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# Targeting Uptake Receptors on Human Plasmacytoid Dendritic Cells Triggers Antigen Cross-Presentation and Robust Type I IFN Secretion

**Jurjen Tel,* Simone P. Sittig,*,[1] Rebecca A. M. Blom,*,[1] Luis J. Cruz,*,[2] Gerty Schreibelt,* Carl G. Figdor,* and I. Jolanda M. de Vries*,[†]**

**Plasmacytoid dendritic cells (pDCs) play a crucial role in initiating immune responses by secreting large amounts of type I IFNs. Currently, the role for human pDCs as professional APCs in the cross-presentation of exogenous Ags is being re-evaluated. Human pDCs are equipped with a broad repertoire of Ag uptake receptors and an efficient Ag-processing machinery. In this study, we set out to investigate which receptor can best be deployed to deliver Ag to pDCs for Ag (cross-)presentation. We show that targeting nanoparticles to pDCs via the C-type lectins DEC-205, DC immunoreceptor, blood DC Ag-2, or the FcR CD32 led to uptake, processing, and (cross-) presentation of encapsulated Ag to both $CD4^+$ and $CD8^+$ T cells. This makes these receptors good candidates for potential in vivo targeting of pDCs by nanocarriers. Notably, the coencapsulated TLR7 agonist R848 efficiently activated pDCs, resulting in phenotypical maturation as well as robust IFN-α and TNF-α production. Taken together, their cross-presentation capacity and type I IFN production to further activate components of both the innate and adaptive immune system mark pDCs as inducers of potent antitumor responses. These findings pave the way to actively recruit human pDCs for cellular cancer immunotherapy.** ***The Journal of Immunology*****, 2013, 191: 5005–5012.**

Plasmacytoid dendritic cells (pDCs) secrete large amounts of type I IFNs (IFN-α/β) in response to viral or bacterial stimuli (1). Type I IFNs are essential in initiating immune responses by stimulating NK cell functions, Th1 responses and DC maturation for efficient cross-priming of $CD8^+$ T cells (2, 3). Nonstimulated pDCs, in contrast, are involved in the induction of regulatory responses and tolerance (4). Human pDCs exploit a repertoire of different pattern-recognition receptors such as TLRs or C-type lectin receptors (CLRs) for the detection of pathogens and the uptake of Ags for subsequent processing and presentation to T cells (5). Previous studies showed that the delivery of Ab-coupled Ags to the CLRs DEC-205, DC immunoreceptor (DCIR), and blood DC Ag-2 (BDCA-2) or to the FcR CD32 (FcγRIIa) leads to Ag-specific $CD4^+$ T cell activation (6–8). However, for a potent antitumor response, both $CD4^+$ and $CD8^+$ T cell activation is essential. Although human pDCs were previously thought to poorly cross-present exogenous Ag to $CD8^+$ T cells, accumulating evidence now suggests that human pDCs are endowed with an efficient cross-presenting machinery (9–14). Just recently, we could demonstrate the potency of pDCs in a small cohort of metastatic melanoma patients, in whom vaccination with activated pDCs induced favorable immune responses and significantly extended overall survival (15). This puts human pDCs forward as potent activators of antitumor responses that efficiently present exogenous Ags to T cells in combination with a high type I IFN production to further activate components of both the innate and adaptive immune system.

Harnessing pDCs for DC-based immunotherapy by direct targeting of pDCs in vivo is an attractive opportunity; it allows the delivery of Ags without extensive handling and ex vivo culturing (16, 17). The potency of harnessing pDCs in vivo was already demonstrated in mice, where targeting the murine pDC-specific receptor Siglec-H induced the priming of Ag-specific $CD8^+$ T cells upon Ag uptake via this receptor (18). Moreover, targeting murine pDCs via BST2 induced Ag-specific $CD4^+$ and $CD8^+$ T cell responses (18, 19). Human pDCs, however, do not express Siglec-H (20), and expression of BST2, although a possible target, is not restricted to human pDCs, but is also found on B cells and upregulated by IFN-α on a range of other cell types (21). In this study, we investigated which receptor can best be harnessed to deliver Ags for Ag (cross-)presentation to human pDCs. For this purpose, poly(lactic-coglycolic acid) (PLGA) nanoparticles (NPs) were used that were decorated with Abs against DEC-205, DCIR, BDCA-2, or CD32 and that encapsulated peptide Ags along with

*Department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen 6500 HB, The Netherlands; and [†]Department of Medical Oncology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen 6500 HB, The Netherlands

[1]S.P.S. and R.A.M.B. contributed equally to this work.

[2]Current address: Endocrinology, Leiden University Medical Centre, Leiden, The Netherlands.

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Address correspondence and reprint requests to Prof. I. Jolanda M. de Vries, Department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail address: j.devries@ncmls.ru.nl

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Abbreviations used in this article: BDCA-2, blood dendritic cell Ag-2; CLR, C-type lectin receptor; DCIR, dendritic cell immunoreceptor; DC-SIGN, dendritic cell–specific ICAM-3–grabbing nonintegrin; DQ-BSA, BSA labeled with an autoquenching fluorescent BODIPY dye; FITC-TT, FITC-labeled tetanus-toxoid$_{830–844}$ peptide; MFI, mean fluorescence intensity; MHC I, MHC class I; MHC II, MHC class II; mIgG1, murine IgG1; NP, nanoparticle; pDC, plasmacytoid dendritic cell; PD-L1, programmed cell death ligand 1; PLGA, poly(lactic-coglycolic acid); TT, tetanus-toxoid$_{830–844}$ peptide.

the TLR7 agonist R848 (Fig. 1). PLGA is a biocompatible polymer that is already widely used as a vaccine carrier (22). Furthermore, the use of these nanocarriers allows the codelivery of several components, such as Ag and adjuvant, circumventing the need for systemic TLR administration and all of the associated side effects. We demonstrate that targeting PLGA NPs via DEC-205, DCIR, BDCA-2, or CD32 to human pDCs led to receptor-specific uptake and processing of the particulate Ag. The processed peptides were loaded in MHC class I (MHC I) and II (MHC II) and (cross-)presented to $CD8^+$ and $CD4^+$ T cells, respectively. Importantly, the coencapsulated TLR7 agonist R848 efficiently activated pDCs, resulting in phenotypical maturation and cytokine production.

## Materials and Methods

### *Cells*

Cells were obtained from buffy coats of healthy individuals in accordance with institutional guidelines. PBMCs were obtained by Ficoll density centrifugation, and monocytes were removed by plastic adherence. Peripheral blood leukocytes were used for pDC isolation by positive selection using anti–BDCA-4–conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). pDC purity was routinely ≥95% as analyzed by flow cytometry by double staining with CD123 and BDCA-2. pDCs were cultured in X-VIVO-15 medium (Cambrex, Verviers, Belgium) supplemented with 2% human serum (Sigma-Aldrich, St. Louis, MO) and activated as indicated.

### *PLGA NPs*

NPs were generated using the copolymer PLGA as described previously (23). $gp100_{272–300}$ peptide, $tetanus\text{-}toxoid_{830–844}$ peptide (TT), TLR-ligand R848 (Axxora, San Diego, CA), and BSA labeled with an autoquenching fluorescent BODIPY dye (DQ-BSA; Molecular Probes, Leiden, The Netherlands) were encapsulated by addition to the NPs (Table I). Encapsulation efficiency was determined by reverse-phase HPLC, as described before (24, 25). NPs were coated with lipid–polyethylene glycol and streptavidin and subsequently coupled to the following biotinylated Abs as described by Cruz et al. (25): anti-DCIR (Dendritics), anti–DEC-205 (eBioscience), anti–BDCA-2 (Miltenyi Biotec), anti-CD32 (AbD Serotec), and anti–DC-specific ICAM-3–grabbing nonintegrin (DC-SIGN).

### *Particle binding and uptake*

To study particle binding, $3 \times 10^4$ pDCs was incubated with 20 μg/ml NPs encapsulating FITC-labeled TT (FITC-TT) (and R848) for different time periods at 4°C. Cells were washed, and fluorescence intensity of FITC was analyzed by flow cytometry while constantly keeping the cells on ice to avoid internalization of particles. Particle uptake was studied by incubating pDCs for different time periods at 37°C with 20 μg/ml NPs encapsulating FITC-TT (and R848) and determining changes in cell-associated fluorescence of FITC on an FACSCalibur (BD Biosciences, San Jose, CA); data were analyzed by CellQuest (BD Biosciences). As a pH-sensitive fluorophore, FITC loses intensity at lower pH values as present in endosomes and lysosomes (19). This could account for the seemingly low percentage of positive cells for DEC-205–, DCIR-, and CD32-targeted NPs after overnight culture. Also, the PLGA surrounding the fluorescent Ag can shield the FITC signal, as supported by the observation that upon in vitro degradation of PLGA, thereby releasing its fluorescent content, fluorescence levels increased 150-fold (23).

### *Confocal microscopy*

Particle uptake after overnight incubation was confirmed by confocal analysis of intracellular FITC signal of the FITC-TT–containing NPs. Cells were fixed on poly-L-lysine–coated glass slides and stained with anti-human HLA-DR/DP (Q5/13) Ab, followed by a secondary goat-anti-mouse IgG Alexa 647 Ab (Molecular Probes, Carlsbad, CA). Cells were imaged with a Bio-Rad MRC 1024 confocal system (Bio-Rad) operating on a Nikon Optiphot microscope and a Nikon 60× planApo 1.4 oil immersion lens (Nikon).

### *Ag degradation*

A total of $2.5 \times 10^4$ pDCs was cultured in 100 μl phenol red free X-VIVO 15 medium (Cambrex) supplemented with 2% human serum in the presence of 0.5 μg soluble DQ-BSA or DQ-BSA encapsulated in Ab-coated NPs. After 4 h at 37°C, the excessive NPs were washed away, and cells were cultured at 37°C up to 72 h. The fluorescence released within pDCs was measured (excitation 485 nm; emission 530 nm) with a CytoFluor II (Applied Biosystems) at various time points. Ag degradation kinetics were determined as described previously (23).

### *Phenotype*

The phenotype of pDCs was determined by flow cytometry after overnight stimulation with 20 μg/ml NPs containing R848 (and either FITC-TT or $gp100_{272–300}$), 10 ng/ml recombinant human IL-3 (Cellgenix Freiburg, Germany), or 4 μg/ml soluble R848 at 37°C. The following mAbs with appropriate isotype controls were used for staining: murine IgG1 (mIgG1)-FITC, mIgG1-PE, mIgG1-allophycocyanin, anti–CD80-PE, anti–CD86-allophycocyanin, anti–MHC I–PE, anti–MHC II–FITC, anti–CD83-PE, anti–programmed cell death ligand 1 (PD-L1)–PE, anti–ICOS ligand–Alexa 488, anti–CCR7-PE (BD Pharmingen, San Diego, CA), and anti–CD40-PE (Beckman Coulter, Mijdrecht, The Netherlands). Flow cytometry analysis was performed on an FACSCalibur (BD Biosciences); the mean fluorescence intensity (MFI) and percentage of positive cells were determined from live cell population based on forward light and side scatter using CellQuest (BD Biosciences).

### *Ag presentation to $CD4^+$ T cells*

Peripheral blood leukocytes from healthy donors were cultured for 8–10 d with TT peptide (3 μg/ml) and IL-2 (50 EU/ml) to increase the pool of TT-specific T cells. Autologous pDCs were cultured overnight at 37°C either with 20 μg/ml NPs containing FITC-TT and R848 or with 4 μg/ml soluble R848 together with soluble TT peptide. The pDCs were then added to the prestimulated peripheral blood leukocytes in a ratio of 1:10. After 4 d, 1 μCi (0.037 MBq)/well [$^3$H]thymidine (MP Biomedicals, Amsterdam, The Netherlands) was added to measure proliferation. [$^3$H]Thymidine incorporation was measured with a beta counter.

### *gp100-specific activation of $CD8^+$ T cells*

pDCs from HLA-$A2.1^+$ donors were loaded with different concentrations of soluble $gp100_{280–288}$ short peptide, irrelevant peptide (either $gp100_{154–167}$ or $tyrosinase_{369–376}$), or $gp100_{272–300}$ long peptide in 96-well round-bottom plates ($7 \times 10^3$ cells/well). After ∼1 h, allogeneic $CD8^+$ $gp100_{280–288}$-specific T cells (26, 27) ($5 \times 10^4$ cells/well) and 4 μg/ml R848 (where applicable) were added. After overnight incubation, CD69 expression by the $CD8^+$ $gp100_{280–288}$-specific T cells was measured by flow cytometry using PE-Cy5–conjugated mouse anti-human-CD69 (BD Pharmingen); IFN-γ production was measured using a standard sandwich ELISA (Pierce Endogen).

Furthermore, pDCs ($1 \times 10^4$ cells/well) from HLA-$A2.1^+$ donors were precultured for 2 h with 6.6 μM $gp100_{280–288}$ short peptide and 4 μg/ml R848, 16.6 μM $gp100_{272–300}$ long peptide and 4 μg/ml R848, or 20 μg/ml NPs containing $gp100_{272–300}$ and R848. $gp100_{280–288}$-specific $CD8^+$ T cells ($5 \times 10^4$ cells/well) were added to the prestimulated pDCs and cultured overnight. CD69 expression by the $CD8^+$ $gp100_{280–288}$-specific T cells was measured by flow cytometry using the following Abs: mIgG1-PE, mIgG1-allophycocyanin, anti–CD8-allophycocyanin, and anti–CD69-PE (BD Pharmingen). Proliferation was measured after 4 d of culturing by adding [$^3$H] thymidine and measuring incorporation with a scintillation counter.

### *Cytokine detection*

pDCs were stimulated with 20 μg/ml R848 containing NPs, 10 ng/ml recombinant human IL-3, or 4 μg/ml R848 overnight at 37°C at a concentration of $1 \times 10^6$ pDCs/ml in a 96-well round-bottom plate. After 16 h of activation, supernatant was collected, and IFN-α and IL-6 production was analyzed by ELISA using murine monoclonal capture and HRP-conjugated anti–IFN-α detection Abs (Bender MedSystems, Vienna, Austria) and anti–IL-6 Abs (Sanquin, Amsterdam, The Netherlands), respectively. TNF-α production was measured using a human TNF-α ELISA kit according to the manufacturer's protocols (BD Biosciences).

The cytokine profile was analyzed by incubating $1 \times 10^4$ prestimulated pDCs with $1 \times 10^5$ TT-stimulated peripheral blood leukocytes and collecting supernatant after 3 d. Production of the cytokines IL-5, IL-6, IL-10, TNF-α, and IFN-γ was analyzed with a human Th1/Th2 Multiplex kit (BenderMed Systems) according to the manufacturer's instructions.

*Cytokine production by pDCs targeted in PBMCs.* A total of $3 \times 10^6$ PBMCs was stimulated with 20 μg/ml NPs containing R848 and FITC and incubated at 37°C. After 6 h, brefeldin A (Sigma-Aldrich) was added to the PBMCs at 2 μg/ml and cells further cultured at 37°C. The next day, cells were harvested, and surface receptors and subsequently intracellular IFN-α and TNF-α (BD Pharmingen) were stained and analyzed by flow cytometry.

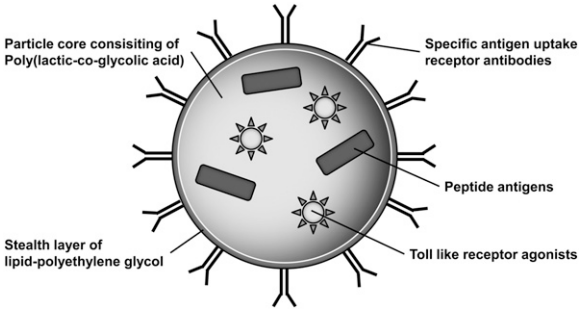


**FIGURE 1.** Schematic depiction of NP. The core of the particle consists of PLGA and comprises variable content, such as an Ag and TLR agonist, as depicted in figure. The particles are shielded by a lipid–polyethylene glycol layer. Biotinylated Abs specific for different Ag uptake receptors are coupled to the particles via streptavidin.

### *Statistics*

All data are expressed as the mean ± SEM. The data were analyzed by one-way ANOVA followed by either Newman–Keuls multiple comparisons test or Dunnett multiple comparisons test.

## Results

### *NPs targeted to different receptors are specifically taken up and degraded by pDCs*

Human pDCs express the receptors DEC-205, DCIR, BDCA-2, and CD32 but not DC-SIGN (7, 8). We investigated whether receptors expressed by pDCs can be used for receptor-specific delivery of nanocarriers and whether pDCs bind, take up, and process Ags targeted to these receptors. To this end, pDCs were incubated with Ab-coated PLGA NPs (Fig. 1) encapsulating either FITC-TT or DQ-BSA (Table I). After incubation for 1 h with FITC-TT–containing NPs at 4˚C, pDCs specifically bound DEC-205–NPs as well as BDCA-2–NPs, but only minimally bound DCIR-NPs and CD32-NPs compared with control DC-SIGN–NPs (Fig. 2A). The targeted NPs were taken up by pDCs after overnight incubation at 37˚C, as demonstrated by the percentage of $FITC^+$ cells, which even reached statistical significance for BDCA-2–NPs (Fig. 2B). We also performed a kinetic analysis of binding and uptake ranging from 1 h to overnight incubation. We observed that the binding (4˚C) of the different NPs over a time course of 16 h was low (Supplemental Fig. 1A). However, we did observe that the DC-SIGN–coated particles bound at a lower rate compared with the NPs targeted to receptors expressed by pDCs. For the uptake (37˚C) of the different NPs, pDCs incubated with BDCA-2–NPs showed a high total percentage of $FITC^+$ pDCs, whereas for the other receptors, the percentage of $FITC^+$ pDCs increased at a lower rate. The lowest percentage of $FITC^+$ pDCs was observed for the negative control DC-SIGN–NPs (Supplemental Fig. 1B). The relatively low percentage of $FITC^+$ cells could potentially be attributed to quenching of FITC in endosomal compartments, as FITC is a pH-sensitive dye, or by shielding by the PLGA surrounding the fluorescent Ag. This notion was supported by the observation that upon in vitro degradation of PLGA, thereby releasing its fluorescent content, fluorescence levels increased 150-fold (23).

Additional evidence for NP internalization was obtained by confocal microscopy. In line with the results obtained by flow cytometry, pDCs targeted via DEC-205, DCIR, BDCA-2, and CD32 had all taken up FITC-TT–containing PLGA NPs (Fig. 2C). Z-stack analysis confirmed that the FITC signal was located within the pDCs (data not shown). These data demonstrate that pDCs can bind and take up NPs coated with Abs specific for DEC-205, DCIR, BDCA-2, and CD32.

To investigate whether human pDCs are able to process particulate Ag delivered via surface receptors, we made use of the model protein DQ-BSA. DQ-BSA is self-quenched; quenching is relieved upon release of DQ-BSA from the NPs within the cell and subsequent processing into fluorescent peptides by cellular proteases. Incubation of pDCs with DQ-BSA–containing PLGA NPs showed an increase in fluorescence over time as measured by spectrophotometry (Fig. 2D). We did not observe, however, a difference of Ag degradation by pDCs targeted via the different receptors, nor with the control DC-SIGN–NPs. Notwithstanding, these findings demonstrate that pDCs process particulate Ags upon receptor-specific delivery.

### *Receptor targeting delivers particulate R848 to TLR7-containing endosomes*

Activation of pDCs is essential for the induction of optimal immune responses and T cell activation. Therefore, we investigated whether the encapsulated TLR7 agonist R848 is able to induce full pDC activation by studying surface receptor expression and cytokine production by pDCs. As expected and in line with our previous study (24), pDCs cultured with IL-3 were minimally activated, in contrast to pDCs stimulated with soluble R848 (Fig. 3A). An interesting observation made throughout our experiments is that a large proportion of pDCs died in vitro when cultured with DC-SIGN–NPs compared with the use of either soluble IL-3 or other receptor-targeted NPs (Supplemental Fig. 2A). pDCs targeted via DEC-205, DCIR, BDCA-2, and CD32 showed significant increased expression of the costimulatory molecules CD40, CD80, and CD86, whereas pDCs incubated with DC-SIGN–NPs did not (Fig. 3A). Remarkably, targeting of R848-containing PLGA NPs via DEC-205, DCIR, BDCA-2, and CD32 induced pDC activation to a similar degree as soluble R848, even though the concentration of particulate R848 is ~100 times lower than the concentration of soluble R848. Furthermore, the targeted delivery of NPs induced a trend toward upregulation of CD83, MHC I, MHC II, ICOS ligand, coinhibitory molecule PD-L1, and CCR7; however, these levels did not reach statistical significance compared with levels induced by control DC-SIGN–NPs (Fig. 3A and data not shown). The notion that encapsulated R848 induces full activation of pDCs is further supported by the production of inflammatory cytokines. Encapsulated R848 delivered via DEC-205, DCIR, BDCA-2, and CD32 induced significant production of IL-6, TNF-α, and IFN-α compared with the production induced by DC-SIGN–NPs (Fig. 3B, Table II). Surprisingly, upon targeting pDCs with R848-containing Ab-coated NPs, they secreted comparable amounts of cytokines as pDCs activated with soluble R848.

Next, we investigated whether we can also deliver the contents of the particles to pDCs in bulk PBMC cultures. To this end, we

Table I. Characteristics of PLGA NPs

| Samples | Ags (μg/mg PLGA) | R848 (μg/mg PLGA) | Size ± SD (nm) | PDI ± SD | ζ Potential ± SD (mV) |
|---|---|---|---|---|---|
| NP (DQ-BSA) | 18.8 ± 3.2 | — | 245.6 ± 16.7 | 0.178 ± 0.116 | −21.5 ± 2.4 |
| NP (FITC-TT + R848) | 8.2 ± 3.6 | 1.90 ± 0.5 | 267.5 ± 12.6 | 0.183 ± 0.136 | −22.7 ± 1.6 |
| NP (gp100 + R848) | 10.4 ± 2.9 | 1.82 ± 0.4 | 249.8 ± 14.6 | 0.123 ± 0.086 | −23.5 ± 1.9 |

PDI, Polydispersity index.

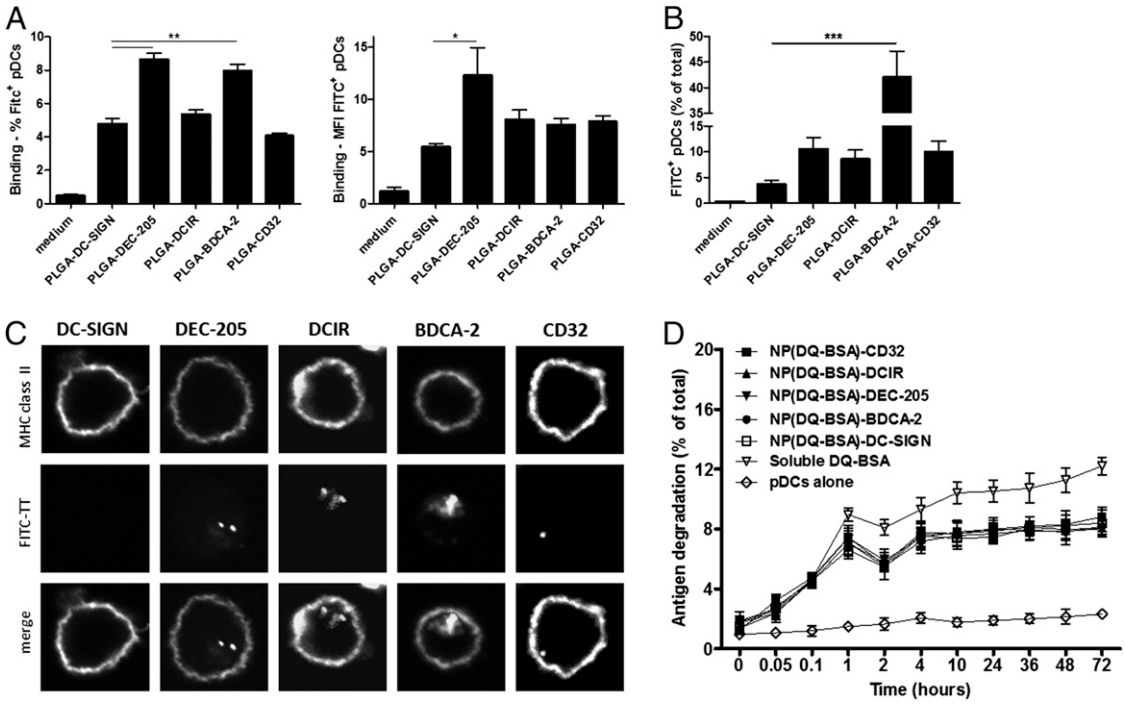


**FIGURE 2.** NPs targeted to different receptors are specifically taken up and degraded by pDCs. (**A**) Human pDCs were incubated with NPs containing FITC-TT for 1 h at 4˚C, washed, and analyzed by flow cytometry to determine binding of NPs. Figures show the percentage of FITC-positive cells and the MFI of the FITC signal. Data show the means ± SEM of six independent experiments. (**B**) pDCs were incubated overnight at 37˚C with NPs containing FITC-TT or with soluble IL-3. Uptake by pDCs was then analyzed by flow cytometry. Data represent at least four independent experiments with means ± SEM. (**C**) Uptake of encapsulated FITC-TT was further confirmed by confocal microscopy. Pictures show signal from surface MHC II (*top panel*) and FITC-TT (*middle panel*) of pDCs incubated with the different NPs encapsulating FITC-TT; the *bottom panel* shows the merge of the two. (**D**) pDCs were incubated with Ab-coated NPs encapsulating the self-quenching model protein DQ-BSA. After 4 h, the excessive NPs were washed away, and fluorescence was measured spectrophotometrically at various time points during 72 h. The fluorescent signal is depicted as the percentage of fluorescence relative to the total amount that could theoretically be generated upon full degradation of the ingested DQ-BSA. Data represent the mean value ± SEM from two independent experiments performed in quadruplicate. Statistical significance (A, B) was determined by one-way ANOVA followed by Dunnett multiple comparison test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

incubated PBMCs overnight with the different NPs. Interestingly, a clear population of pDCs could only be detected when the NPs could deliver their contents to pDCs via receptors they express (Supplemental Fig. 2B), but not for DC-SIGN–NPs. This indicates that the survival of pDCs in bulk culture, like in isolated pDC cultures, depends on the acquisition of specific stimuli. This finding is in accordance with previous studies demonstrating that pDCs need a stimulus to survive. Similar to the experiments with isolated pDCs, also in total PBMCs pDCs only produced the cytokines TNF-α and IFN-α when targeted through DEC-205, DCIR, BDCA-2, or CD32 (Supplemental Fig. 3). Thus, particulate R848 can reach TLR7 containing endosomes in pDCs, both isolated and in bulk, thereby inducing full pDC activation without affecting TLR-induced cytokine secretion.

### *Receptor targeting induces Ag-specific CD4$^+$ T cell activation and IFN-γ production*

Next, we investigated the capacity of pDCs to induce TT-specific CD4$^+$ T cell responses after targeted delivery of Ag encapsulated in Ab-coated PLGA NPs. pDCs preincubated with soluble TT and soluble R848 induced significantly higher proliferation of autologous TT-responsive T cells compared with unstimulated T cells (peripheral blood leukocytes only) (Fig. 4A). This indicates that pDCs efficiently process and present soluble TT to CD4$^+$ T cells, resulting in an Ag-specific proliferative recall responses. Importantly, pDCs incubated with DEC-205–, DCIR-, BDCA-2–, or CD32-targeted PLGA NPs containing both TT and R848 also induced significantly higher proliferation of autologous TT-responsive T cells compared with unstimulated T cells or T cells cocultured with DC-SIGN–NP-targeted pDCs (Fig. 4A). Notably, the recall response after targeted delivery of particulate TT and R848 is higher than after delivery of soluble Ags, even though a substantially lower amount of both Ag and TLR ligand is comprised in the NPs. Cytokine analysis of IFN-γ, TNF-α, IL-10, IL-6, and IL-5 revealed that TT-specific CD4$^+$ T cells cocultured with NP-targeted pDCs predominantly produced large amounts of IFN-γ (Fig. 4B). Taken together, pDCs incubated with NPs targeted to the receptors DEC-205, DCIR, BDCA-2, or CD32 are able to process and present the particulate Ag to CD4$^+$ T cells, inducing proliferation of Ag-specific CD4$^+$ T cells that predominantly produce IFN-γ.

### *pDCs cross-present soluble and receptor-targeted Ags*

Cross-presentation of exogenous Ags to cytotoxic CD8$^+$ T cells is essential for the induction of antitumor immunity (28). We studied the capacity of human pDCs to cross-present tumor Ags derived from soluble and particulate gp100 peptide. gp100 is a melanoma-associated tumor Ag that is commonly used as target Ag in immunotherapy of melanoma. pDCs presented the soluble short $gp100_{280–288}$ peptide, which directly binds extracellular HLA-A2 molecules, to $gp100_{280–288}$-specific CD8$^+$ T cells, as shown by the expression of the early T cell activation marker CD69 (Fig. 5A) and the secretion of IFN-γ (Fig. 5B). Moreover, pDCs effectively cross-presented the soluble long $gp100_{272–300}$ peptide, which needs to be taken up and processed before it can be loaded onto MHC I molecules. In accordance with previous studies (14, 29), we observed that the TLR7 agonist R848 enhanced the ability of the pDCs to

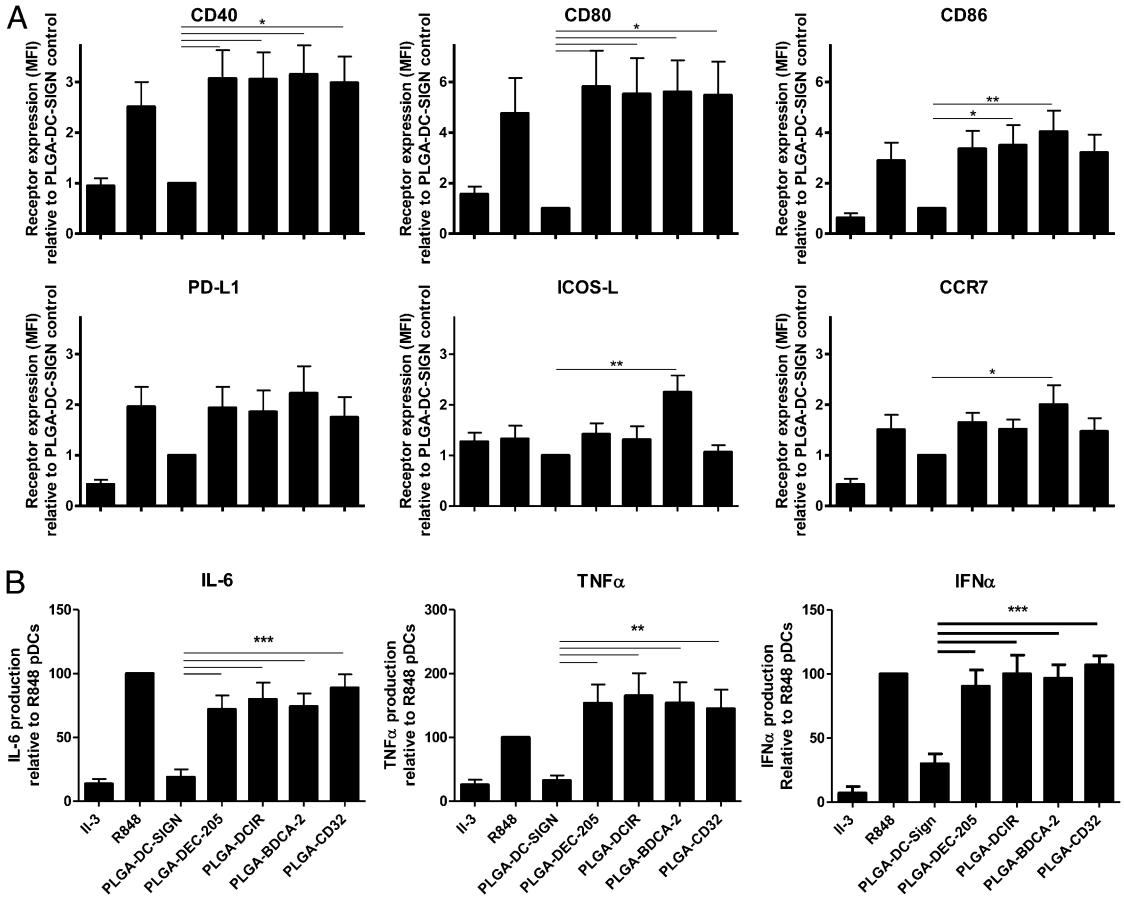


**FIGURE 3.** Targeted delivery of particulate R848 induces pDC activation. (**A**) Human pDCs were incubated overnight with NPs containing the TLR7 ligand R848. Expression of the surface molecules CD40, CD80, CD86, PD-L1, ICOS-1, and CCR7 were measured by flow cytometry. Figures show the relative receptor expression (MFI) compared with the DC-SIGN–NP control. (**A**) IFN-α, TNF-α, and IL-6 secretion of pDCs after overnight incubation with NPs was determined by ELISA. Data were collected from at least four independent experiments, and relative means ± SEM compared with R848-stimulated pDCs are shown. Statistical significance was determined by one-way ANOVA followed by Dunnett multiple comparison test. $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$. ICOS-L, ICOS ligand.

specifically prime IFN-γ–secreting $CD8^+$ T cells (Fig. 5B). To determine which uptake receptor could be harnessed for facilitating optimal Ag cross-presentation and cross-priming of $CD8^+$ T cells by pDCs, we used Ab-coated PLGA NPs with encapsulated $gp100_{272–300}$ peptide and R848. Fig. 5 illustrates that pDCs incubated with $gp100_{272–300}$ containing DC-SIGN–NPs only induced minimal $CD8^+$ T cell activation. Interestingly, $gp100_{272–300}$ containing NPs targeted to pDCs via DEC-205, DCIR, BDCA-2 or CD32 significantly increased gp100-specific T cell activation (Fig. 5C) and proliferation (Fig. 5D), even though the concentration of particulate $gp100_{272–300}$ is ~120 times lower than the concentration of soluble $gp100_{272–300}$. These data reveal that DEC-205, DCIR, BDCA-2, and CD32 are all possible targets for the delivery of Ags to human pDCs and that Ags delivered via these receptors are cross-presented by pDCs and lead to the priming of Ag-specific $CD8^+$ T cells.

Table II. Production of IL-6, TNF-α, and IFN-α by human pDCs after overnight incubation with IL-3, R848, or the different Ab-coated NPs

| | IL-6 (pg/ml) | TNF-α (pg/ml) | IFN-α (pg/ml) |
|---|---|---|---|
| IL-3 | 447 ± 69 | 564 ± 124 | 11 ± 4 |
| R848 | 5999 ± 2857 | 4287 ± 1358 | 773 ± 295 |
| Anti–DC-SIGN–NP | 1514 ± 605 | 860 ± 336 | 332 ± 167 |
| Anti–DEC-205–NP | 2948 ± 585 | 4036 ± 1541 | 736 ± 276 |
| Anti–DCIR-NP | 3270 ± 904 | 3429 ± 1220 | 661 ± 275 |
| Anti–BDCA-2–NP | 4276 ± 1612 | 3776 ± 1137 | 712 ± 285 |
| Anti–CD32-NP | 5186 ± 2012 | 3824 ± 1034 | 721 ± 279 |

Data are shown as mean ± SEM.

## Discussion

In this study, we set out to investigate which uptake receptor expressed by human pDCs can best be targeted for the delivery of Ag for (cross-)presentation. We showed that targeting DEC-205, DCIR, BDCA-2, and CD32 successfully delivered the content of NPs to pDCs, leading to specific activation, cytokine production, as well as (cross-)presentation of particulate Ag to both $CD4^+$ and $CD8^+$ T cells. Our findings further support the notion that human pDCs can cross-present exogenous Ag (9–11, 13, 14), a process essential for the induction of antitumor immunity (28). In this study, pDCs cross-presented $gp100_{272–300}$ peptide either in soluble form or encapsulated in NPs that were targeted to uptake receptors DEC-205, DCIR, BDCA-2, and CD32. Together, this puts human pDCs forward as interesting targets for the induction of potent adaptive immune responses against tumors. This is further underlined by our recent findings, in which vaccination with activated pDCs induced favorable immune responses and a significantly prolonged overall survival in a small cohort of melanoma patients (15).

After encountering soluble TLR ligands, human pDCs downregulate their surface expression of DCIR and BDCA-2, thereby reducing the internalization of Ags (6, 30). Although CD32 expression remains unchanged upon TLR-induced activation, Ag

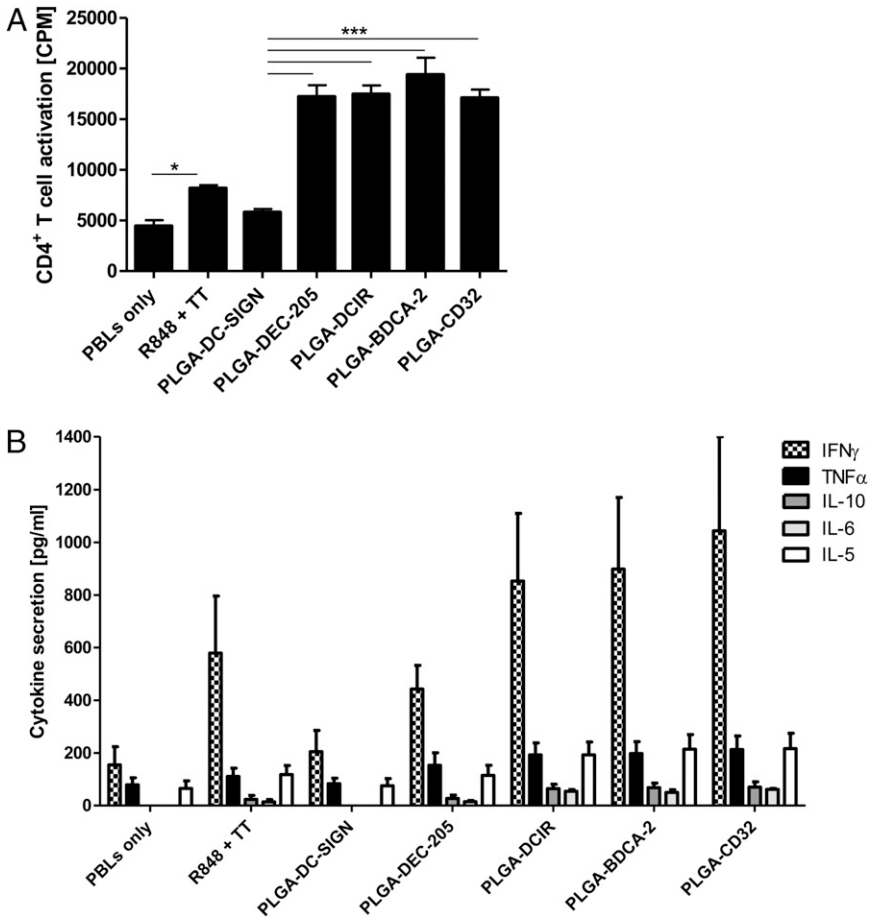


**FIGURE 4.** Targeted Ag delivery to pDC surface receptors induces Ag-specific $CD4^+$ T cell activation and IFN-γ production. pDCs were incubated overnight with NPs containing FITC-TT and R848 or a combination of soluble R848 and TT peptide as a control. Subsequently, autologous TT-responsive peripheral blood leukocytes were added. Four days later, T cell proliferation was measured by [$^3$H]thymidine incorporation (**A**), and cytokine production was determined in supernatants (**B**). Data show mean values ± SEM of three independent experiments. Statistical significance was determined by one-way ANOVA followed by Newman–Keuls multiple comparison test. $*p < 0.05$, $***p < 0.0001$.

internalization via this receptor is impaired (6). In contrast, DEC-205 expression increases upon pDC activation and still efficiently captures Ags for subsequent processing and presentation (8). However, the delivery of Ag without TLR ligands as adjuvants would likely induce tolerogenic responses, because immature pDCs minimally express costimulatory molecules that are needed for T cell activation (31, 32). Furthermore, our data support the importance of TLR ligands as adjuvants in the functional activation of $CD8^+$ T cells, as pDCs pulsed with Ag alone did not induce IFN-γ–secreting T cells. Therefore, the simultaneous delivery of both Ags and TLR ligands is critical for the successful induction of Ag-specific immune responses via uptake receptors in vivo. In accordance with earlier findings in monocyte-derived DCs, we found that the simultaneous delivery of coencapsulated Ags and TLR ligands is effective to achieve successful pDC activation as well as Ag (cross-)presentation and T cell activation (33–35).

Previously, we and others have shown that the cross-linking of uptake receptors modulated TLR-induced signaling. In those studies, the secretion of cytokines was determined after the cross-linking of receptors followed by the addition of soluble TLR ligands. Cross-linking CD32 on human pDCs did not inhibit TLR-induced IFN-α secretion (6, 36, 37); however, cross-linking DEC-205, DCIR, or BDCA-2 impaired TLR7- and/or TLR9-induced IFN-α secretion, and cross-linking DCIR and BCDA-2 also impaired TLR7- and/or TLR9-induced TNF-α production (7, 8, 38). The amplitude of cytokine secretion in response to TLR ligands can be regulated by signaling of the uptake receptors through ITAMs or ITIMs. Receptors that signal through ITAMs usually activate cells, whereas ITIM-bearing receptors inhibit cell activation. For the regulation of cytokine production by pDCs, this principle does not always hold true (reviewed in Ref. 39). For example, BDCA-2–induced signaling through the ITAM motif inhibited type I IFN secretion by pDCs (38, 40), as did DCIR-induced signaling through the ITIM motif (7). Interestingly, we did not observe such an inhibition of TNF-α or IFN-α secretion when R848 was delivered to pDCs via DEC-205, DCIR, BDCA-2, or CD32 in PGLA NPs. This might indicate that the timing of signaling through these receptors and the slow release of TLR ligands from NPs in the TLR7-containing endosomes circumvent the negative modulatory effect of receptor cross-linking. Furthermore, also the mode of entry of the TLR ligand, soluble versus particulate and targeted, is an important factor that deserves further investigation.

Delivery of NPs to pDCs via DEC-205, DCIR, BDCA-2, or CD32 resulted in comparable functional outcomes in our experiments. When opting for exclusive in vivo targeting of human pDCs, BDCA-2 would serve as an interesting receptor, as it is exclusively expressed by human pDCs. DEC-205 and CD32, in contrast, are expressed by a wider range of immune cells (41, 42) and therefore would be suited for a more general approach of Ag delivery. Additionally, we showed in this study that NPs can deliver Ag to pDCs via DCIR resulting in Ag cross-presentation. Recently, Klechevsky et al. (43) showed that targeting DCIR with fusion constructs specifically delivered Ags to various cell types including myeloid DCs, resulting in cross-presentation and cross-priming of $CD8^+$ T cells. In accordance with their study, we also observed that the addition of soluble R848 enhanced the capacity of pDCs to cross-prime $CD8^+$ T cells. However, in contrast to their and other studies, in this study, we used nanocarriers that enabled us to simultaneously deliver TLR agonists and Ags to human pDCs. This circumvents the need for systemic TLR agonist administration and the

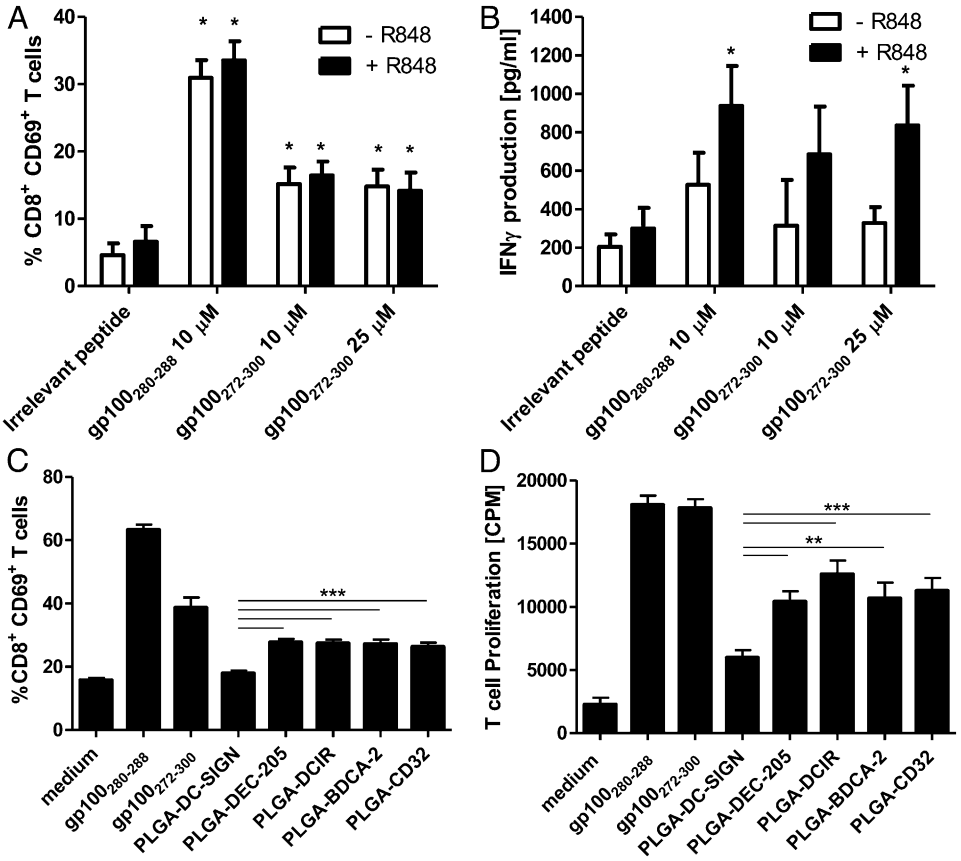


**FIGURE 5.** pDCs cross-present soluble Ag and receptor-targeted particulate Ag to $CD8^+$ T cells. (**A** and **B**) Specific $CD8^+$ T cell activation with soluble gp100 Ag. pDCs were incubated with 10 μM irrelevant peptide ($gp100_{154–167}$ or $tyrosinase_{369–376}$), 10 μM gp100 short peptide ($gp100_{280–288}$), or with 10 μM or 25 μM gp100 long peptide ($gp100_{272–300}$). Next, DCs were cocultured overnight with allogeneic $CD8^+$ T cells expressing a $gp100_{280–288}$-specific TCR in the presence of 4 μg/ml R848. Ag-specific T cell activation was assessed by analysis of CD69 expression (A) and IFN-γ production (B). Data show mean values ± SEM of at least four independent experiments. (**C** and **D**) Specific $CD8^+$ T cell activation with gp100 long peptide containing NPs. Human pDCs were preincubated with NPs containing long $gp100_{272–300}$ and R848 for 1 h. $gp100_{280–288}$-specific $CD8^+$ T cells were added to the prestimulated pDCs and cultured overnight. Ag-specific T cell activation was assessed by analysis of CD69 expression (C); proliferation was measured after 4 d by [$^3$H] thymidine incorporation (D). Data were collected from at least three independent experiments, and means ± SEM are shown. Statistical significance was determined by one-way ANOVA followed by Newman–Keuls multiple comparison test. $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$.

associated side effects (44–46). We believe that exploiting NPs that target such Ag uptake receptors that are expressed by both pDCs and myeloid DCs has great potential, as they interact synergistically, thereby increasing Ag-specific immune responses induced by these cells (47–50). Furthermore, we believe that nanocarriers, such as the PLGA particles described in this study, pose a powerful and versatile tool, as they can be decorated with different Abs for targeting of different cell populations. Moreover, these nanocarriers allow the incorporation of different substances, such as adjuvants for cell activation and fluorophores or radioactive labels for cell-tracking purposes. Additionally, the encapsulation of different forms of Ags (e.g., peptides, proteins, or glycolipids) ensures the activation of Ag-specific $CD4^+$ and $CD8^+$ T cells as well as NKT cells.

In conclusion, the CLRs DEC-205, DCIR, and BDCA-2 as well as the FcR CD32, with their distinct properties regarding expression by different immune cells, are all potential targets for the delivery of NPs to pDCs in vivo. Our data indicate that PLGA NPs might be a promising tool to efficiently load pDCs with tumor Ag and simultaneously activate them by coencapsulated TLR ligands, resulting in fully activated pDCs capable of stimulating antitumor responses via both production of IFN-α and direct induction of Ag-specific $CD4^+$ and $CD8^+$ T cell responses. These exciting findings pave the way to actively recruit human pDCs for cellular in vivo immunotherapeutic strategies.

## Disclosures

The authors have no financial conflicts of interest.

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