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Pneumococcal Interaction with Human Dendritic Cells: Phagocytosis, Survival, and Induced Adaptive Immune Response Are Manipulated by PavA

Nadja Noske,*†‡ Ulrike Kämmerer,§ Manfred Rohde,¶ and Sven Hammerschmidt2*†‡

Dendritic cells (DCs) ingest and process bacteria for presenting their Ags to T cells. PavA (pneumococcal adherence and virulence factor A) is a key virulence determinant of pneumococci under in vivo conditions and was shown to modulate adherence of pneumococci to a variety of nonprofessional phagocytic host cells. Here, we demonstrated the role of PavA for the interaction of human DCs with live pneumococci and analyzed the induced host cell responses upon ingestion of viable pneumococci. Expression of PavA protected pneumococci against recognition and actin cytoskeleton-dependent phagocytosis by DCs compared with isogenic pavA mutants. A major proportion of internalized pneumococci were found in membrane-bound phagosomes. Pneumococcal phagocytosis promotes maturation of DCs, and both wild-type pneumococci and PavA-deficient pneumococci triggered production of proinflammatory cytokines such as IL-1β, IL-6, IL-8, IL-12, and TNF-α and antiinflammatory IL-10. However, cytokine production was delayed and reduced when DCs encounter pneumococci lacking PavA, which also results in a less efficient activation of the adaptive immune response. Strikingly, purified PavA reassociates to pneumococci but not DCs and reduced phagocytosis of the pavA mutant to levels similar to those of wild-type pneumococci. Additionally, pavA mutants covered with exogenously provided PavA protein induced a DC cytokine profile similar to wild-type pneumococci. In conclusion, these results suggest that PavA is key factor for live pneumococci to escape phagocytosis and to induce optimal cytokine productions by DCs and adaptive immune responses as well. The Journal of Immunology, 2009, 183: 1952–1963.

Streptococcus pneumoniae (pneumococci) are commensals of the human respiratory tract and colonize up to 70% of the individuals without causing clinical symptoms. However, these apparently harmless colonizers are also well known as serious human pathogens that transmigrate into the lungs, enter the bloodstream, and cross the blood-brain barrier (1). As a consequence, this versatile pathogen causes infections ranging from severe local infections, including pneumonia, sepsis, and meningitis (2). In healthy individuals, the mucosal surfaces with their epithelial cells and the secreted mucus constitute a physical barrier that prevents pathogens to gain access into deeper tissues. Here, the pathogens are also faced by the production of antimicrobial agents, such as defensins (3, 4). Additionally, mucosal tissues are scattered with sentinel professional phagocytes and APCs including dendritic cells (DCs).5 DCs are involved in the coordination of immune defenses upon stimulation in response to microbial signals (5). Immature DCs efficiently phagocytose or macrophagocytose bacteria and process them into cell surface-presentable Ags. During this process, the immature DCs mature and convert into potent APCs. Maturation of DCs is characterized by changes in surface expression of MHC, adhesion and costimulatory molecules, and cytokine production as well (6). Upon maturation, DCs migrate from the place of Ag uptake into tissue-draining lymphoid organs such as lymph nodes or spleen. Maturation of DCs diminishes their capacity to internalize and process Ags but greatly enhances their ability to prime naïve T cells. As a consequence, DC responses upon bacterial infections initiate adaptive immune responses (7). However, pathogens have evolved various strategies to escape host immune responses. Regarding DCs, their subversion by pathogens and exploitation as a Trojan horse to disseminate within the host—as recently shown for HIV and Chlamydia—is under debate (8–10). Pneumococci are encased by a capsular polysaccharide (CPS), and the CPS is recognized as a sine qua non for invasive diseases (11). The capsule protects pneumococci against uptake into professional phagocytes and complement-mediated opsonophagocytosis (12, 13). Another potent virulence factor of pneumococci interfering with both eukaryotic cell function and the immune system is the pore-forming cytolsin pneumolysin (14, 15). TLR4 recognizes pneumolysin as a pathogen-associated molecular pattern, thereby providing protection against pneumococcal infections (16). Moreover, pneumolysin production enhances mucosal clearance of pneumococci by stimulating neutrophil recruitment and

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promotes bacterial Ag delivery to the nasal-associated lymphoid tissue (17, 18). The cell wall of pneumococci is decorated with numerous virulence factors that execute their function in different host niches (19, 20). Remarkably, atypical surface proteins, lacking a leader peptide or even a motif for membrane anchoring such as the PavA protein (pneumococcal adherence and virulence factor A), have been found to be key virulence factors of *S. pneumoniae*. Deficiency in PavA impairs adherence of pneumococci to host epithelial and endothelial cells and attenuates virulence of pneumococci in mouse infection models (21, 22). Although pneumococci lacking PavA produce a capsule and their cytotoxin pneumolysin, these mutants were massively attenuated in a mouse sepsis model and, additionally, intracranial infections of mice resulted in rapid clearance from the CNS (21, 22). It is suggested that PavA function is not directly involved in adhesion or virulence, but is rather modulating other, yet unidentified, important virulence determinants of *S. pneumoniae* (22).

In naive mice immature bone marrow-derived myeloid DCs (BMDCs) pulsed with heat-killed pneumococci stimulated humoral responses specific for the surface proteins PsPsa and PsA, and the surface-exposed polysaccharides phosphorylcholine and CPS as well (23). Pneumolysin induces a rapid and caspase-independent apoptosis, while in a delayed onset and associated with maturation of DCs, a caspase-dependent apoptosis is induced that does not require uptake of bacteria (24).

However, the mechanisms of recognition, ingestion, and intracellular fate of live pneumococci by DCs and the contribution of specific bacterial components of pneumococci on induced adaptive immune responses have not been studied in great detail. In the present study we demonstrate the key role of PavA for internalization and processing of viable pneumococci by immature human blood-derived DCs. Additionally, we show that the presence of pneumococcal PavA is required to stimulate optimal cytokine production by DCs and adaptive immune responses.

**Materials and Methods**

**Bacterial strains, culture conditions, and protein purification**

*S. pneumoniae* strains ATCC 11733 (serotype 2), R800 (nonencapsulated), D39 (serotype 2), D39Δply, NCTC 10319 (serotype 35A), and the pneumolysin (ply) as well as the PavA-deficient mutants D39ΔpavlA, S.p. 35AΔply, S.p. 35AΔpavA, and S.p. 35AΔplyΔpavlA, respectively, were reported recently (22, 25, 26). Bacteria were grown to mid-log phase (OD600 0.3) in Todd-Hewitt broth (Oxoid) supplemented with 0.5% yeast extract (THY) and antibiotics when appropriate, or cultured on blood agar. Full-length pavA (derived from R800) was subcloned from clone UB1155 (21) by digestion with BamHI and PstI into the similarly digested expression vector pMALc2x (New England Biolabs). Maltose-binding protein (MBP) and MBP-PavA were produced in Escherichia coli JM109 and purified according to the manufacturer’s guidelines by affinity chromatography using an amylose resin (New England Biolabs) followed by anion exchange column (Hitrap Q HP) purification.

**Isolation of human DCs from peripheral blood cells and maturation of DCs**

Human monocyte-derived DCs were isolated and cultured from PBMCs by a standard protocol (27). Monocytes were either extracted from human peripheral blood, from buffy coat (PBMC) suspensions (German Red Cross, Wiesendall, Germany), or from an highly enriched monocyte concentrate that was obtained after employing a combination of leukapheresis of blood cells and centrifugal elutriation as previously described in detail (28). DC precursors were cultured for 5–8 days in RPMI 1640 medium (PAAB Laboratories) supplemented with 10% FBS, 2 mM glutamine and penicillin/streptomycin (100 IU/ml and 100 μg/ml; PAAB Laboratories), 20 IU/ml purified recombinant human GM-CSF, and 16 IU/ml IL-4 (Stratman Biotech). Cytokines and medium were replaced every second day. Purity of immature DCs was >90% as indicated by FACS analyses. Primary Abs used for DCs were: human CD11c (IgG1), CD25 (IgG1), CD83 (IgG2), CD86 (IgG2), or HLA-DR (IgG1) for MHC-class II. Non specific binding of Abs was calculated by incubating DCs with the appropriate isotype anti-IgG2 or anti-IgG1 (Caltag Laboratories, BD Pharmingen, or Miltenyi Biotec). The Abs were conjugated with PE or FITC. Surface marker expression was quantified using a FACSCalibur (BD Biosciences).

**Infection of human DCs**

For our antibiotic protection assays DCs were seeded into 96-well cell culture plates (Greiner Bio-One) at a density of 1 × 10⁵ cells/well. To perform immunofluorescence staining or electron microscopy, DCs were seeded on glass coverslips (diameter, 12 mm) in 24-well cell culture plates (Greiner Bio-One). For flow cytometric analysis and transmission electron microscopy the cells were used in 6-well cell culture plates (Greiner Bio-One) at a density of 5 × 10⁶ cells/ml. The attachment of DCs to the surface of the dishes occurred within 1 h at 37°C under 5% CO₂. The prepared DCs were infected with a multiplicity of infection (MOI) of 50 pneumococci per permissive cell in RPMI 1640 supplemented with 1% penicillin G and 100 μg of gentamicin for 1 h at 37°C under 5% CO₂. Intraacellular viable bacteria were recovered after washing with PBS by a saponin-mediated lysis (1% (w/v)) for 10 min at 37°C. The amount of released pneumococci per well was enumerated by plating serial dilutions on blood agar plates. For immune fluorescence microscopy pneumococci-infected DCs were fixed with 3.7% paraformaldehyde. To distinguish between adherent (extracellular) and intracellular (phagocytosed) pneumococci the bacteria were labeled with Alexa Fluor 488 and/or Alexa Fluor 568 (Invitrogen) as previously reported in detail (26, 30). Image acquisition of representative experiments was performed with confocal laser scanning microscopy (CLSM; Leica TCS SP5) and the CLSM software.

**Electron microscopy**

Preparations of samples for field emission electron microscopy (FESEM) and transmission electron microscopy (TEM) were performed identical to the procedures described recently (31).

**Real-time RT-PCR**

RNA of DCs was purified with the RNAeasy purification kit (QIAGEN). Equal amounts of purified RNA (calculated using the NanoDrop ND-1000, the SuperScript III reverse transcriptase (Invitrogen), and oligo(dT) primers (Invitrogen) were used to synthesize cDNA. PCR reactions were done with IL-specific oligonucleotides and hsp60 primers (Table I) and real-time PCR was performed on an ABI PRISM 7000 (Applied Biosystems) using SYBR Green quantitative PCR (Roche Diagnostics) according to the manufacturers’ instructions.

**Cytokine assays**

Cytokine concentrations in the DC cell culture supernatants were measured with the Lumienx technology (BioSource Europe) according to the manufacturer’s instructions. A human inflammatory five-plex kit (GM-CSF, TNF-α, IL-6, IL-8, and IL-1β) and beads for IL-12 (IL-12p40 and IL-12p70), IL-10, and IFN-γ were used for quantification (BioSource Europe), and 50 μl of a 1/10 dilution was used in the assays. Evaluation of the data was performed with the MasterPlexGT software (MiralBio).

**T cell activation assay of oxidative mitogenesis variant (OxMt) of the classical MLR**

To obtain strong signals in a nonradioactive detection assay, allogeneic T cells used as reporter cells in the mixed leukocyte reaction were prepared following the method of Hill et al. (32). T cells were isolated from PBMCs by using sheep RBCs and washed twice with ice-cold PBS. T cells (1 × 10⁷) were then resuspended in 975 μl of ice-cold PBS, carefully mixed with 25 μl of filtered sodium periodate (5 μg/ml; Sigma-Aldrich; dissolved in ice-cold PBS), and incubated on ice for 20 min. After washing...
To elucidate the interference of the amount of surface-expressed virulence factor PavA on immunomodulation after phagocytosis by DCs negatively correlates with the expression capsular polysaccharide on phagocytosis by DCs, we studied the phagocytosis of various pneumococcal strains. A recent study indicated that pneumococcal strains NCTC 10319 and ATCC 11733 produce lower capsular poly-saccharide than pneumococcal strains ATCC 11733 (serotype 2), D39 (serotype 2), and nonencapsulated R800 (25). Immature DCs (iDCs) were infected for 30 min with the pneumococcal strain NCTC 10319 (referred to as *S. pneumoniae* type 35A) to analyze the impact of the amount of surface-expressed PavA protein binding assays

Binding of FITC-labeled pneumococci to immobilized purified MBP or MBP-PavA, which were immobilized in wells of a 96-well microtiter plate (polystyrene surface), was measured as described previously (26). Binding of soluble MBP or MBP-PavA to pneumococci or DCs was analyzed by flow cytometry using the following Abs: polyclonal anti-MBP antiserum (polystyrene surface), was measured as described previously (26). Binding of FITC-labeled pneumococci to immobilized purified MBP or MBP-PavA to pneumococci or DCs was analyzed by flow cytometry using the following Abs: polyclonal anti-MBP antiserum (polystyrene surface), was measured as described previously (26). Binding of FITC-labeled pneumococci to immobilized purified MBP or MBP-PavA (22) (1/200), and secondary anti-rabbit FITC conjugate (MoBiTec).

**Statistical analysis**

All data are reported as mean ± SD unless otherwise noted. Results were statistically analyzed using the paired two-tailed Student’s test, and a value of *p* < 0.05 was accepted as indicating significance.

**Results**

**Impact of the capsular polysaccharide on phagocytosis of pneumococci by human DCs**

To elucidate the interference of the amount of surface-expressed capsule on phagocytosis by DCs, we studied the phagocytosis of various pneumococcal strains. A recent study indicated that pneumococcal strains NCTC 10319 and ATCC 11733 produce lower amounts of CPS compared with the mouse virulent strain D39 (25). Immature DCs (iDCs) were infected for 30 min with the encapsulated pneumococcal strains ATCC 11733 (serotype 2), NCTC 10319 (serotype 35A), D39 (serotype 2), its nonencapsulated mutant D39ΔpavA, and with the nonencapsulated strain R800. The results of the antibiotic protection assay and double immunofluorescence microscopy demonstrated that pneumococcal uptake by DCs negatively correlates with the expression capsular polysaccharide (Fig. 1). Impaired phagocytosis was observed for D39, while internalization of nonencapsulated R800 and strains NCTC 10319 and ATCC 11733 was highly efficient (Fig. 1). Phagocytosis of the nonencapsulated mutant D39ΔpavA was significantly enhanced compared with its isogenic wild-type D39 (Fig. 1). Due to a high phagocytosis rate, we selected the pneumococcal strain NCTC 10319 (referred to as *S. pneumoniae* type 35A) to analyze the impact of the virulence factor PavA on immunomodulation after phagocytosis by DCs. This pneumococcal strain is further characterized for its interaction with nonprofessional host cells and produces all known pneumococcal virulence factors (22, 29, 33).

**PavA impedes phagocytosis of pneumococci by immature DCs**

To investigate the effect of PavA on phagocytosis of live pneumococci by human DCs, infections were conducted with wild-type *S.p.* 35A, isogenic *pavA* mutant *S.p.* 35AΔpavA, pneumolysin-deficient mutant *S.p.* 35AΔply, or the double mutant *S.p.* 35AΔplyΔpavA (21, 22). First, pneumococcal uptake by DCs was scored over time by double immunofluorescence microscopy (Fig. 2, A and B). In general, the number of intracellular bacteria was clearly dependent on the period of infection and the MOI. After 30 min a maximum of intracellular bacteria was reached and extended...
infections resulted in similar (45 min) or lower numbers (>60 min; data not shown) of intracellular bacteria (Fig. 2A). When using a MOI of 50 bacteria per DC, which was explored for further analysis, the number of bacteria present in each DC varied between 4 and 9 after 30 min for the wild-type and ply mutant (data not shown). Enumeration of intracellular bacteria by immunofluorescence microscopy showed no significant differences between wild-type and pneumolysin-deficient pneumococci when DCs were infected for 15, 30, or 45 min (Fig. 2A). In contrast, 30 and 45 min after infection the number of intracellular PavA- and pneumolysin-deficient pneumococci was significantly higher compared with wild-type S. p. 35A and S. p. 35AΔply (Fig. 2A and B). These results were confirmed when applying the antibiotic protection assay after infecting the DCs with a MOI of 50 bacteria per cell (Fig. 2, C and D). After 30 min of DC infection the rates of internalized and recovered pneumococci were at least 2-fold higher for the pavA mutant S. p. 35AΔpava or S. p. 35AΔplyΔpava compared with the isogenic wild-type or ply mutant S. p. 35AΔply (Fig. 2, C and D). Similar to the immunofluorescence microscopic analysis, the antibiotic protection assay showed no significant differences between wild-type and pneumolysin-deficient bacteria (Fig. 2C). Strikingly, the enhanced ingestion of PavA-deficient pneumococci was also observed for the serotype 2 strain ATCC 11733 and the highly encapsulated and mouse virulent pneumococcal strain D39 (Fig. 2D). In conclusion, these data suggest that the expression of PavA impedes uptake of S. pneumoniae by human DCs. Additionally,
these data revealed that pneumococcal uptake by DCs is not influenced by pneumolysin. To avoid pneumolysin-induced cytolytic or cytotoxic effects, pneumococci devoid of pneumolysin (Δply) were used for long-term infection experiments.

The intracellular fate of S. pneumoniae in DCs is not influenced by PavA expression

To assess survival of internalized pneumococci postinfection and the role of PavA in this scenario, the intracellular fate of S.p. 35AΔply and its isogenic pavA mutant were compared. DCs were infected with pneumococci and 30 min postinfection extracellular pneumococci were killed by antibiotic treatment and eliminated. After removing the antibiotics the viability of intracellular pneumococci was investigated for the indicated time points (up to 6 h postinfection) by plating the intracellular bacteria on blood agar plates. Similar to our other experiments, enumeration of CFU on blood agar plates revealed significantly higher numbers of intracellular viable bacteria for the pavA mutant at early times points compared with S.p. 35AΔply (Fig. 2, C and E). Herein, we were especially interested in the ability of pneumococci to survive within the DC. The results revealed killing of both strains by DCs. Apparently, the time-dependent decrease in recovered bacteria was not significantly different when comparing PavA-deficient pneumococci with the PavA-expressing bacteria (Fig. 2E). The major proportion of phagocytosed bacteria was processed 3 h postinfection by DCs. In conclusion, these data suggest that PavA expression is important for pneumococci to diminish significantly phagocytosis by DCs but cannot significantly improve the intracellular fate of pneumococci within phagosomes of DCs.

**Pneumococcal uptake in membrane-bound phagosomes of DCs relies on actin cytoskeleton dynamics**

The influence of the actin cytoskeleton dynamics on pneumococcal phagocytosis by DCs was investigated in the presence of the pharmacological inhibitor cytochalasin D, which inhibits actin polymerization. In the presence of cytochalasin D, phagocytosis of pneumococci was diminished as determined by enumeration of recovered intracellular pneumococci (Fig. 3A). Additionally, illustrations by CLSM indicated that adherence was not affected by cytochalasin D treatment of DCs, while this treatment resulted in loss of intracellular pneumococci as indicated by CLSM (Fig. 3B). The uptake and localization of pneumococci was further analyzed by FESEM and TEM (Fig. 3C). Illustrations by FESEM showed the formation of membranous structures that engulf the bacteria during uptake, indicative for the essential role of the host cell actin cytoskeleton dynamics during pneumococcal uptake by DCs (Fig. 3C). Intracellular localization studies by TEM 30 min postinfection of DCs revealed that the major proportion of pneumococci is located in membrane-bound phagosomes (Fig. 3C). Strikingly, both pneumolysin-deficient strains S.p. 35AΔply and S.p. 35AΔplyΔpavA were also localized in the cytoplasm, and electron micrographs suggest that pneumococci exit membrane-bound phagosomes independently from pneumolysin production (Fig. 3C). CLSM and the use of polyclonal Abs against

**FIGURE 3.** Phagocytosis of pneumococci by human DCs and intracellular fate of bacteria as determined by electron microscopy and immunofluorescence microscopy. A, DCs were pretreated with cytochalasin D (0.05 mmol), and the number of intracellular pneumococci were determined by the antibiotic protection assay. *p < 0.05. B, Immunofluorescence microscopy of adherent (yellow) and intracellular (red) pneumococci after infection of cytochalasin D pretreated (CytD) or untreated DCs (none). C, Electron microscopic visualization of pneumococcal adherence to and invasion into DCs and intracellular trafficking inside DCs. a and b, Wild-type S.p. 35A (NCTC 10319) and S.p. 35AΔply trigger cytoskeletal rearrangements resulting in uptake by membrane ruffles; c and d, ultrathin sections depict S.p.35AΔply and its isogenic pavA-mutant S.p. 35AΔplyΔpavA (d) inside membrane-bound compartments (indicated by arrows), most probably inside phagosomes, after 30 min of infection. As has been demonstrated by confocal microscopy, ultrathin sections also reveal that the isogenic pavA mutant is more invasive compared with S.p. 35AΔply. e and f and inset of f, After longer infection times both strains are able to exit the membrane-bound compartments (phagosome, phagolysosome) to reside free inside the cytoplasm of DCs. Bars represent 1 μm.
Lamp1 (lysosomal-associated membrane protein 1), a membrane marker protein of late endosomes and phagolysosomes, confirmed that intracellular pneumococci were mainly located in phagolysosomes (Fig. 3D).

**Pneumococci induce maturation of DCs independently of PavA expression**

To investigate the host cell response after pneumococcal infection, we assessed the expression of surface marker proteins on DCs. Immature human DCs, positive for CD11c, were infected for 30 min with live pneumococci (S.p. 35AΔply). The changes of maturation-associated surface marker expression of CD11c+ DCs infected with S.p. 35AΔply or S.p. 35AΔplyΔpavA was measured by flow cytometry. The total fluorescence values were monitored after gating the main population of events, subtracting the corresponding isotype background of the used Ab used to detect the surface marker, and after selecting a threshold for the control of untreated human DCs. For each flow cytometric analysis, values were recorded for 10,000 events and the means ± SD of at least three independent experiments are shown. To demonstrate the expression levels of the markers, the total fluorescence quantity value (geometric mean fluorescence intensity multiplied with the percentage of positive events) was used in A and B, respectively. The representative histograms in C show the log fluorescence intensity on the x-axis, and the y-axis shows the numbers of events after 18 h of stimulation. A, DCs were infected for 30 min with pneumococci (S.p. 35AΔply). After killing the extracellular bacteria by antibiotic treatment, the stimulation was continued for the indicated time points (2, 8, and 18 h). B and C, Maturation profiles of DCs infected for 30 min with S.p. 35AΔply or its pavA mutant S.p. 35AΔplyΔpavA. Bacteria were killed by antibiotic treatment, and analysis of surface marker expression was performed after 18 h by flow cytometry.

A

![Graph A](https://via.placeholder.com/150)

**FIGURE 4.** Profiles of surface marker expression by DCs stimulated with pneumococci. The profile of surface marker expression of HLA-DR (MHC class II), CD25, CD83, and CD86 indicative of maturation of CD11c+ DCs infected with S.p. 35AΔply or S.p. 35AΔplyΔpavA was measured by flow cytometry. The total fluorescence values were monitored after gating the main population of events, subtracting the corresponding isotype background of the used Ab used to detect the surface marker, and after selecting a threshold for the control of untreated human DCs. For each flow cytometric analysis, values were recorded for 10,000 events and the means ± SD of at least three independent experiments are shown. To demonstrate the expression levels of the markers, the total fluorescence quantity value (geometric mean fluorescence intensity multiplied with the percentage of positive events) was used in A and B, respectively. The representative histograms in C show the log fluorescence intensity on the x-axis, and the y-axis shows the numbers of events after 18 h of stimulation. A, DCs were infected for 30 min with pneumococci (S.p. 35AΔply). After killing the extracellular bacteria by antibiotic treatment, the stimulation was continued for the indicated time points (2, 8, and 18 h). B and C, Maturation profiles of DCs infected for 30 min with S.p. 35AΔply or its pavA mutant S.p. 35AΔplyΔpavA. Bacteria were killed by antibiotic treatment, and analysis of surface marker expression was performed after 18 h by flow cytometry.
significantly up-regulated in a time-dependent manner (Fig. 4A). The up-regulation of the MHC class II complex (HLA-DR) was prominent, as indicated by the highest total fluorescence intensity, followed by CD86 and CD25, and CD83 (Fig. 4A). To elucidate whether the sole association of pneumococci is sufficient for the up-regulation of DC surface markers, DC infections were conducted in the presence of cytochalasin D. The results revealed no up-regulation of DC-specific marker proteins, suggesting that internalization of pneumococci is a prerequisite for induction of DC maturation (data not shown). Remarkably, no significant differences in DC maturation were observed when surface marker expression of DCs infected with pavA mutant was compared after 18 h to infections with the PavA-producing strain S. p. 35AΔply (Fig. 4, B and C). In conclusion, uptake of pneumococci induced maturation of DC, and this process resulted in slightly higher expression of CD86 and MHC class II compared with control DCs pulsed with LPS (0.5 μg/ml) (Fig. 4C). These data show that maturation of DCs is induced by internalized pneumococci but does not rely on PavA expression.

PavA expression by pneumococci is required to induce a maximum host inflammatory response

The activation process of DCs involves, in addition to the up-regulation of surface markers, the expression and release of cytokines. These host cell immune responses are essential to stimulate and prime the adaptive immune system. To explore whether infections with pneumococci induce or modulate release of IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-γ, and TNF-α by DCs, the APCs were infected for 30 min with a MOI of 50 of live S. p. 35AΔply. Similar to our previous experiments, extracellular bacteria were killed by applying the antibiotics for 1 h and, in a first approach, cytokine mRNA expression was quantified after 18 h for IL-1β, IL-6, IL-8, IL-10, IL-12p35, IL-12p40, IFN-γ, and TNF-α by employing the real-time reverse-transcription PCR technique (qPCR). RPS9 served as the housekeeping gene. The qPCR revealed that phagocytosis of S. pneumoniae induced higher levels of immune regulatory cytokine mRNA expression in DCs compared with unstimulated DCs (Fig. 5A). Additionally, the levels of mRNA expression were significantly reduced for DCs infected with PavA-deficient pneumococci compared with DCs infected with PavA-positive S. p. 35AΔply bacteria (Fig. 5B).

The up-regulation of the cytokine gene products was further analyzed using the Luminex technology (BioSource Europe). The amounts of cytokines in cell culture supernatants of infected DCs were measured at indicated time points postinfection (Fig. 6A). The supernatant of LPS-pulsed DCs (48 h) was used as control (data not shown). The concentrations of the immune regulatory proteins increased in a time-dependent manner, and after 18 and 48 h postinfection, we measured also the release of the antiinflammatory IL-10 (Fig. 6A). Highest effects on cytokines/chemokines secreted by DCs pulsed with viable pneumococci were shown for IL-8, followed IL-6, IL-12, TNF-α, IL-1β, and IL-10. No production or release of IFN-γ was measured, which confirms the purity of the population of DCs used (34). Compared with the supernatant of LPS-pulsed DCs, the relative amounts of the cytokines measured 18 h postinfection were higher in S. p. 35AΔply-infected DC cultures, with the only exception of IL-8 (data not shown). These data show a pneumococcal-induced proinflammatory host cell response, which is followed by an antiinflammatory response as indicated by IL-10 release. However, compared with the LPS-induced cytokine release, the proportion of IL-12 and IL-10 shifted to a reduced proinflammatory overbalance for both pneumococcal strains (Fig. 6C). Similar to the PavA-positive S. p. 35AΔply strain, the corresponding pavA mutant induced release of IL-1β, IL-6, IL-8, IL-12, TNF-α, and, to a minor degree, IL-10. Again, IFN-γ release was not measured (data not shown). The deficiency of PavA in pneumococci resulted in a significantly decreased cytokine release compared with S. p. 35AΔply as indicated after 18 h postinfection of DCs (Fig. 6B).
Induction of T cell proliferation by DCs pulsed with pneumococci is influenced by PavA expression

Stimulated and Ag-presenting DCs activate proliferation of naive T cells, thereby linking the innate immune response with the adaptive immune response of the host. To assess the impact of the pneumococcal PavA protein on subsequent T cell proliferation, activation of T cells was measured after pulsing human DCs with viable \( S.p. \) \( 35A\Delta p l y \) or its isogenic \( p a v A \) mutant. Human DCs were infected with pneumococci and 30 min postinfection extracellular bacteria were killed. The infections were continued for 18 h, representing a time point with significant levels of DC-released cytokines. Proliferation of T cells stimulated with LPS-matured DCs, iDCs, or infected DCs was measured using a nonradioactive OxMi T cell proliferation test. iDCs and LPS-pulsed DCs (0.5 \( \mu g/ml \) \( E. coli \) LPS; Sigma-Aldrich) were used as controls. \( \ast, p < 0.05 \) for \( S.p. \) \( 35A\Delta p l y \) relative to its isogenic \( p a v A \) mutant (\( S.p. \) \( 35A\Delta p l y \Delta p a v A \)).

PavA protein reassociates to pneumococci and complements the defects of the \( p a v A \) mutant

Binding studies were performed to investigate whether recombinant PavA reassociates to the pneumococcal cell wall, as has been shown for the pneumococcal enolase, and/or binds to DCs. The PavA protein was produced as a MBP fusion protein (MBP-PavA) and purified under nondenaturing conditions. The functional activity of the recombinant PavA was confirmed by its ability to bind to immobilized fibronectin (data not shown). First, binding of FITC-labeled pneumococci was investigated for immobilized MBP-PavA. The results showed a dose-dependent binding of wild-type pneumococci (data not shown) and pneumococci deficient in PavA (Fig. 8A) to immobilized PavA protein. Specific binding of pneumococci to immobilized MBP was not detected (Fig. 8A). Additionally, we assessed binding of soluble PavA protein (MBP-PavA) to viable pneumococci by flow cytometry. Strikingly, soluble PavA binds to viable pneumococci as shown for \( p a v A \) mutant \( S.p. \) \( 35A\Delta p l y \Delta p a v A \) and similarly to \( S.p. \) \( 35A\Delta p l y \) (Fig. 8B). Binding of MBP alone was not detected (Fig. 8B). These results suggest that PavA binds directly to the pneumococcal cell surface. Moreover, flow cytometric analysis shows that PavA is not recognized by iDCs (Fig. 8B). In infection experiments with immature human DCs we investigated whether exogenously added recombinant PavA protein has the capability to inhibit phagocytosis of the \( p a v A \) mutant, so that the level of internalized pneumococci resembles that of \( S.p. \) \( 35A\Delta p l y \). The \( p a v A \) mutant was preincubated with recombinant PavA protein and DCs were infected for 30 min. Remarkably, the internalization rates of pneumococci deficient for...
pavA gene expression but complemented with MBP-PavA protein were similar to S. pneumoniae and human DCs was determined by flow cytometry. Bound proteins were detected using anti-MBP or anti-PavA antiserum and anti-rabbit FITC conjugate. The dot plots show the percentage of positive events, and the log fluorescence intensity is shown on the x-axis. C. Number of intracellular surviving pneumococci in the absence or presence of bacterial-bound MBP-PavA protein. Pneumococci were pretreated for 30 min with the recombinant proteins (20 μg/10^8 bacteria). DCs seeded in wells of a 96-well cell culture plate were infected for 30 min with a MOI of 50 bacteria (5 × 10^5). The numbers of intracellular pneumococci were determined using the antibiotic protection assay and quantitative plating. Results represent the means ± SD of at least three independent experiments, *p < 0.005. D. Impact of bacterial PavA pretreatment on pneumococci-induced cytokine release by DCs. Human iDCs were infected for 30 min with S.p. 35AΔply or its isogenic pavA mutant with a MOI of 50. The pavA mutant was employed without further treatment or after preincubation with MBP and MBP-PavA. Cytokines were determined after 18 h of stimulation with the Luminex technology using a multiplex bead-based cytokine assay. The graphs show the results of representative experiments (done in triplicates) and the amount of cytokines that are mostly affected. Values (automatically processed by the software without showing the SD) are the means of three DC charges as calculated by the MasterPlexQT software of the Luminex instrumentation.

Discussion

Pneumococcal infections are relatively common, however, compared with high nasopharyngeal carrier rates in healthy adults and in children infections are less common than expected. Nevertheless, pneumococci are the most common bacteria associated with community-acquired pneumonia, which is often accompanied by bacteraemia (2). Binding to epithelial cells of host mucosal surfaces and immune evasion mechanisms are essential steps for pneumococci to initiate nasopharyngeal colonization and enable bacterial dissemination into submucosal host niches or the bloodstream. Encapsulation protects pathogens against phagocytosis by professional phagocytes and allows pneumococci to survive and multiply during nasopharyngeal colonization or in the bloodstream (35).
In this study we have for the first time characterized the interaction of viable pneumococci with human monocyte-derived DCs and the induced inflammatory response reliant on bacterial PavA expression. We have demonstrated that pneumococcal phagocytosis by DCs is independent of pneumolysin production but is significantly influenced by the capsular polysaccharide and PavA expression. Pneumolysin is a cell-modulatory toxin at sublytic concentrations and induces cytokine synthesis and CD4+ T cell activation (17). Interestingly, a pneumolysin variant lacking hemolytic and complement-activating activity showed enhanced virulence compared with pneumococci lacking pneumolysin. It has been suggested that pneumolysin is recognized by TLR4 and that the relatively rare occurrence of invasive disease after asymptomatic pneumococcal colonization is due to pneumolysin TLR4-induced robust inflammatory host cell responses (16). After exposure of >3 h to BMDCs, heat-killed pneumococci elicit apoptosis where the rapid caspase-induced apoptosis depends on pneumolysin expression (24). In our experiments DCs were pulsed at a maximum for 1 h with viable wild-type pneumococci or isogenic mutants. The stimulation and infection of DCs for up to 2 h with viable pneumococci did not induce necrotic or apoptotic death in the major proportion of DCs. However, to determine cytokine profiles or adaptive immune responses infections were continued for up to 18 h. Flow cytometric analysis with annexin V and propidium iodide showed that infections with viable and pneumolysin-expressing wild-type pneumococci induced significant necrosis, while infections with ply mutants did not (data not shown). Hence, the use of wild-type bacteria precludes the analysis of pneumococci-induced cytokines and T cell responses. Similar to in vitro studies with nonprofessional and professional phagocytes such as macrophages (25, 29), ingestion of encapsulated pneumococci by DCs was massively impaired. These results are in accordance with Neisseria meningitidis whose adherence to and uptake by DCs are significantly impaired for encapsulated bacteria (36). In contrast to strain D39, which is a high-encapsulated and mice-virulent strain, nonencapsulated pneumococci or serotypes producing lower amounts of CPS including serotype 35A (25) were efficiently phagocytosed by DCs. Interestingly, uptake experiments with FITC-labeled pneumococcal CPS from types 9N and 14 demonstrated phagocytosis of CPS material by immature human DCs into lysosomal compartments. No DC maturation or IL-10 or IL-12 production was induced by these CPS (37). A recent study demonstrated that the pneumococcal CPS of a serotype 1 activates the results of the OxMi confirm the finding that PavA is a relevant virulent factor for the induction of a proper immune answer. In agreement with earlier studies, these data support the idea that PavA is pivotal for the functional activity of other unidentified pneumococcal virulence determinants (22). These unknown virulence determinants contribute presumably to protection against phagocytosis by DCs and improve intracellular survival of the bacteria. Moreover, comparisons of the induced cytokine levels support the hypothesis that PavA modulates especially immunogenic bacterial proteins. Therefore, the unidentified proteins will represent promising vaccine candidates or target structures for novel therapeutic interventions. In Listeria monocytogenes the PavA homolog Fbp54 decreases the protein levels of the adhesin internalin B and toxin listeriolysin O and has been suggested to act as a chaperone (52). Listeriolysin O is a highly immunogenic virulence determinant of Listeria and mediates the escape of the bacteria from the vacuole into the cytosol (53). In pneumococci PavA does not affect levels of pneumolysin or other known virulence factors (21, 22). Here, we show for the first time that exogenously added recombinant PavA is able to complement the deficiency of PavA in PavA-deficient pneumococci. PavA has been identified on the
pneumococcal surface despite lacking a signal peptide and membrane anchor domain (22). Our binding assays indicated a reassociation of purified PavA to the pneumococcal cell surface while binding to DCs was not observed. Remarkably, the exogenously added and reassociated PavA protein prevents phagocytosis of pavA-deficient pneumococci, which are otherwise taken up in high numbers compared with PavA-expressing bacteria. Additionally, the immune response of DCs resembles that of DCs infected with PavA-positive pneumococci. It is noteworthy that purified PavA protein does not induce maturation of DCs. Again, these data demonstrate the important role of PavA for full virulence of pneumococci. Moreover, the lack of a functional PavA causes less severe immune responses in the host, and the virulence factors modulated by a functional PavA represent immunogenic factors that may have a high potential as vaccine candidates. As a consequence, the loss of function of PavA in S. pneumoniae improves survival of the host and results in less severe immune responses to pneumococcal infections.

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Disclosures
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References