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Human Plasmacytoid Dendritic Cells Phagocytose, Process, and Present Exogenous Particulate Antigen

Jurjen Tel,*† Annechien J. A. Lambeck,*† Luis J. Cruz,* Paul J. Tacken,* I. Jolanda M. de Vries,*‡ and Carl G. Figdor*

Plasmacytoid dendritic cells (pDCs) play a major role in shaping both innate and adaptive immune responses, mainly via their production of large amounts of type I IFNs. pDCs are considered to primarily present endogenous Ags and are thought not to participate in the uptake and presentation of Ags from the extracellular environment, in contrast to their myeloid counterparts, which efficiently endocytose extracellular particulates. In this study, we show that human pDCs are able to phagocytose and process particulate forms of Ag entrapped in poly(lactic-co-glycolic acid) microparticles. Furthermore, pDCs were also able to sense TLR ligands (TLR-Ls) incorporated in these particles, resulting in rapid pDC activation and high IFN-α secretion. Combining a tetanus toxoid peptide and TLR-Ls (CpG C and R848) in these microparticles resulted in efficient pDC activation and concomitant Ag-specific T cell stimulation. Moreover, particulate Ag was phagocytosed and presented more efficiently than soluble Ag, indicating that microparticles can be exploited to facilitate efficient delivery of antigenic cargo and immunostimulatory molecules to pDCs. Together, our results show that in addition to their potency to stimulate innate immunity, pDCs can polarize adaptive immune responses against exogenous particulate Ag. These results may have important consequences for the development of new immunotherapeutic strategies exploiting Ag and TLR-Ls encapsulated in microparticles to target APC subsets. The Journal of Immunology, 2010, 184: 4276–4283.

Dendritic cells (DCs) are considered key players in processing and presenting both endogenous and exogenous Ags, thereby triggering the immune system (1). Human plasmacytoid DCs (pDCs) constitute a rare subset of circulating blood DCs and are considered as immunomodulating cells capable of shaping the immune response (2). pDCs differ from myeloid DCs in their ability to produce large amounts of type I IFNs (IFN-α and IFN-β), thereby providing an immunostimulatory environment for both innate and adaptive immune cells. Type I IFNs are known to be important for antiviral immunity, but several studies show that they also play a role in bacterial infections and cancer immunity (3, 4). Although unstimulated pDCs promote an unbiased Th or a Th2 response, activated pDCs promote DC activation and trigger a Th1 response (5–7). Recently, it was shown that not only viruses, but also whole live bacteria can induce pDC activation, resulting in Th1 polarization of naive CD4+ T cells (8, 9). Furthermore, pDCs can interact synergistically with myeloid DCs by enhancing their priming ability in a contact-dependent manner, resulting in increased levels of Ag-specific CTLs (3, 10). Finally, activated pDCs abrogate cancer cell replication and induce tumor cell death (11).

Altogether, these findings indicate that pDCs can play a role in inducing immune responses against viruses as well as bacteria and cancerous cells. In addition, it implies that pDCs might be of use in immunotherapeutic regimens to evoke or stimulate Ag-specific immune responses (12).

Nonetheless, human pDCs are considered to poorly present exogenous Ags. Although pDCs are well capable of presenting endogenous Ags, they appear to be less efficient in presenting Ags captured from the extracellular environment (2). pDCs can take up and present soluble Ags from their environment, but this seems to require specific receptor-mediated endocytosis, as their macroinocytic capacity is poor (13, 14). We recently reported that pDCs are able to internalize soluble exogenous Ag bound to Ag-specific Abs via the FcγRIIa receptor, resulting in CD4+ T cell activation (15).

Apart from processing exogenous soluble Ag, it remains highly controversial whether human pDCs phagocytose and process nonopsonized exogenous particle-like structures independent from Fc receptors (2). Although some studies show that pDCs phagocytose apoptotic cells (16, 17), several report that pDCs hardly take up particulates, such as apoptotic cells (18), latex beads (17, 19), zymosan granules (14), or extracellular bacteria (3). Human pDCs have been shown to prime functional T cell responses on phagocytosis of apoptotic debris (16). However, Piccioli and coworkers (3) postulate that pDCs cannot phagocytose bacteria (Staphylococcus aureus and Escherichia coli) and require myeloid DCs to respond to bacterial stimuli. On the contrary, other studies show that pDCs can be activated by extracellular bacteria (S. aureus and Streptococcus pyogenes), but it was not established whether this was due to uptake of entire bacteria or mere bacterial components (8, 9). Furthermore, most vaccines, such as live attenuated or inactivated pathogen-based formulations, lipid emulsions, biocompatible and biodegradable nano- and microparticles, immunostimulatory complexes, virosomes, and virus-like particles, are particulate in nature and favor delivery of antigenic
cargo and/or immunostimulatory molecules to phagocytic APCs, such as myeloid and monocyte-derived DCs (moDCs) (20–23). Until now, it is not known whether pDCs can play a direct role in the immune response to such particulate vaccines. This prompted us to perform a study to elucidate whether pDCs play a direct role in taking up and processing Ags and sensing stimuli derived from exogenous particle-like structures, such as bacteria or particulate vaccines.

In this study, we exploit poly(lactic-co-glycolic acid) (PLGA) microparticle-encapsulated Ag to investigate whether pDCs are able to phagocytose and present particulate Ag. We not only demonstrate that human pDCs take up microparticles via phagocytosis and efficiently process the encapsulated Ag, but also that pDCs sense TLR ligands (TLR-Ls) incorporated within these particles, resulting in pDC maturation and IFN-α production. Furthermore, human pDCs induce Ag-specific T cell activation on phagocytosis of microparticles, containing both Ag and TLR-Ls.

Materials and Methods

Materials

PLGA (Resomer RG 502 H, lactide/glycolide molar ratio 48:52 to 52:48) was purchased from Boehringer (Ingelheim, Germany). Solvents for peptide synthesis and PLGA preparation (dichloromethane, 2-propanol, N,N’-dimethylformamide, and ethyl acetate) were obtained from Merck (Darmstadt, Germany). Polyvinyl alcohol (PVA) was purchased from Sigma-Aldrich (St. Louis, MO). The phospholipid 2-(4,4-difluoro-5-(4-

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phenyl-1, 3-butadieynyl)-4-bora 3a, 4a diaza-5 indacene-3 pentanoyle 1-hexadecanoyl-sn-glycero-3-phosphocholine, and DQ-BSA were procured at Molecular Probes (Leiden, The Netherlands). R848 and CpG C were purchased from Axorra (San Diego, CA). Cytochalasin D and chloroquine were purchased from Sigma-Aldrich.

Synthesis of FITC-tetanus toxoid

The tetanus toxoid (TT) epitope comprises the 830–844 region of tetanus toxoid. The TT peptide was synthesized as described previously according to standard protocols of solid-phase peptide synthesis, using the Fmoc/tert butyl strategy (24). The N-terminus of the TT peptide was modified with FITC through linking via a Lys-Lys cathepsin-like cleavage site as described previously (25).

Microparticle preparation

Several PLGA microparticle formulations containing FITC-TE-TT-peptide or DQ-BSA and TLR-Ls (R848 and CpG C) were prepared by double emulsion method (26). In brief, 50 mg PLGA in 1 ml ethyl acetate containing FITC-TE-TT-peptide (2 mg in 100 μl H2O) or DQ-BSA (2 mg in 100 μl H2O-DMSO) (R848 40.8 mg in 50 μl H2O-DMSO 9/1) and CpG C (0.1 mg in 50 μl H2O) were emulsified under sonification during 60 s. This first emulsion was rapidly added to 1 ml 1% PVA/7% ethyl acetate in distilled water and vortexed vigorously during 30 s. Next, the emulsion was dispersed in 1 ml 1% PVA/7% ethyl acetate in distilled water and vortexed 3 min and then stirred overnight to evaporate ethyl acetate. The microparticles were then washed three times with distilled water through centrifugation (3000 × g at 4°C) and supernatant replacement. Finally, the microparticles were lyophilized during 3 or 4 d. The prepared microparticles were completely spherical as characterized by SEM (Supplemental Fig. 1).

Dynamic light scattering and ζ-potential

Particle size and polydispersity of the microparticles were measured by dynamic light scattering (DLS). DLS measurements were performed on an ALV light-scattering instrument equipped with an ALV5000/60 × 0 Multipurpose correlator and an Oxxius SLM-532 150 mw DPSS laser operated at a wavelength of 532 nm. A refractive index matching bath of filtered cis-decalin surrounded the cylindrical scattering cell, and the temperature was controlled at 21.5°C ± 0.3°C using a Haake F3-K thermostat. For each sample the autocorrelation function, g2(τ), was recorded 10 times at a detection angle of 90°. For each measurement the diffusion coefficient, D, was determined using the second-order cumulant and the corresponding particle diameter was calculated assuming that the particles were spherical in shape. Microparticles of 2 μm and with a relatively monodisperse diameter were produced (Table 1). ζ potential of the microparticles was determined on a Malvern ZetaSizer 2000. The ζ potential of the particles ranged from −40 to −45 mV, largely due to the negatively charged carboxylic acid groups of the PLGA polymer (Table 1).

Determination of Ag in the microparticles

Entrapment efficiency of FITC-TT peptide was determined by digesting 10 mg microparticles in 3 ml 0.8 N NaOH overnight at 37°C, filtering with a 0.2 μm sterile syringe filter, and measuring fluorescence relative to a standard curve (492 nm excitation and 20 nm emission) using a CytoFluor II (Applied Biosystem, Foster City, CA). DQ-BSA entrapment was determined by Coomassie protein assay (Pierce, Rockford, IL). Ag loading was determined by dividing the amount of encapsulated Ag by the theoretical amount assuming that the entire added amount of Ag was encapsulated. The entrapment efficiency of Ag was 80–90% (Table I).

Determination of TLR-Ls in the microparticles

The encapsulation efficiency of TLR-Ls was determined by HPLC. HPLC analysis was performed at room temperature using a Shimadzu system (Shimadzu, Kyoto, Japan) equipped with a reverse-phase Symmetric C18 column (250 mm × 4.6 mm). The flow rate was fixed at 1 ml/min and detection was obtained by UV detection at 220 nm. A linear gradient of 0–100% of acetonitrile (containing 0.05% trifluoroacetic acid) in water (containing 0.045% trifluoroacetic acid) was used for the separation of R848 and CpG C. The peak of R848 was well separated from that of the CpG C in the established chromatographic condition. The retention times of the CpG C and R848 were ~17 and 26 min, respectively. The regression analysis was constructed by plotting the peak/area ratio of R848 or CpG C versus concentration (μg/ml). The calibration curves were linear within the range of 1–10 μg/ml for R848 and 10–40 μg/ml for CpG C. The correlation coefficient (R2) was always greater than 0.99, indicating a good linearity. R848 and CpG C were extracted by dispersing 5 mg microparticles in 500 μl 85% acetonitrile in water, followed by centrifugation at 12,000 rpm for 10 min. The PVA was precipitated and the supernatant was subsequently assayed for RP-HPLC. The amount of R848 and CpG C was calculated by interpolation into the standard curves as described previously. The encapsulation efficiency of R848 and CpG C was 40–60% (Table I).

Cells

PBMCs were obtained from buffy coats of healthy individuals after informed consent and were purified using Ficoll density centrifugation. Monocytes were removed via adherence and pDCs were purified from the nonadherent cell population (PBLS) by positive isolation using anti-BDCA-4–conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The pDC purity was routinely up to 95%, as assessed by double staining BDCA-2/CD123. The pDCs were cultured in X-VIVO-15 medium (Cambrex, Verviers, Belgium), supplemented with 2% human serum.

Flow cytometry

Uptake of particulate or soluble Ag and pDC phenotype were determined by flow cytometry. The pDCs (105 cells/well in 100 μl medium) were cultured with 1 mg/ml PLGA microparticles (0.25 mg/ml) at 37°C with PLGA particles (0.25 mg/ml) containing encapsulated Ag (FITC-TE-TT or DQ-BSA) and TLR-Ls (CpG C and R848) or with equal amounts of soluble Ag or soluble CpG C and R848. If no activation stimuli were added, pDCs were cultured with 10 ng/ml IL-3 as a survival stimulus. The following primary Abs and the appropriate isotype controls were used for pDC phenotyping: anti-CD80 (Becton Dickinson, Mountain View, CA) and anti-CD86 (BD Pharmingen, San Diego, CA). PE-labeled goat–anti-mouse IgG mAb was used as secondary Ab. Cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA).

Confocal microscopy

Uptake of particulate FITC-TE-TT was confirmed by confocal microscopy. Cells were fixed on poly-t-lysine coated glass slides and stained with anti-human MHC class II Ab (clone Q5/13), followed by a secondary goat–anti-mouse IgG Alexa 647 mAb (Molecular Probes, Carlsbad, CA). Cells were then incubated with a Bio-Rad MRC 1024 confocal system operating on a Nikon Optiphot microscope and a Nikon 60× planApo 1.4 oil immersion lens. Pictures were analyzed with Bio-Rad Lasersharp 2000 and Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) software.

Ag processing

Ag processing was measured with DQ-BSA, a protein strongly labeled with a fluorescent BODIPY dye. DQ-BSA microparticles (0.25 mg/ml) were added to pDCs (105). Cells were cultured at 37°C and fluorescence was measured.
measured spectrophotometrically in a CytoFluor II or by flowcytometry on a FACSCalibur.

IFN-α and IL-6 ELISA
IFN-α and IL-6 production was analyzed with murine monoclonal capture and HRP-conjugated anti–IFN-α Abs (Bender MedSystems, Vienna, Austria) and anti–IL-6 Abs (Sanquin, Amsterdam, The Netherlands) respectively, using standard ELISA procedures.

Ag presentation assay
PBLs from healthy donors were cultured for 8–10 d with TT(303-344) peptide (3 μg/ml) and IL-2 (50 EU/ml) to increase the number of TT-responsive cells. Autologous pDCs (10⁵) were incubated overnight at 37˚C with different PLGA particle formulations (0.25 mg/ml) or equal amounts of soluble R848 and CpG C or soluble FITC-TT. Subsequently, pDCs were added to the prestimulated PBLs at a ratio of 1:10 in 6-fold. After 4 d, proliferative responses were determined by adding tritiated thymidine (1 μCi [0.037 MBq]/well; MP Biomedicals, Amsterdam, The Netherlands) to the cell cultures. Tritiated thymidine incorporation was measured after 16 h in a scintillation counter. In addition, cytokine production (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 [p70], TNF-α, TNF-β, IFN-γ) was measured in the supernatants of the Ag presentation assay after 4 d with a human Th1/Th2 Multiplex kit (Bender MedSystems) according to manufacturer’s instructions.

Results
Human pDCs phagocytose particulate Ag more efficiently than soluble Ag

To investigate whether pDCs can phagocytose particulates, we produced distinct combinations of PLGA microparticles (Table I), containing TT(303-344) peptide covalently linked to FITC (FITC-TT) or the fluorescent-labeled protein DQ-BSA. The pDCs were incubated in the presence or absence of particulate FITC-labeled TT-peptide or DQ-BSA. Incubation of pDCs with particulate FITC-TT resulted in an increase in FITC⁺ pDCs in time (Supplemental Fig. 2). After overnight incubation, ~50% of the pDCs were FITC⁺, indicating that pDCs had taken up particulate FITC-TT (Fig. 1A, 1B). Incubating pDCs with particulate DQ-BSA resulted in 94% fluorescent pDCs, demonstrating that virtually all pDCs have taken up particulate DQ-BSA (Fig. 1A). Differences in the percentage of fluorescent pDCs are presumably due to the higher fluorescent labeling of DQ-BSA compared with FITC-TT. To demonstrate that particulate FITC-TT is actually taken up and not simply binding to the pDCs, cells were incubated with cytochalasin D prior to the addition of particulate FITC-TT to block phagocytosis. As expected, cytochalasin D-treated pDCs displayed a strongly reduced FITC signal, indicating diminished uptake of particulate Ag (Fig. 1A, 1B). Additional evidence for internalization was obtained by confocal microscopy. The majority of the pDCs showed diffuse fluorescent staining, indicative of intracellular release of FITC-TT from the PLGA particle (Fig. 1C). On z-stack analysis, we could confirm that detected FITC signals were indeed located within the pDC (Fig. 1C). In addition, the capacity of pDCs and moDCs to take up particulate DQ-BSA was compared. Although pDCs are clearly able to take up particulate Ag, their uptake capacity is lower than moDCs (Supplemental Fig. 4A, 4B).

To investigate whether uptake of particulate Ag is more efficient than soluble Ag, pDCs were incubated overnight with an equal amount of either particulate or soluble FITC-TT. Incubation with particulate FITC-TT resulted in 60% FITC⁺ pDCs, whereas incubation with soluble FITC-TT resulted in only 14% FITC⁺ pDCs (Fig. 1D). In addition, the mean fluorescence intensity of the pDCs is four times higher after addition of particulate compared with soluble FITC-TT (data not shown). These data reveal that pDCs are well equipped to take up particulate Ag and that particulate Ag is phagocytosed considerably more efficiently than soluble Ag.

Particulate Ag is processed by human pDCs
To determine whether pDCs are able to process phagocytosed particulate protein, the model protein BSA, labeled with a fluorescent BODIPY dye (DQ-BSA), was used. DQ-BSA is labeled to such high degree that the fluorescence is self-quenched. Quenching is relieved on release of Ag from the PLGA particles within the cell and subsequent processing of the Ag into fluorescent peptides by cellular proteases. Incubation of pDCs with particulate DQ-BSA resulted in an increase of fluorescent cells in time as analyzed by flowcytometry, demonstrating uptake and processing of particulate DQ-BSA (Fig. 2A). Ag processing was also measured by spectrophotometry. Incubation of pDCs with particulate DQ-BSA resulted in an increase in fluorescence indicative of Ag degradation, whereas the fluorescence of particulate DQ-BSA incubated without pDCs did not increase (Fig 2B). In addition, processing of exogenous Ag was analyzed after CpG C-induced pDC activation. We previously demonstrated that pDC activation diminishes processing and presentation of exogenous immune complexes (27). In accordance with this result, CpG C-induced activation strongly diminished pDC fluorescence on incubation with both particulate DQ-BSA or particulate FITC-TT as measured by flowcytometry (data not shown). As expected, pDC activation also strongly diminished fluorescence as measured by spectrophotometry, demonstrating that uptake of extracellular particulate Ag is decreased on pDC maturation (Fig 2B).

Human pDCs are activated via delivery of particulate TLR-Ls
Activation of pDCs is essential for effective Ag presentation and activation of naive T cells (10). Therefore, we investigated if pDCs are also able to sense encapsulated particulate TLR-Ls rather than soluble TLR-Ls. We decided to activate pDCs exploiting TLR-Ls that trigger TLRs located in the endosomal compartments (28). Binding of specific viral components, such as unmethylated CpG-rich DNA motifs or dsRNA, to these TLRs is known to upregulate MHC and costimulatory molecules and production of type I IFN.
As pDCs predominantly express TLR-7 and -9, PLGA particles containing the TLR-7 agonist R848 and the TLR-9 agonist CpG C were generated. Indeed, exposing pDCs to these PLGA particles induced activation as reflected by strong upregulation of CD80 (Fig. 3A, 3B) and CD86 expression (Fig. 3A, 3C) and production of high levels of IFN-α (Fig. 3D, mean IFN-α production: 5566 pg/ml) and IL-6 (Fig. 3E, mean IL-6 production: 2156 pg/ml). As expected, pDCs cultured with IL-3 or with PLGA particles containing only Ag (FITC-TT) did not acquire an activated profile. Activation of pDCs by PLGA-encapsulated TLR-Ls could be blocked by cytochalasin D, indicating that activation depends on phagocytosis of the PLGA microparticles (Fig. 3B–E). In addition, pretreatment with chloroquine, which blocks phagosomal acidification and thereby inhibits TLR triggering, also blocked pDC activation on incubation with particulate TLR-Ls. Incubation of pDCs with particle-free CpG C or R848 in combination with cytochalasin D or chloroquine also inhibited phenotypical maturation and IFN-α secretion (data not shown). Furthermore, blocking of particulate TLR-L–induced pDC activation both by cytochalasin D and chloroquine was concentration-dependent.

**FIGURE 1.** PDCs phagocytose particulate Ag. Human pDCs were incubated with or without PLGA particles containing FITC-labeled TT peptide or DQ-BSA. Uptake of particulate Ag by pDCs was measured by flowcytometry after 24 h. In addition, uptake of particulate FITC-TT was blocked by incubating pDCs with the phagocytosis blocker cytochalasin D (2.5 or 10 μM). Representative dot plots of the fluorescence intensity of pDCs are shown. Values in the lower right quadrant represent the percentage of fluorescent pDCs (A). Combined data of three independent experiments. Data are mean values ± SEM (B). Uptake of particulate FITC-TT by pDCs was further confirmed by confocal scanning laser microscopy. Depicted are the merged pictures of the fluorescence of particulate FITC-TT (green) and MHC class II staining (red). pDCs only (upper left), pDCs incubated with particulate FITC-TT (C, magnification ×60). Uptake of particulate versus soluble Ag. Human pDCs were incubated with particulate or soluble FITC-TT and uptake was measured by flowcytometry after 24 h. Data are mean values of three independent experiments ± SEM (D). Statistical significance was determined by ANOVA and Newman-Keuls testing. **p < 0.01; ***p < 0.001.

**FIGURE 2.** Human pDCs process phagocytosed particulate Ag. Human pDCs were incubated with PLGA particles containing the self-quenched model protein DQ-BSA for 2, 6, or 24 h. Fluorescence, caused by uptake and subsequent degradation of DQ-BSA, was measured by flowcytometry. The gray peak represents pDCs incubated without particulate DQ-BSA. Values represent the percentage of fluorescent pDCs (A). Human pDCs were incubated with particulate DQ-BSA (■) and fluorescence was measured spectrophotometrically during 48 h. As a control, fluorescence of pDCs only (●), particulate DQ-BSA only (▲) or pDCs activated with CpG C before adding particulate DQ-BSA (▼) was measured. Two independent experiments were performed with similar results. Data are mean fluorescence for one of the experiments (B).
dependent (Supplemental Fig. 3, data not shown). Importantly, particulate TLR-Ls consistently induced a comparable, with a tendency to higher, activation state compared with the same amount of soluble TLR-Ls in six independent donors, as reflected by the expression of the costimulatory markers CD80 and CD86 (Fig. 4A) and IL-6 production (Fig. 4B). These results demonstrate that pDCs are efficiently activated on phagocytosis of particulate TLR-Ls.

Uptake of particulate Ag and TLR-Ls by human pDCs induces T cell proliferation

Previously we have demonstrated that DCIR and FcγRII-mediated uptake of KLH protein induces efficient Ag presentation by human pDCs (15, 30). In this study, we showed that human pDCs are capable of processing phagocytosed particulate Ag and in addition can be activated by particulate TLR-Ls. We next investigated whether pDCs are capable of presenting ingested particulate Ag and induce T cell responses.

The pDCs incubated with PLGA particles, containing FITC-TT and TLR-Ls, induced higher proliferation of autologous TT-responsive T cells when compared with pDCs incubated with empty PLGA particles and soluble TLR-Ls (Fig. 5A). This indicates that pDCs efficiently present particulate Ag to T cells, resulting in Ag-specific proliferative recall responses. Furthermore, particulate FITC-TT and TLR-Ls induced higher TT-specific T cell proliferation than soluble FITC-TT coincubated with soluble TLR-Ls, indicating that pDCs are more efficient in inducing specific immune responses toward particulate than soluble Ags. Although the uptake capacity of particulate Ag by pDCs was substantially lower compared with moDCs, pDCs induced only a 2-fold lower Ag-specific proliferative T cell response than moDCs (Supplemental Fig. 4). Furthermore, CD4+ T cells were prestimulated with autologous pDCs incubated with PLGA particles containing FITC-TT and TLR-Ls or with empty PLGA particles and soluble TLR-Ls as a control. On restimulation of the CD4+ T cells with autologous PBMCs loaded with TT-peptide, pDCs incubated with PLGA particles containing FITC-TT and TLR-Ls induced higher proliferative T cell responses compared with the control. These data demonstrate that the induced T cell responses are indeed Ag specific (Fig. 5C).

To gain more insight in the polarization of the induced T cell responses, Th1 and Th2 cytokines were measured. We observed that responding T cells produced both Th1 and Th2 cytokines (IFN-γ, IL-6, IL-10) and the proinflammatory cytokine IL-8 (Fig. 5B). The other analyzed Th1/Th2 cytokines were not detected or only detected in a small part of the culture supernatants. We observed the highest cytokine production in the T cell cultures incubated with pDC activated with coencapsulated Ag and TLR-Ls (mean IFN-γ: 262 pg/ml; IL-6:193 pg/ml; IL-8: 3274 pg/ml; IL-10: 169 pg/ml). T cells incubated with pDCs activated with empty PLGA and soluble TLR-Ls produced 30–40% less cytokines. Also, T cell cultures incubated with pDCs activated with soluble Ag and TLR-Ls produced 30–60% less
cytokines, indicating that uptake of particulate Ag/TLR-Ls is more potent in inducing T cell proliferation and cytokine production than their soluble counterparts.

Discussion
Human pDCs are regarded as immunomodulating cells and have been described to induce immunity (31), as well as to promote regulatory T cell-mediated immunosuppression (32, 33). In this study, we investigated whether pDCs can play a role in immune responses against exogenous particulates. Up to now, it was highly controversial whether pDCs are able to respond to stimuli entrapped in particle-like structures (3, 8, 9, 14, 16–19). We demonstrate that pDCs are clearly capable of phagocytosing Ag entrapped within PLGA microparticles and subsequent processing of the encapsulated Ag. Furthermore, pDCs sense TLR-Ls entrapped in microparticles, resulting in efficient pDC activation as reflected by upregulation of costimulatory molecules and high IFN-α production. Finally, phagocytosis of a particulate vaccine, containing both Ag and TLR-Ls, results in induction of Ag-specific CD4+ T cell responses. Our results provide evidence that human

FIGURE 4. Particulate TLR-Ls are superior to soluble TLR-Ls in activating human pDCs. Human pDCs were incubated with particulate TLR-Ls (CpG C and R848) or an equal amount of soluble TLR-Ls. Expression of the maturation markers CD80 and CD86 (A) and IL-6 production (B) was measured after 24 h. Values of CD80 and CD86 represent the relative receptor expression compared with IL-3 pDCs. Data are mean values of at least three independent experiments ± SEM. Statistical significance was determined by Wilcoxon signed rank testing (A) or by ANOVA and Newman-Keuls testing (B). *p < 0.05; **p < 0.01.

FIGURE 5. Human pDCs present particulate Ag, resulting in Ag-specific T cell responses. Human pDCs were incubated with PLGA particles containing FITC-TT peptide and the TLR-Ls CpG C and R848 (PLGA-TT-R848-CpG C) for 18–24 h. As a control, pDCs were incubated with PLGA particles containing no peptide or TLR-Ls (PLGA) in combination with soluble TLR-Ls or a combination of soluble TT-peptide and soluble TLR-Ls. Subsequently, autologous TT-responsive PBLs were added. After 4 d, T cell proliferation was measured by [3H]thymidine incorporation (A). Cytokine production was measured after 4 d by cytokine bead array (B). CD4+ T cells were prestimulated with autologous pDCs incubated with PLGA-TT-R848-CpG C, with soluble FITC-TT and soluble TLR-Ls or with empty PLGA and soluble TLR-Ls as a control. Subsequently, CD4+ T cells were restimulated with autologous PBMCs loaded with TT-peptide. After 4 d, T cell proliferation was measured by [3H]thymidine incorporation (C). Data were calculated by subtracting the CPM of the background (T cells incubated with IL-3 pDCs) from the CPM of the experimental wells. Data are mean values of two (C) or three (A, B) independent experiments ± SEM. Significance was determined by ANOVA and Newman-Keuls testing. *p < 0.05; **p < 0.01; ***p < 0.001.
pDCs can play a role in induction of adaptive immune responses induced by stimuli derived from phagocytosed exogenous particle-like structures, such as vaccine preparations, but possibly also bacteria and other microorganisms.

The observation that uptake of particulate Ag by pDCs was partially blocked by cytochalasin D, an inhibitor of actin polymerization, demonstrates that the microparticles are at least in part taken up via phagocytosis, an actin-dependent process. Similar results were obtained with moDCs (34), indicating that internalization of microparticles by moDCs as well as pDCs is a dynamic process involving actin filaments. Endocytic receptors most likely mediate the internalization of PLGA microparticles (34), but the nature of these receptors and the exact mechanism remain to be elucidated.

Several factors may explain the disparity between studies investigating whether pDCs are able to take up particle-like structures. First, the composition of the studied particulates (apoptotic cells, beads, bacteria, or PLGA microparticles) is diverse and uptake might require different receptors and uptake mechanisms, some of which may not be exploited by pDCs. Furthermore, some studies compared the phagocytic capacity of pDCs to that of moDCs and found that pDCs showed very little to no uptake compared with moDCs (3, 14, 18). We also found that moDCs were superior to pDCs in phagocytosing particulate Ag, notwithstanding that almost all pDCs engulfed particles. Because of this 5- to 10-fold lower phagocytic capacity of pDCs compared with moDCs, the methods used to quantify particle uptake must be relatively sensitive and may explain why some studies concluded that pDCs cannot phagocytose. This is reflected in our experiments where PLGA particles containing highly fluorescent DQ-BSA were detected in a higher percentage of pDCs after 24 h than PLGA particles containing the less fluorescently labeled FITC-PTT peptide. Furthermore, it should be noted that uptake of PLGA-encapsulated fluorescent molecules is likely underestimated, because fluorescence is quenched or shielded by the PLGA surrounding the fluorescent Ag. This notion was supported by the observation that on in vitro degradation of PLGA, thereby releasing its fluorescent content, fluorescence levels increased 150-fold (35).

In accordance with studies performed with moDCs (21, 36, 37), human pDCs take up and present particulate Ag more efficiently than soluble Ag, resulting in enhanced T cell stimulation. This is presumably caused by controlled release of encapsulated Ag from the PLGA particle resulting in prolonged and more efficient Ag presentation compared with soluble Ag (21, 37). Furthermore, ex vivo Ag loading of moDCs using microparticles is also more efficient than external loading of peptides in MHC molecules. Because of the rapid turnover of MHC-peptide complexes on the cell surface, the duration of T cell stimulation in vivo is prolonged on uptake of particulate Ag compared with external loading of peptides (37). Finally, particulate TLR-Ls induced efficient pDC activation comparable to activation induced by soluble TLR-Ls. Taken together, microparticles are well suited to deliver antigenic cargo and immunostimulatory molecules to pDCs, thereby inducing the activation of pDCs expressing all the necessary characteristics for optimal T cell activation. To this end, PLGA particles may be equipped with Abs directed against endocytic pDC-specific surface receptors to target these cells in vivo (38–40). Targeting Ags to different subsets of DCs is only recently explored (41) and has already proven to enhance and modulate immune responses (30, 38). Our observation that pDC can phagocytose particulate Ag may also contribute to the previously described ability of pDCs to enhance myeloid DC mediated Ag presentation (10). In the end, a combined particulate vaccine could be created that targets different appropriate Ags and immunostimulatory molecules to different subsets of DCs, that can subsequently act synergistically in inducing an optimal immune response.

PDCs have been found in several types of tumors: head and neck cancer, ovarian cancer, primary melanoma cancer, and breast cancer (4). In tumors, the presence of pDCs correlated with an unfavorable prognosis and is thought to promote T cell tolerance rather than T cell activation. Most likely, pDCs are not properly activated on uptake of tumor-derived substances because of the lack of danger signals (12). Recent studies have shown that in vivo TLR triggering with TLR-Ls, such as CpG, induced pDC activation, resulting in antitumor immunity and partial tumor regression (42–44). Furthermore, activated human pDCs pulsed with a tumor Ag primed IFN-γ secreting Ag-specific CTLs (45). We also show that pDCs produce high levels of IFN-α and IL-6 and induce proliferative CD4+ T cell responses against a universal Th epitope on phagocytosis of PLGA microparticles, containing peptide Ag and TLR-Ls. The Th peptide is frequently used in clinical trials as a Th cell epitope to increase immunogenicity of a vaccine (46). Altogether, this indicates that PLGA microparticles might be a promising tool to efficiently load pDCs with tumor Ags and helper epitopes and simultaneously activate them by coencapsulated TLR-Ls, resulting in fully functional pDCs capable of stimulating antitumor responses via both production of IFN-α and direct induction of Ag-specific T cell responses.

The in vivo function of pDCs as APCs remains controversial and functional outcomes of pDC-T cell interactions largely depend on the immunological context of the encounter (47). Activated pDCs have been demonstrated to promote Th1 (31, 47) as well as Th2 responses (32, 33), underlining their remarkable functional plasticity. The pDCs were shown to prime specific CD4+ and CD8+ lymphocytes against endogenous and exogenous Ags (15, 48), viruses (49) and tumor Ags (45). By contrast, when activated by CpG-B pDCs demonstrate a strong immune suppression and induce the differentiation of allogeneic CD4+CD25+ T cells into CD4+ CD25+ regulatory T cells (32). There is evidence that the presence of IFN-α stimulates the differentiation of pDCs into Th1-inducing pDCs, whereas the absence of IFN-α and presence of inflammatory signals stimulate differentiation in Th2-inducing pDCs inhibiting the Th1-immune response (50). In our study, pDCs stimulated with PLGA-encapsulated Ag and TLR-Ls produced high amounts of IFN-α and induced T cell-mediated production of both Th1 (IFN-γ) and Th2 cytokines (IL-10, IL-6). Perhaps, the fact that the model Ag TT used here is a recall Ag, and therefore likely stimulated memory T cells, may cause this cytokine profile. It is well conceivable that by both the antigenic load and the pDC activating stimuli encapsulated in these PLGA microparticles, T cell activation and the resulting cytokine profile can be modulated. The ability of human pDCs to cross-present Ags to CD8+ T cells remains controversial (16, 51). Whether human pDCs can cross-present particulate Ags and induce functional CD8+ T cells is yet unknown and needs further investigation.

In conclusion, our findings reveal the capacity of pDCs to respond to particulate stimuli, indicating that pDCs can play a role in immune responses induced by particulates. This calls for further studies on the role pDCs play in clearing bacterial infections and handling current particulate vaccines. Furthermore, Ab coating of these particulate “vaccine carriers” offers perspectives for targeting specific subsets of APCs and may lead to the design of novel vaccine strategies actively recruiting pDCs for cellular immunotherapy.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary figure 1. Morphology of PLGA microparticles as analyzed by scanning electron microscopy.

Supplementary figure 2. Kinetics of uptake of particulate FITC-TT.

Human pDCs were incubated with or without PLGA particles containing FITC-labeled TT peptide. The uptake of particulate FITC-TT was measured by flowcytometry at different time points. The upper row of dotplots show the fluorescence intensity of pDCs after incubation with particulate FITC-TT. The lower row of dotplots show the fluorescence intensity of the matching IL-3 cultured pDCs.

Supplementary figure 3. Concentration dependant blocking of particulate TLR-Ls induced pDC activation by cytochalasin D.

Human pDCs were incubated with different concentrations cytochalasin D before adding PLGA particles containing TLR-Ls CpG C and R848 (PLGA-TT-R848-CpG C). Expression of the maturation markers CD80 (A), CD86 (B) and IFN-α production (C) was measured after 24 hrs. Values of CD80 and CD86 represent the relative receptor expression compared to IL-3 pDCs. Data are mean values of at least 3 independent experiments ± SEM. Statistical significance was determined by ANOVA and Newman-Keuls testing. *P<0.05, **P<0.01, ***P<0.001

Supplementary figure 4. Uptake and presentation of particulate antigen by pDCs versus monocyte-derived DCs (moDCs).

Human pDCs (black lined histogram) and immature moDCs (grey lined histogram) were incubated overnight with PLGA containing DQ-BSA. Fluorescence, caused by uptake and subsequent degradation of DQ-BSA, was measured by flowcytometry. The gray filled histogram represents pDCs incubated without particulate DQ-BSA. (A). Human pDCs (▲) or immature
monocyte-derived DCs (♦) were incubated with particulate DQ-BSA and fluorescence was measured spectrophotometrically during 48 hrs. As a control, fluorescence of pDCs only (■) and immature monocyte-derived DCs only (▼) was measured (B). Human pDCs and moDCs were incubated with PLGA particles containing FITC-TT peptide and the TLR-Ls CpG C and R848 (PLGA-TT-R848-CpG C) for 18-24 hrs. Subsequently, autologous TT-responsive PBLs were added. After 4 days, T cell proliferation was measured by ³H-thymidine incorporation. Data were calculated by subtracting by the CPM of the background (TT-responsive PBLs incubated with IL-3 pDCs or moDCs) from the CPM of the experimental wells (C).