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Clinically applicable CD304 (BDCA-4)$^+$ human plasmacytoid dendritic cells – a new era in dendritic cell–based immunotherapy





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Introduction

Dendritic cells (DCs) constitute a family of antigen-presenting cells defined by their morphology, phenotype, and unique capacity to process exogenously encountered antigens and to present them to naive T cells. Following infection or inflammation, DCs undergo a complex process of maturation and migrate to lymph nodes to present antigens and activate T cells. This decisive role in inducing immunity was the rationale for DC-based immunotherapy, in which DCs loaded with tumor antigens were injected into cancer patients to stimulate T cells to eradicate tumors[1,2]. Now, after more than a decade, we know that monocyte-derived DCs (Mo-DCs) can induce immune responses in a significant number of patients, in particular when both CD4$^+$ and CD8$^+$ T cell responses are obtained, as also recently shown by our DC vaccination studies[3]. Nevertheless, the number of objective clinical responses has been limited, hampering its implementation as a novel form of standard treatment[4]. Although the evidence of clinical responses is still scarce, expectations are high because the clinical responses that are induced are often long lasting. It remains unclear whether DCs differentiated *ex vivo* from monocytes or CD34$^+$ progenitors are the optimal source of DCs for the induction of potent immune responses[5]. Because of the limited number of naturally circulating DCs, virtually all vaccination studies for more than a decade have been based on DCs differentiated *ex vivo* from monocytes or CD34$^+$ progenitors. Recently, it was proposed that these "artificial" DCs may be less effective than their natural counterparts that circulate in the blood because of the extensive culture period. Moreover, compounds required to differentiate precursors into DCs might negatively affect DC function. Especially their migratory capacity towards T cell areas in lymph nodes might decrease due to exhaustion of the DCs. We reported on the first clinical study of therapeutic vaccination against cancer exploiting naturally occurring plasmacytoid DCs (pDCs) purified by using MACS® Technology[6].

Materials and methods

Patient characteristics and clinical protocol

Sixteen distant metastatic melanoma patients (according to the 2001 American Joint Committee on Cancer staging system) were enrolled in this feasibility study. The primary endpoint was toxicity related to vaccination and immunological response. The trial was approved by the local Institutional Review Board (Committee on Research Involving Human Subjects Arnhem-Nijmegen) and in accordance with the declaration of Helsinki. Written informed consent was obtained from all patients. The clinical trial registration number is NCT01690377.[6]

Isolation of pDCs with the CliniMACS® System

The pDCs were directly isolated from apheresis products using the fully enclosed CliniMACS® Plus Instrument and GMP-grade magnetic bead–coupled Anti-BDCA-4 antibodies (CliniMACS CD304 (BDCA-4) Reagent).[6] The purity of pDCs after immunomagnetic isolation was determined by flow cytometry. The following primary monoclonal antibodies (mAbs) and the appropriate isotype controls were used: CD45-FITC, CD303 (BDCA-2)-PE and CD123-APC (all Miltenyi Biotec).[6] The procedure resulted in clinically applicable purified pDCs, which had an average purity of 75% and a yield between 13×10^6 and 33×10^6 cells (fig. 1). Following apheresis and CliniMACS Cell Separation, pDCs were cultured overnight at a concentration of 10^6 cells/mL in X-VIVO™ 15 medium

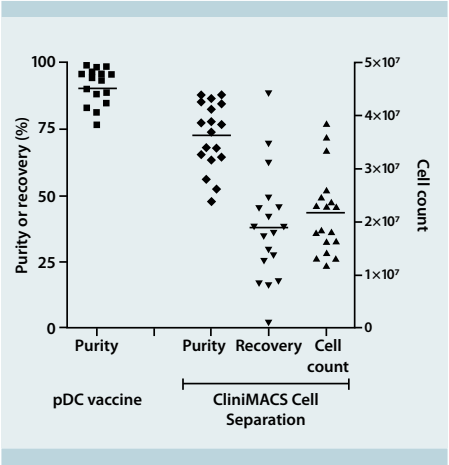

Figure 1 pDC isolation based on the CliniMACS System is feasible. The right panel in the graph shows the purity (%), recovery (%), and number of cells obtained in 18 isolations from patient samples. The left panel shows the purity (%) of the administered pDC vaccines. The pDC vaccines were checked for contaminating cells by determining the presence of T cells (CD3) and B cells (CD19). T and B cells were routinely less than 3% and 10% of all cells, respectively (data not shown). Data were adapted from reference 6.

(Lonza) containing 2% pooled human serum, supplemented with 10 ng/mL recombinant human interleukin-3. The pDCs were subsequently activated for 6 hours by addition of FSME-IMMUN® (1:10 v/v) (Baxter AG). During the last 3 hours of activation, pDCs were loaded with the melanoma-associated peptides $gp100_{154}$, $gp100_{280}$, and tyrosinase[7]. Purity of the vaccine was determined by flow cytometry (fig. 1). The peptide-loaded pDCs were administered intranodally in a clinically tumor-free lymph node region under ultrasound guidance[8].

RNA isolation and quantitative PCR

To determine mRNA expression of IFN-α and RIG-I, blood was drawn before vaccination and at 4 and 24 hours after vaccination. RNA isolation from patient samples was performed using the PAXgene® Blood RNA Kit (Qiagen) according to the manufacturer's instructions. RNA isolations from PBMCs from healthy volunteers were done using the ZR RNA isolation kit (Zymo Research) according to the manufacturer's instructions. mRNA levels for the genes of interest were determined by quantitative PCR (qPCR) with a Bio-Rad® CFX apparatus (Bio-Rad) with SYBR® Green (Applied Biosystems). Analysis was done using the Bio-Rad CFX Manager™ v1.6 software, and expression levels were determined relative to PBGD expression.[6]

^{111}In-oxinate labeling and scintigraphic imaging

pDCs activated for 6 hours with FSME were labeled with 5 MBq ^{111}In-oxinate (GE Healthcare) in 0.1 M Tris-HCl (pH 7.0) for 15 minutes at room temperature as described previously[9]. *In vivo* planar scintigraphic images (256×256 matrix, 174 and 247 keV ^{111}In photopeaks with 15% energy window) of the injection depot and corresponding lymph node basin were acquired with a gamma camera (Siemens e.cam) equipped with medium energy collimators, 15 minutes, 24 hours, and 48 hours after injection. Migration was quantified by region-of-interest analysis of the individual nodes visualized on the images and expressed as the fraction of ^{111}In-labeled DCs that had migrated from the injection depot to following lymph nodes after 15 minutes, 24 hours, and 48 hours.[6]

Immunomonitoring of patients

Four days after the third vaccination, a DTH skin test was performed and frequencies of anti-vaccine $CD8^+$ T cells in the blood were estimated using mixed lymphocyte peptide cultures (MLPCs) as described previously[6].

From positive DTH sites, half of the biopsy was manually cut and cultured in Gibco® RPMI 1640 (Life Technologies) containing 7% HS and IL-2 (100 U/mL, Proleukin®, Chiron). Every 7 days, half of the medium was replaced by fresh medium. After 2 to 5 weeks of culturing, T cells were tested for specificity against gp100 and tyrosinase. DTH-derived cells were stained with tetrameric MHC complexes containing the $gp100_{154\text{-}167}$, $gp100_{280\text{-}288}$, or $tyrosinase_{369\text{-}376}$ peptides (Sanquin) combined with CD8 staining.

For the MLPCs, PBMCs isolated before and after one cycle of three pDC injections were thawed, divided into three groups, and incubated for 1 hour at room temperature in Gibco IMDM (Life Technologies) with 1% HS and 2 µM of the peptides $tyrosinase_{369}$ (YMDGTMSQV), wild type $gp100_{154}$ (KTWGQYWQV), or wild type $gp100_{280}$ (YLEPGPVVTA). These pulsed cells were then washed, pooled, and distributed at 2×10^5 cells/0.2 mL in round-bottom microwells in IMDM with 10% HS, L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L), 1-methyl-L-tryptophan (100 µM), IL-2 (20 U/mL), and IL-7 (10 ng/mL). On day 7, 50% of the medium was replaced by fresh medium containing IL-2 and peptides at 4 µM. Tetramer labeling was performed on day 14 as described previously.[6] Anti-$gp100_{154}$ T cell clones were derived that represented either the spontaneous anti-gp100 T cells present prior to vaccination in patients 2, 5, 6, 10, and 11, or the pDC-induced anti-gp100 T cells present after vaccination in patients 1, 4, 8, and 12. Tetramer-positive $CD8^+$ T cells were sorted at 1 cell/well and restimulated weekly with irradiated HLA-$A2^+$ EBV-transformed B cells pulsed with the $gp100_{154}$ peptide at 2 µM, and irradiated allogeneic PBMCs as feeder cells, in medium supplemented with IL-2 and IL-7.

Matched historical controls

Matched historical controls were identified from records of metastatic melanoma patients from the Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands), The Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital (Amsterdam, The Netherlands), and University Hospital Essen (Essen, Germany) who had received first-line dacarbazine (DTIC) chemotherapy at 850–1000 mg/m² i.v. at 3 weekly intervals, between March 2000 and March 2010[6].

Statistical analysis

Significant differences from controls were determined according to paired Student's t-test or by one-way ANOVA analysis followed by the Tukey's post-hoc test. Differences between pre- and post-vaccination were evaluated with a Wilcoxon signed-rank test. Kaplan-Meier probability estimates of overall survival were calculated, and statistical differences between the survival of the groups were determined with a log-rank test. Statistical significance was defined as $p<0.05$. SPSS 19.0 was used for survival analyses.

Results and discussion

Clinical study with natural pDCs

We have embarked on exploiting pDCs that naturally circulate in the peripheral blood for cancer immunotherapy. Although it is well known that intratumoral pDCs, when inactive, are associated with tumor progression, activated interferon alpha (IFN-α)-producing pDCs can activate NK cells and induce antigen-specific T and B cell responses. Our finding that commonly used prophylactic vaccines against infectious diseases could simply be used as adjuvants to activate pDCs to secrete significant amounts of IFN-α has certainly expedited the use of naturally circulating DCs in the clinic[10].

We initiated the first study exploiting pDCs to treat advanced stage IV melanoma patients. Designed as a safety study, we treated 15 patients in three cohorts with dose-escalating pDC vaccines ranging from 0.3 to 3 million cells per injection. The vaccines were well tolerated and no signs of severe toxicity (common toxicity criteria grade 3–4) were observed. Six vaccinated patients developed grade 1 flu-like symptoms and one patient reported grade 2 non-treatment related pain resulting from progressive subcutaneous metastasis. In none of the vaccinated patients did we detect antibodies to the murine antibody used during the isolation procedure (data not shown). We conclude that it is feasible and safe to administer activated and tumor-peptide loaded pDCs to patients. As the number of cells was low and no data existed on their migratory capacity *in vivo*, we decided to inject the cells intranodally to maximize the possibility to raise an immune response. Forty-eight hours after intranodal injection, a significant proportion of ^{111}In-labeled pDCs remained at the injection site, and distinct amounts were detected in distant lymph nodes (fig. 2A). To verify whether activated pDCs secreted significant quantities of type I IFNs *in vivo*, we investigated gene expression of IFN-α and the IFN-induced gene RIG-I in blood mononuclear cells before and after vaccination. We compared these expression levels to those of cells from healthy individuals. In line with their ability to secrete large amounts of type I IFNs, transcription of IFN-α was clearly induced four hours after vaccination and decreased 20 hours later (fig. 2B), indicating a temporal systemic induction of type I IFNs. As expected,

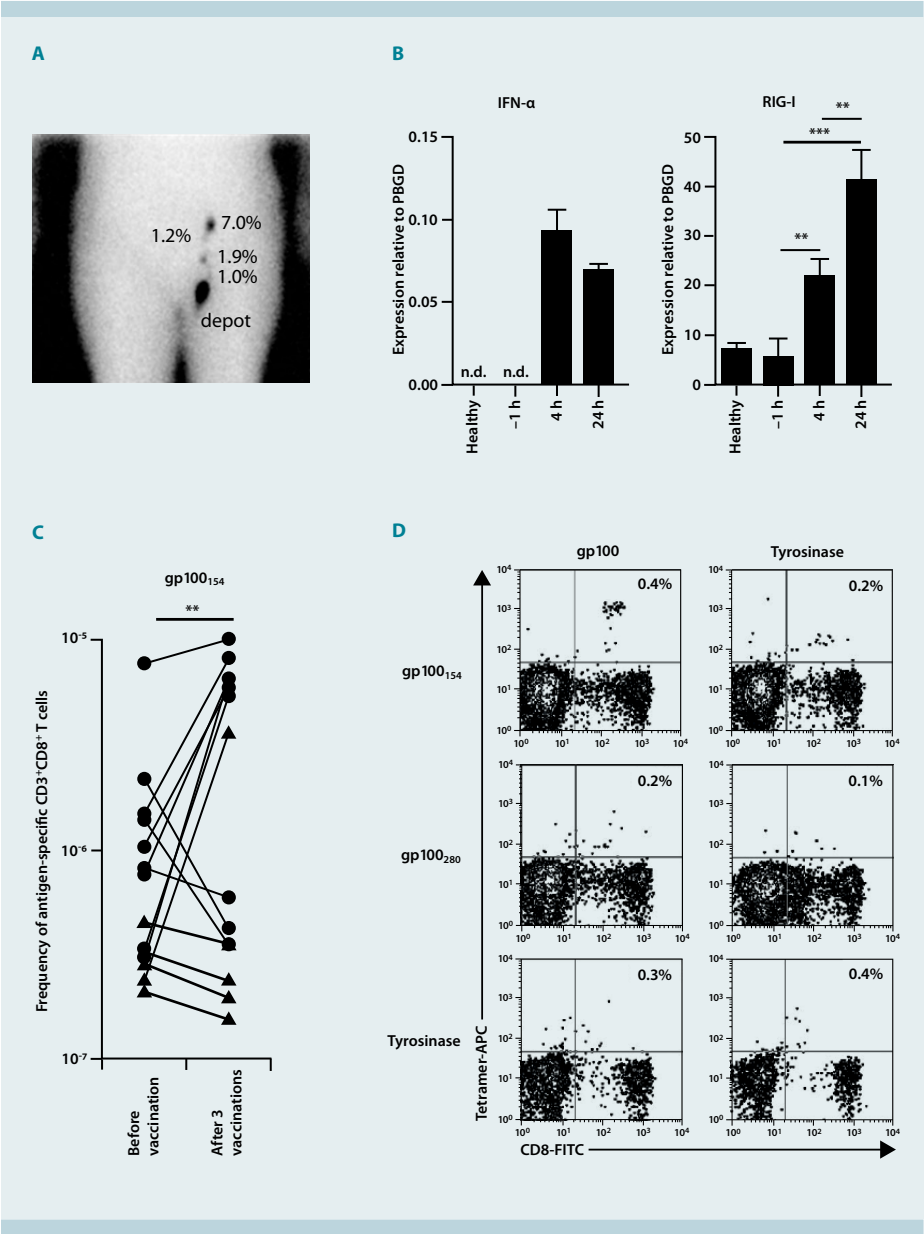

Figure 2 **Activated pDCs are mature and migrate to distinct lymph nodes *in vivo*.** **(A)** Migration and biodistribution of ^{111}In-labeled pDCs visualized by scintigraphical imaging. Forty-eight hours after administration, 11% of injected pDCs were distributed over up to 4 distant lymph nodes away from the injection depot. **(B)** The graphs show the IFN-α and RIG-I gene expression levels in the blood relative to PBGD expression at the indicated times after each vaccination. Healthy indicates steady-state expression levels of indicated genes in 8 healthy individuals (n.d.: not detected; ** $p<0.01$; *** $p<0.001$). **(C)** pDC vaccine–related CD8$^+$ T cell responses were detected in the blood after MLPCs. The graph shows the frequencies of gp100$_{154}$ tetramer$^+$CD8$^+$ T cells in the blood before and after one cycle of vaccination. **(D)** pDC vaccine-related CD8$^+$ T cell responses were detected in biopsies taken from DTH skin tests. Two weeks after the third pDC injection, a DTH skin test was performed by intradermally injecting pDCs loaded with either the gp100 or the tyrosinase peptides. Biopsies taken 2 days later were cultured for 3–4 weeks in low-dose IL-2, and proliferating T cells were stained with specific tetramers. Dot plots show DTH-infiltrating lymphocytes stained with gp100$_{154}$ tetramers (** $p<0.01$). Data were adapted from reference 6.

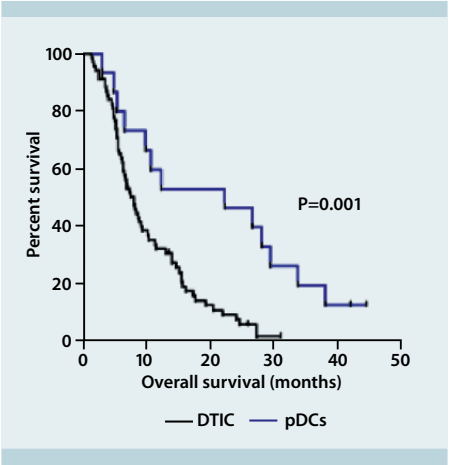

Figure 3 pDC vaccination improves overall survival. Clinical outcome of pDC vaccination was compared to a group of carefully matched historical control patients who received dacarbazine as first-line treatment. Median overall survival data showed a remarkable improvement compared to matched control patients: 22.0 months versus 7.6 months. Furthermore, the overall survival of pDC-vaccinated patients was significantly improved compared to matched controls. Statistical significance between the survival of the groups was determined by a log-rank test, p=0.001. Data were adapted from reference 6.

we observed increases in the expression of the IFN-induced gene RIG-I after four hours, which further increased after 24 hours (fig. 2B). We used tetramers to detect the presence of tumor antigen–specific $CD8^+$ T cells in blood, and in biopsies taken from skin delayed-type hypersensitivity (DTH) reactions. *Ex vivo* tetramer staining was negative both before and after vaccination (data not shown). Therefore, we resorted to an *in vitro* restimulation of blood mononuclear cells under limiting dilution conditions over two weeks with the three antigenic peptides, before screening all microcultures for the presence of $CD8^+$ $tetramer^+$ cells. This procedure allowed us to estimate the frequencies of blood $CD8^+$ T cells that recognize a given antigen and proliferate *in vitro* in response to this antigen. As shown in figure 2C, 7 out of 15 patients showed a significant increase in the frequency of $gp100_{154}$-specific $CD8^+$ T cells. Furthermore, we detected anti-$gp100_{154}$ $CD8^+$ T cells in DTH biopsies in two patients after vaccination (fig. 2D). We conclude that vaccination with small numbers of peptide-loaded and activated pDCs can induce tumor-specific $CD8^+$ T cell responses in metastatic melanoma patients[6].

Although the initial endpoint of this study was safety and feasibility, we obtained some surprising clinical results: the median overall survival showed a remarkable improvement compared to matched control patients: 22.0 months (95% CI: 1.8–42.2) versus 7.6 months (95% CI: 5.8–9.4) (fig. 3). We observed highly improved clinical responses with these freshly isolated naturally circulating DCs when compared to conventional Mo-DCs: 7 out of 15 stage IV melanoma patients are still alive two years after start of treatment. Interestingly, we obtained these results even with a tenfold lower dose, demonstrating the potency of these natural pDCs. Interestingly, Celli *et al.* recently reported that as little as one hundred DCs are needed to elicit a T cell response, indicating that indeed perhaps only small amounts of DCs are needed for the induction of anti-tumor responses[11].

Conclusion

Taken together, our results demonstrate that 1) the pDCs injected into a single lymph node distributed into downstream nodes, 2) after each vaccination even small numbers of injected pDCs induced a systemic type I IFN signature, and 3) injected pDCs primed tumor-specific $CD8^+$ T cells. These findings indicate that it is worthwhile to further explore the potential of pDCs and other naturally circulating DCs for cancer immunotherapy. It will also be interesting to see if other DC subsets, such as the myeloid DCs, are equally or even more effective.

Acknowledgments

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