysis showed that internalization of PPS or PPS-TT did not inhibit phenotypic maturation of DCs in response to LPS (data not shown). Levels of HLA-DR and CD40 were upregulated in response to LPS following PPS and PPS-TT internalization, although expression was lower than that achieved by LPS stimulation alone. The control antigen TT did not influence LPS-induced DC maturation (differences between TT-plus-LPS and LPS results: HLA-DR, $P = 0.22$; CD40, $P = 0.2$). When supernatants were analyzed for cytokine production, dramatic differences were, however, seen (Fig. 5). Uptake of PPS or PPS-TT predisposed DCs to produce very high levels of IL-10 upon exposure to LPS compared to LPS alone ($P = 0.008$ and $P = 0.07$, respectively). In contrast, IL-12 secretion remained the same or was slightly reduced as IL-10 increased, but this reduction was not statistically significantly different.

DISCUSSION

This study has demonstrated that pneumococcal capsular polysaccharides interact directly with human DCs. We have shown that they are internalized and traffic to late endosomal compartments and that uptake is likely to involve both macropinocytosis and receptor-mediated endocytosis. While Zamze and colleagues (22) have recently shown that pneumococcal

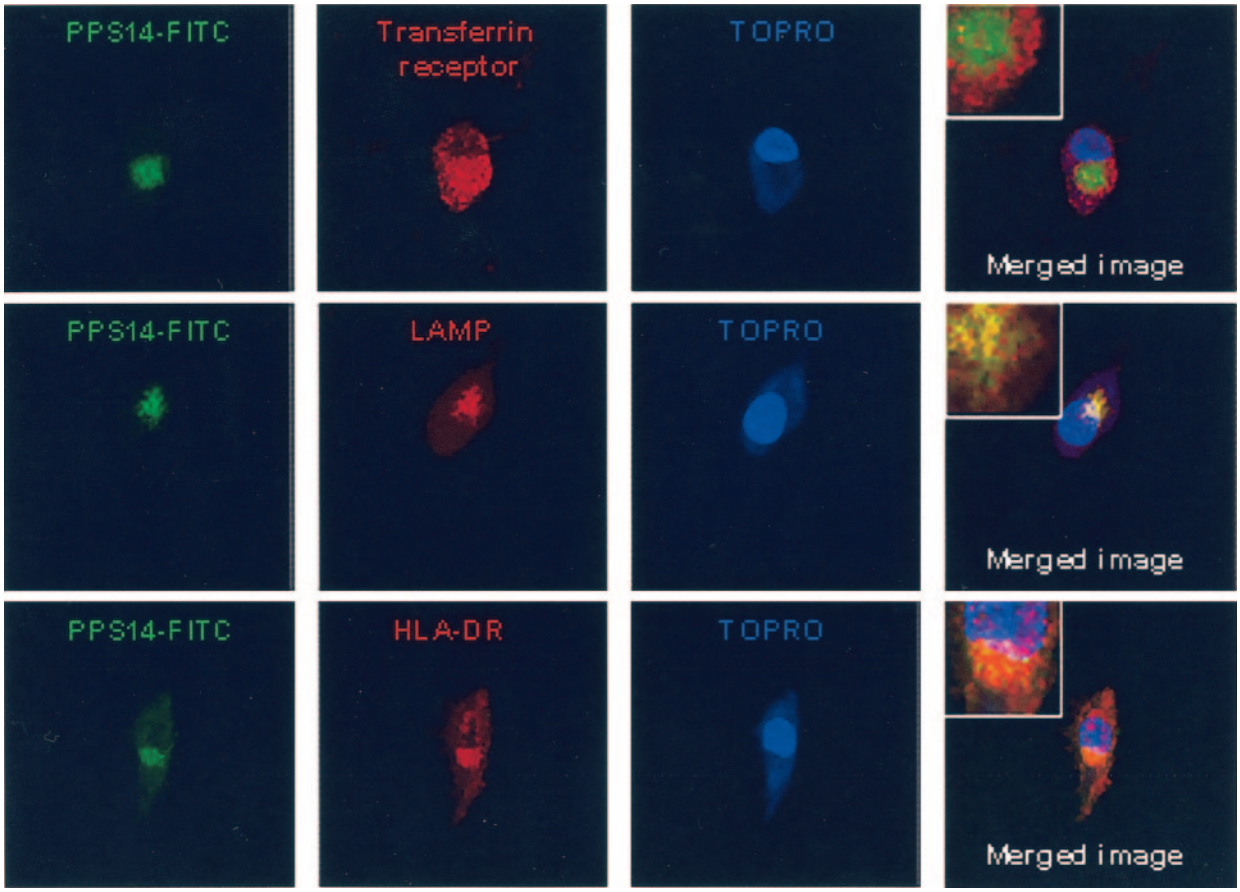

FIG. 3. Intracellular processing of pneumococcal polysaccharide type 14. Immature dendritic cells were incubated with 60 μg/ml FITC-labeled PPS14 (green) for 24 h, fixed, stained with specific markers for various endocytic organelles as indicated (Alexa 568, red) and a DNA label (TO-PRO, blue), and processed for confocal microscopy. Yellow staining in the merged images indicates colocalization of Alexa 568 and FITC. Insets in the merged images are close-ups of the regions of interest.

polysaccharides bind the macrophage mannose receptor (MR) in vitro, it is likely that they also bind some of the many other receptors with affinity for carbohydrate-containing ligands present on the DC surface, such as DC-SIGN or other C-type lectins. Despite uptake and trafficking, we have been unable to consistently elicit T-cell responses either by incubating human-derived adult PBMCs with purified polysaccharides and measuring cytokine production or by studying T-cell proliferation following incubation with polysaccharide-pulsed DCs using carboxyfluoroscein succinimidyl ester analysis (results not shown).

Uptake of pneumococcal polysaccharides does not in itself induce DC maturation or cytokine production. In this respect, PPS are similar to most other antigens, including many proteins, in that they do not induce DC maturation alone. Turley et al. (21) have shown that such antigens are stored in their native form inside DCs until the DCs receive a second signal that induces their maturation. Only then will processing and MHC loading commence. Interestingly, Colino and colleagues (4) have recently shown that intact heat-killed *S. pneumoniae* cells induce phenotypic maturation of DCs and the secretion of tumor necrosis factor alpha, IL-12, and IL-10 and that pulsed bone marrow-derived DCs were then able to support both antipneumococcal protein and polysaccharide responses after transfer to naïve mice. The difference between the effect of intact heat-killed pneumococci on murine DCs in Colino's studies and the results of our studies in human DCs and purified capsular polysaccharides is likely to be due to the choice of antigen, as heat-killed bacteria are also a stimulus for the maturation of human DCs on the basis of results of studies in our laboratory (results not shown). This is likely to be mediated by noncapsular components, and heat-killed bacteria are likely to contain many surface-exposed molecules that are stimulatory for DCs.

Exposure of DCs to pneumococcal polysaccharides in the presence of a second signal such as LPS dramatically altered the response of DCs in our studies. Activation of monocyte-derived DCs with LPS normally induces strong upregulation of MHC class II and costimulatory and adhesion molecules as well as the release of a variety of pro- and anti-inflammatory cytokines, among them IL-12 and IL-10. Preincubation with pneumococcal polysaccharides still allows phenotypic maturation of DCs but predisposes them to release very high levels of IL-10 upon stimulation with LPS. This cytokine has potent immunosuppressive effects on a large number of components of the immune system, among them negative feedback regulation of the DCs themselves, as well as an important role in the induction of adaptive regulatory T cells, which can suppress

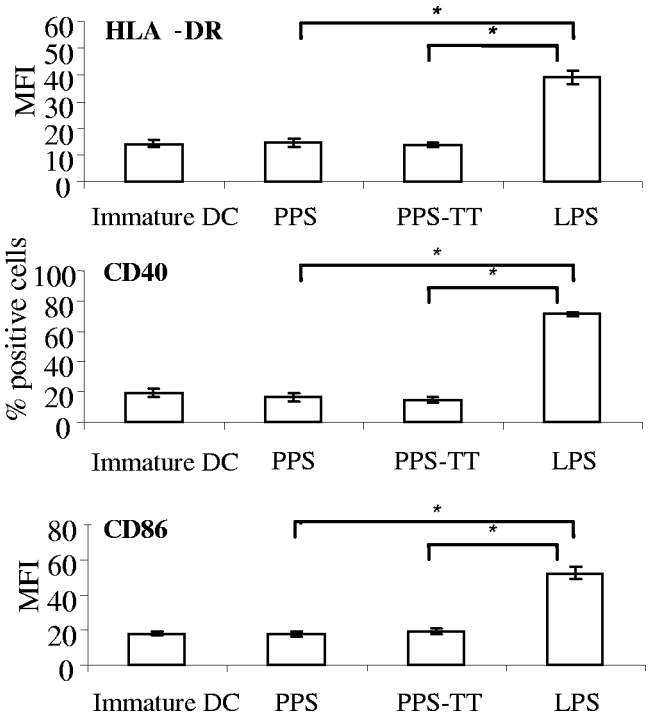

FIG. 4. Effect of PPS/PPS-TT on DC phenotype. Immature DCs were incubated overnight with PPS or PPS-TT (types 1, 6B, 9N, 14, 19F, or 23F) or LPS, and expression of HLA-DR, CD40, and CD86 was analyzed by flow cytometry. As there were no differences between the various serotypes in surface marker expression, data were pooled and represent the means of six different serotypes and of five independent experiments. *, $P < 0.001$ using Student's paired t test. MFI, mean fluorescence intensity.

effector cells in an antigen-specific manner. Thus, secretion of high levels of IL-10 by DCs after PPS/LPS stimulation may have profound effects on DC-induced T-cell differentiation. In a recent paper by Sen and colleagues (18), purified pneumococcal polysaccharides were shown to contain impurities that bind TLR2 ligands, and in other model systems these may be critical costimulators. In our studies, PPS alone did not stimulate IL-10, although TLR2 ligands may have a role in the subsequent secretion of IL-10 following LPS stimulation.

Recently, similar imbalances between IL-10 and IL-12 production by DCs have been described in the context of mycobacterial infection. Macrophages infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis, release large amounts of the mycobacterial cell wall component lipoarabinomannan (LAM). LAM binds its main in vivo receptor DC-SIGN with high affinity (7) but is also recognized by other receptors such as the MR, CD11b, and CD11c (14). Binding of LAM to DC-SIGN and MR delivers a negative signal to DCs, resulting in the release of high levels of IL-10 and reduced levels of IL-12, respectively, in response to LPS (7, 17). LAM is structurally very similar to naturally occurring pneumococcal polysaccharides. CD11b is part of the CR3 receptor (CD11b/CD18, also called Mac-1). CR3 is another receptor which is targeted by a microbial molecule, in this case *Bordetella pertussis*-derived filamentous hemagglutinin, to induce high levels of IL-10 production and inhibit IL-12 production in DCs in response to LPS (16). Interestingly, Colino and Snapper (5) have suggested that IL-10 has a critical role in optimizing the DC induction of in vivo humoral responses to *S. pneumoniae.* In their studies of murine APC function, they were able to show the critical role of IL-10 in supporting both antiprotein and antipolysaccharide pneumococcal responses following pulsing of DCs by whole, heat-killed bacteria. They hypothesize that IL-10 has a critical role in limiting the time frame during which DCs can respond to continued exposure to bacteria by delaying the onset of apoptosis.

High levels of IL-10 are also released by DCs in the respiratory tract after exposure to an inhaled, innocuous antigen,

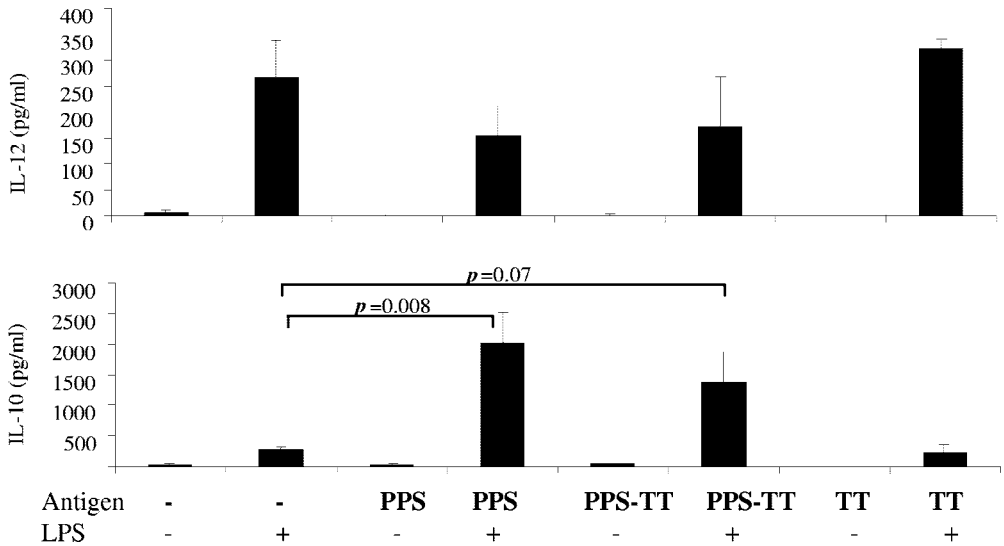

FIG. 5. Effect of PPS/PPS-TT/TT on LPS-induced DC cytokine production. Immature DCs were incubated for 24 h with PPS, PPS-TT, or TT in the absence or presence of 0.5 μg/ml LPS. IL-12 and IL-10 levels in the culture supernatants were measured by standard enzyme-linked immunosorbent assay. Two different serotypes were used (14 and 19F). As there were no differences between the various serotypes in cytokine production, data were pooled and represent the means of two different serotypes and nine independent experiments.

such as ovalbumin. Exposure results in the rapid migration of pulmonary DCs to the draining lymph node. DCs mature during transit but produce high levels of IL-10 rather than IL-12, resulting in the induction of adaptive regulatory cells which will delete or anergize antigen-specific T cells (1). Such DCs are omnipotent cells that have the capacity to induce effector type 1 and 2 responses, but they are predisposed to production of IL-10 by exposure to suppressive factors present in the local microenvironment of the respiratory tract. In our studies, incubation of DCs with polysaccharides and LPS also results in a mature, IL-10-producing DC population, a phenotype associated with priming of adaptive regulatory cells. Exposure to a vaccine containing purified polysaccharide of meningococcal serogroup C results in subsequent hyporesponsiveness to a second dose (8). Recently, Torling and colleagues have shown a similar effect for the pneumococcal polysaccharide vaccine administered to the elderly (20). The mechanism explaining this hyporesponsiveness remains elusive. While it is possible that large doses of polysaccharides may cause apoptosis of antigen-specific B cells, thus reducing the number of B cells available to respond in a subsequent challenge, it is unclear why the B-cell pool should not be replenished, particularly as the phenomenon with meningococcal serogroup C has been described in young individuals and, furthermore, can be overcome by the use of a conjugate vaccine (3). Induction of anergy or immunoregulation by polysaccharides in the way described in these studies may help to explain the hyporesponsiveness observed after repeated vaccination with pure polysaccharides, although the immunomodulating effect of preexisting polysaccharide-specific IgM (binding via inhibitory Fc receptors) may also be a factor.

Our studies illustrate that the polysaccharide capsule of the pneumococcus is not only a bacterial virulence factor and a target for protective antibodies but may also subtly influence immune responses to it via interaction with dendritic cells in a unique way not previously described for encapsulated bacteria.

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