IFN-α Skews Monocytes into CD56+ expressing Dendritic Cells with Potent Functional Activities *in vitro* and *in vivo*

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Short title: IFNα skews monocytes into CD56+ dendritic cells

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Abstract

The anti-tumor effect of IFN-α is mediated by the activation of CTLs, NK cells and the generation of highly potent antigen-presenting dendritic cells (IFN-DCs). Here, we show that IFN-DCs generated *in vitro* from monocytes express CD56 on their surface, a marker which has been thought to be specific for NK cells. FACS analyses of CD56+ and CD56- IFN-DCs showed a nearly identical pattern for most of the classical DC markers. Importantly, however, only CD56+ IFN-DCs exhibited cytolytic activity up to 24% that could almost completely be blocked (-81%) after co-incubation with anti-TRAIL. Intracytoplasmatic cytokine staining revealed that the majority of IFN-DCs independently of their CD56 expression were IFN-γ positive as well. In contrast, CD56+ IFN-DCs showed stronger capacity in stimulating allogenic T cells compared to CD56- IFN-DC.

Based on these results, five patients with metastasized medullary thyroid carcinoma were treated for the first time with monocyte-derived tumor antigen-pulsed IFN-DCs. After a long term follow-up (in mean 37 months) all patients are alive. Immunohistochemical analyses of DTH skin reaction showed a strong infiltration with CD8+ cells. In two patients no substantial change in tumor morphology was detected. Importantly, by analyzing peripheral blood mononuclear cells, these patients also showed an increase of antigen-specific IFNγ-secreting T cells.

In summary, we here describe for the first time that cytotoxic activity of IFN-DCs is mainly mediated by an IFN-DC subset showing partial phenotypic and functional characteristics of NK cells. These cells represent another mechanism of the anti-tumor effect induced by IFN-α.

abbreviations: DC dendritic cell, DTH delayed-type hypersensitivity; IFN-DC interferon-α generated DC, IKDC interferon-producing killer DCs, MTC medullary thyroid carcinoma

Introduction

IFN-α is a cytokine belonging to type I IFNs and has been most frequently used in patients with certain types of cancer. For example, patients with hematologic malignancies and solid tumors such as melanoma, renal carcinoma, Kaposi's sarcoma and neuroendocrine malignancies have been treated. Despite many years of work in preclinical as well as in clinical settings, the mechanisms underlying the IFN-induced antitumor response are not well understood. It was thought that the direct inhibitory effects on tumor cell growth and function were the major mechanisms of the IFN-mediated antitumor responses in patients. However, early experiments in mouse tumor models have shown that IFN-α plays an important role in the activation of a long-lasting antitumor response [1]. Subsequent studies have also provided evidence for a role of type I IFNs in the differentiation of the Th1 subset, as well as in the generation of CTL and in the promotion of the *in vivo* proliferation and survival of T cells [2-5]. In parallel to these mechanisms, natural killer cells (NK cells) may also be activated by IFN-α leading to a strong cytolytic activity of these cells [6]. On the *in vivo* level, the magnitude of naturally occuring IFN-α is secreted from IFN-producing cells (IPCs), now known as plasmocytoid dendritic cells (pDCs) [7]. Therefore, DC may directly trigger cytolytic activity of NK cells. On the other hand, the interaction between NK cells and DCs has been described to be reciprocal and could lead to the maturation and functional activation of monocyte-derived DCs [8-10].

In mice, a new immune cell-subset, termed as interferon-producing killer DCs (IKDCs) has been described for the first time [11,12]. These cells are hybrid cells that unify DC- and NK-functions [11,12]. IKDC are distinct from conventional DCs and plasmocytoid DCs as they show the molecular expression profile of both NK cells and DCs. They produce substantial amounts of interleukin-12 (IL-12) and IFN-γ, depending on activation stimuli. Most interestingly, IKDCs directly kill typical NK targets mediated by the TNF-related apoptosis-inducing ligand (TRAIL) pathway. Most recently, it has been proposed that these cells may belong to the NK cell lineage and could represent a subtype of activated NK cells with the capability to gain APC-function [13-15]. Up to now, IKDCs have only been described in rodents, however, not yet in humans.

We here describe that monocyte-derived DCs generated with IFN-α and GM-CSF, express high levels of CD56 on their surface, a marker thought only to be present on NK cells [16] and NKT cells [17]. Former studies already stated that IFN-α-generated DCs reveal direct cytolytic activity [18,19], however, without identifying a certain DC subset. In addition, Banchereau and coworkers already reported on the *in vivo* use of CD34+ progenitors which were activated

with type I interferon and which were used for the treatment of stage IV melanoma patients [20] leading to the induction of tumor antigen-specific recall memory CD8+ T cells in the majority of patients [20]. In our present study, we describe that CD56+ monocyte-derived IFN-DCs reveal a direct cytolytic activity *in vitro*, whereas CD56- IFN-DCs do not. In addition, we demonstrate that the cytolytic activity is mediated by the expression of tumor necrosis factor-related apoptosis inducing ligand (TRAIL). Intracytoplasmatic cytokine staining revealed that most of CD56+ IFN-DCs as well as CD56- IFN-DCs are IFN-γ-producing as too. Mixed leucocyte reactions, however, revealed a slightly stronger capacity in stimulating allogenic T cells compared to CD56- IFN-DCs. For evaluating the clinical effectiveness of monocyte-derived IFN-α-generated DCs, we also used these cells for the first time for the treatment of a small number of cancer patients with metastasized medullary thyroid carcinoma (MTC). MTC belongs to the group of neuroendocrine cancers specifically characterized by the expression of calcitonin, 32 amino acid long tumor-specific peptide. In the past, we aleady described that calcitonin may serve as specific tumor antigen useful for vaccination strategies in patients with MTC [21]. Following IFN-DC treatment, we now report that two patients do not show any substantial changes in tumor morphology after long-term follow-up.

Material and Methods

Cell separation and dendritic cell generation

For *in vitro* experiments PBMCs from volunteers were used (study number of the local ethical review board: 2608/05). All magnetic separations were performed using microbead technology (Miltenyi Biotec, Bergisch Gladbach, Germany). Untouched $CD14^+$ peripheral blood monocytes were immunomagnetically purified by using a depletion cocktail of biotinylated antibodies and anti-biotin mAb-conjugated microbeads. As verified by flow cytometry analysis a purity of >98% of $CD14^+$ cells was obtained by a secondary purification using anti-CD3 and CD56 microbeads. Monocytes were cultured in RPMI 1640 medium (Life Technologies, Heidelberg, Germany) at 2.5×10^6/ml in the presence of GM-CSF (1000 U/ml; Leukine®, Immunex, Seattle, WA) and IFN-α (1000 U/ml; Roferon-A®, Roche, Mannheim, Germany) without fetal calf serum. After 3 days of culturing, CD56+ IFN-DCs were purified with CD56 mAb-conjugated microbeads leading to a purity of the $CD56^+$ cells of ≥98%. CD56+ and CD56- IFN-DCs were then used for further analyses. For *in vitro* comparison IL4-DCs were generated as formerly described [22].

For *in vivo* use in cancer patients, DCs were generated in a GMP facility (permission received from the Regional Council Düsseldorf: No. 24.30-04.01-001) from monocytes from each patient from an initial leucapheresis (≥ 4.5×10^9 nucleated cells) followed by Ficoll density gradient centrifugation and an adherens step (1-2.5 x 10^8 DCs / 15 ml RPMI for 2 hours). Adherent cells were cultured for three days with GM-CSF and IFN-α as described above. After 72 hours, DCs were pulsed with full-length human calcitonin (Cibacalcin®; Novartis, Basel, Switzerland, 100 µg/ml), known to be a MTC cell-specific antigen 21. After 2 hours, cells were harvested, washed four times with isotonic NaCl and resuspended in 100 µl NaCl 0.9%. In all preparations, cell viability was >95% as evaluated by the trypan blue exclusion method.

Phenotypic analysis of DCs by flow cytometry analysis:

All monoclonal antibodies (mAbs) used for flow cytometry were purchased from BD PharMingen (Heidelberg, Germany) if not otherwise indicated. All antibodies were FITC-, PE-, PerCP Cy5.5-, or APC-conjugated, respectively, and measured in parallel to appropriate isotype controls. Several DC marker were characterized by using anti-CD80, -CD83, -CD86, -CD40, -CD11c, -CD123, -CD209, -CD14, -CD1a, and -HLA-DR, as well as anti-BDCA 1-4 (CD1c, CD303, CD141, and CD304; Miltenyi Biotec) mAbs. Additionally, for

characterization of NK cell surface markers mAbs towards CD56, CD16, CD94, CD161 and CD337 (both from BD PharMingen) and anti-NKG2A, NKG2D, NKp44, and NKp46 (purchased from Beckman Coulter, Krefeld, Germany) were used. Annexin V (Abcam**,** Cambridge, UK) was used for the detection of apoptotic cells. A positive control for Annexin V staining was implanted by incubation cell samples with camptothecin (6µM, 4 hours at 37°C).

For detection of intracellular antigens, cells were treated with Brefeldin A ('golgi-plug') for 3 hours and permeabilized using the Cytoperm-/ Permwash-kit (BD PharMingen) according to the manufacturer's instruction. For intracellular staining, anti-TRAIL (clone 2E5) was used, purchased from Alexis (Lörrach, Germany). Samples were analyzed using a FACSComp device (BD Biosciences, Heidelberg, Germany). Data were analyzed using CellQuest^{PRO} software (BD Biosciences). A minimum of 10.000 events was measured from each DC preparation before administration.

Cytotoxicity assay

To determine cytotoxic activity of CD56+ DCs, TRAIL-sensitive K562 cells ($1x10^6$) were labeled with 100 µCi of ^{51}Cr for 1 hour at 37°C. Cells were washed three times, re-suspended in complete medium, and incubated (10^4 cells / well) with varying numbers of effector cells including CD56+ and CD56- IFN-DCs as well as NK cells (for 18 hours). In some experiments anti-TRAIL RIK-2 mAb (10 µg/ml) or TRAIL ligand (eBioscience; San Diego, CA, USA), respectively, were added. Supernatants were collected and measured by a Wizard Automatic Gamma Counter (Wallac, Turku, Finland). Data were expressed as the mean ± SD of triplicate wells. The percentage of cytotoxicity was calculated as: cytotoxicity (%) = [(experimental group cpm - spontaneous cpm) / (total cpm - spontaneous cpm)] x 100.

Mixed leukocyte reaction

Allogeneic $CD3^+$ T lymphocytes were purified using anti-CD3 conjugated magnetic microbeads and seeded into 96-wells plates at 5 x 10^5 cells per well. Monocyte-derived cells were added to each well in triplicate at different stimulator-to-responder ratios. After 5 days, 1 µCi of [^{3}H]-thymidine (Amersham Pharmacia, Uppsala, Sweden) was added to each well and incubation was continued for additional 18 hours. Cells were finally collected by a Packard filtermate harvester onto Unifilter-96 (Perkin Elmer, Boston, MA, USA) and thymidine uptake was quantified by scintillation counting using a Trilux Mikro Beta (Wallac, Turku, Finland).

Determination of cytokine production by DCs

Intracellular cytokine staining for IFN-γ of CD56+ and CD56- IFN-DCs was performed as following: After 3 days of culturing, brefeldin A was added to cell cultures. Cell surface staining was then conducted with PE-labeled mouse anti-CD1c, PerCP-labeled mouse anti-CD45 and APC-labeled mouse CD56 antibodies, followed by cell permeabilization and staining with FITC-labeled anti-IFN-γ antibodies (PharMingen), respectively. After 30 min cells were thoroughly washed and samples were analyzed by FACS analysis. A minimum of 10.000 events was gathered from each sample.

Measurement of IL-12 in the supernatant was performed by ELISA as described by the manufacturer (Quantikine-ELISA, R&D Systems, Wiesbaden, Germany). Supernatents were collected from an one-day culture of pure CD56- and CD56+ IFN-DC, respectively, as well as of co-cultures with K562 tumor cells.

Electron microscopy

For ultrastructural analysis (electron microscopy) pellets of all samples were fixed in 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 3 h, postfixed for 90 min in 2% OsO_4 in 0.1 M cacodylate (pH 7.3), dehydrated in ethanol, and embedded in epoxy resin. Ultra thin sections (70 nm) were stained with uranyl acetate and lead citrate and examined at 75 kilovolts under a Hitachi electron microscope H-600.

Patients

Table 1 depicts a summary of patient's characteristics. Patients with histological proven medullary thyroid carcinoma (MTC), radiological established disease with pulmonary and hepatic metastases, and postoperative elevated plasma calcitonin levels >500 pg/ml (normal range <10 pg/ml; MTC-specific tumor marker of residual disease) were included into the study.

Treatment of patients and clinical response

All five MTC patients received two subcutaneous injections with a median of 1.9 x 10^8 IFN-DCs (± 0.8 x 10^8 DCs, range 0.7 – 4.0 x 10^8 DCs) per vaccine. Patients were followed up for 18-46 months (mean 37 months). Metastatic lesions were evaluated by CT scans and ultrasonic examinations. WHO definitions were used for stable disease with a change of less than 25% in tumor size and tumor markers without occurrence of new lesions for a minimum of six weeks. Measurements of CEA (Roche, Mannheim, Germany) and calcitonin (Roche

Diagnostics, Mannheim, Germany) were performed by commercial kits according to the manufacturer's instructions. Since the cut-off for calcitonin detection is about 2000 pg/ml (depending on standard curve) all sera were diluted 1:10 with isotonic sodium chloride before assaying. To minimize intra-assay variability all calcitonin measurement were performed three times and results were given as means (± SD). Calcitonin measurements were done in one run at the end of the follow-up period. A change of tumor markers of more than 25% difference for at least 3 months was considered as progression 23.

Intracellular cytokine staining

Autologeous, cryopreserved PBMCs (1 x 10^6 cells/ml) of treated patients were cultured in RPMI in the presence of full-length calcitonin (32 aa; Cibacalcin®; Novartis, Basel, Switzerland) or human albumin, respectively (each 100 µg/ml). Intracellular cytokine staining was performed in principle as described above. Following cell surface staining with PerCP-labeled mouse anti-CD4 antibodies, intracellular staining was performed with PE-labeled anti-IL4 or FITC-labeled anti-IFN-γ antibodies (PharMingen), respectively.

Delayed-type hypersensitivity and immunohistochemistry.

Delayed-type hypersensitivity (DTH) skin tests were documented following treatment with calcitonin-pulsed DCs. DCs were injected intradermally into the upper arm. A positive skin-test reaction was defined as > 5 mm diameter erythema and induration 24 hours after intradermal injection. In one patient (No.1) we additionally tested DTH reactivity by intradermal injection of pure calcitonin (10 µg in 100 µl isotonic NaCl) followed by skin biopsy. In this patient a biopsy (diameter 5 mm) of the DTH site was taken 24 hours after injection. Serial paraffin-embedded sections were stained with a monoclonal antibody against human CD8 (concentration: 1:200; Clone C8/144B, Dako, Hamburg, Germany) in a moist chamber at 37°C for 60 min. Bound antibody was detected using avidin-biotin complex (ABC) peroxidase method (ABC Elite Kit, Vector Laboratories, Burlingame, USA). The staining reaction was performed with 3,3´-diaminobenzidine and H_2O_2. Accurate negative controls were performed.

Statistical analysis.

The results were analyzed for statistical significance by paired and un-paired *t*-test, respectively, depending on the data used for calculation using GraphPad Prism 4.0 computer software (GraphPad Software Inc., San Diego, CA).

Results

Generation of dendritic cells from monocytes in the presence of IFN-α

To investigate the effect of IFN-α on DC differentiation from human monocytes, we cultured purified CD14+ monocytes with clinical grade GM-CSF and IFN-α. These cells will be referred to as IFN-DCs hereinafter. As recently described [24,25], the monocyte marker CD14 was down-regulated in IFN-DC whereas typical DC lineage markers CD1a and CD11c were expressed on these cells on high levels (in mean 74% (± 8%) and 97.9% (± 7%), respectively). We next analyzed the expression of MHC molecules (HLA-DR), co-stimulatory molecules (CD40, CD80, and CD86), and the maturation marker CD83. With the exception of extracellular CD83, which has already been described to be only weakly expressed on IFN-DCs [25], all markers were highly positive (Fig. 1). Most interesting, however, 56% (± 14%) of IFN-DCs were positive for CD56. This is absolutely important, as up to now CD56 expression has only been described to be present on NK cells and NKT cells, however not on antigen presenting cells such as DCs.

Phenotypical and morphological analyses of CD56+ and CD56- IFN-DCs

After positive selection of CD56+ IFN-DCs (Fig. 2), both IFN-DC subtypes were phenotypically characterized by FACS analyses. Both cell populations showed an almost identical phenotypical pattern in regard to DC lineage markers CD1a and CD11c as well as the MHC class II molecule HLA-DR and the co-stimulatory molecules CD80, and CD86. The maturation marker CD83, which was only weakly expressed in IFN-DC was lower in CD56+ cells ($p = 0.02$), whereas CD40 as well as CD123 were significantly higher expressed (CD40: $p = 0.02$; CD123: $p = 0.0096$; Fig. 3). Neither CD56+ IFN-DCs nor CD56- IFN-DCs expressed CD16. Interestingly, the reduction of CD14 was lower in CD56- cells, probably an evidence for incomplete maturation ($p= 0.029$; Fig. 3). These results indicate that CD56+ and CD56- IFN-DCs are not substantially two distinct cell populations but may originate from identical precursor cells.

FACS analyses revealed that CD56+ IFN-DC are bigger in size (as calculated by forward scatter analyses with a mean of 635.0 ± 25.1 relative units) compared to CD56- IFN-DCs (577.9 ± 26.6; Fig. 4). CD56+ and CD56- IFN-DCs were also visualized by using electron microscopy. Here too, CD56+ IFN-DCs appeared to be slightly larger in size compared to CD56- IFN-DC, although cell types showed similar phenotypes with numerous of

pseudopodia. The cytoplasm contained few organelles (mitochondria) and the shape of the nucleus did not show marked differences (Fig. 4).

TRAIL expression and cytotoxic potential of CD56+ IFN-DCs

Previous reports have already demonstrated that a variety of lymphoid and myeloid cells, including T cells, NK cells, monocytes and interferon-producing killer DC (IKDC) can express TRAIL and kill TRAIL-sensitive target cells under certain circumstances [11,12,18,26-28]. To determine whether CD56+ IFN-DC also exhibit tumoricidal activity, these cells were co-cultured for 18 hours with K562 cells at different DC / target ratios. At higher effector / target cell ratios the lysis activity of CD56+ IFN-DC cells were up to 24% (± 4%) (Fig. 5A).

This lysis activity could be partially blocked (-81% ± 2%) with anti-TRAIL indicating that one of the lysis mechanism of these cells is mediated by TRAIL (Fig. 5B). Intracytoplasmatic cytokine analyses of CD56+ IFN-DCs revealed that in mean 95.2% ± 2.7% of all CD56+ IFN-DCs were positive for TRAIL (representativ FACS analysis is given in Fig. 5C). CD56- IFN-DCs also showed a TRAIL expression. This was, however, less pronounced compared to CD56+ IFN-DCs as only 84.7% ± 11.3% were TRAIL positive. The minimal lysis activity (7%) of CD56- IFN-DCs could partially blocked 62% ± 13% with anti-TRAIL.

Cytokine production by IFN-DC

For specific determination of IFN-γ-secreting cells intracellular cytokine staining was performed. After three days of culture of CD14+ monocytes with IFN-α the majority of cells (99.2 ± 0.2%) were IFN-γ positive (representative FACS analysis is shown in Figure 6A). For specification we could demonstrate that the majority of cells (90.1 ± 1.6%) were BDCA1 positive. Importantly, IFN-γ-producing IFN-DCs consisted of CD56+ as well as of CD56- IFN-DCs indicating that these are not two distinct cell populations rather.

After three days of culture with IFN-α, neither CD56- nor CD56+ IFN-DCs produced any significant amounts of IL-12 measured within the supernatents of cells (Fig. 6B). Importantly, however, after additional 24 hours of co-culture with K562 tumor cells, significant amounts of IL-12 (49 pg/ml ± 6%) were secreted while IL12 production of CD56- IFN-DCs was slightly lower, however, still detectable (41 pg/ml ± 5%; Fig. 6). These differences did, however, not reach statistical significancy.

MLR with CD56+ IFN-DC as antigen presenting cells (APC)

To investigate the function of CD56+ IFN-DCs as stimulators of naive $CD3^+$ T cells, MLR was performed. The results were compared with stimulatory capacity of CD56- IFN-DC, IL4-DCs and monocytes, respectively. As formerly described [29], at all stimulator / responder ratios, IFN-DCs were found to be more potent for stimulating T cells compared to immature monocytes ($p < 0.0001$) and, most important, compared to IL4-DCs ($p < 0.0001$; Fig. 7). Detailed analyses of CD56+ and CD56- IFN-DC revealed that CD56+ IFN-DC showed a slightly stronger capacity in stimulating allogenic T cells compared to CD56- IFN-DC at all DC / T cell ratios (Fig. 7). These differences also reached statistical significancy ($p = 0.036$).

Vaccinations with IFN-DC in patients with medullary thyroid carcinoma

Based on these result an *in vivo* immunotherapy trial was started in five patients with metastasized medullary thyroid carcinoma (MTC). The patient's characteristics are given in Table 1. IFN-DC vaccinations were well tolerated by all patients without experiencing any adverse effects or any clinical signs of autoimmune reaction. To study the *in vivo* immune response we analyzed delayed-type hypersensitivity (DTH) skin reactivity. After the 2^{nd} vaccination all patients developed a significant DTH reaction (>1 cm in diameter) characterized by the appearance of erythema and induration at the injection site. In one patient (No.1) we additionally tested DTH reactivity by intradermal injection of pure calcitonin (10 µg in 100 µl isotonic NaCl) without any IFN-DCs. Forty-eight hours after antigen injection there was a strong perivascular and epidermal infiltration with $CD8^+$ T lymphocytes (Fig. 8a). This clearly indicates the ability of IFN-DCs to induce a Th1-like antigen-specific immune response *in vivo*.

During follow-up, of in mean 37 months there were three patients (No.1, 2 and 5) who experienced a progression determined by computed tomography and by measurement of serum tumor marker. In two patients (patients 3 and 4), however, neither substantial changes in tumor morphology nor in serum tumor markers were detected. After a long term follow-up of 44 months serum CEA in patient 4 showed a slight decrease (332 µg/l after treatment compared to 377 µg/l before treatment), whereas in the CT scan of the lung only a tiny increase was detected (Fig. 8b). Pulmonary CT scan of patient 3 showed a stable disease whereas the tumor markers CEA and calcitonin showed a small increase of less than 25% (52 µg/l after treatment vs. 43 µg/l before treatment for CEA and 2,090 pg/ml vs. 2,240 pg/ml for calcitonin, respectively). Importantly, by analyzing peripheral blood mononuclear cells these patients also showed an increase of calcitonin-specific IFN-γ secreting T cells (Fig. 9).

Discussion

Type I interferons (IFNs) are cytokines exhibiting antitumor effects, including multiple activities on immune cells. The major mechanisms mediating this process is the differentiation of the Th1 subset [2,3], the generation of cytotoxic T cells (CTLs) [4] and the activation of natural killer cells (NK cells) [6]. In the last couple of years another mechanism, namely the differentiation of monocytes into dendritic cells by interferon-α (IFN-DCs) have been proposed to be responsible as well [30,31]. When compared with the classical interleukin 4-generated DCs (IL-4-DCs), IFN-DCs exhibit a typical DC morphology and express a similar level of co-stimulatory and class II MHC molecules [31,32]. In contrast, IFN-DCs reveal a significantly higher level of MHC class I molecules as well as other DC markers including CD1a [33], CD208 (DC-LAMP) [34], and CD197 (CCR7) [35].

Depending on their maturation stage, IFN-DCs also secrete large amounts of inflammatory cytokines such as IL-1ß, IL-6, IL-10, IL-18, and TNF-α [29]. In addition, IFN-DCs induce a higher amount of long-lived CTLs against certain tumor antigens compared to classical DCs [36]. Most important, in the context of cytotoxicity, these cells also reveal direct tumoricidal activity via the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) which was shown to be strongly up-regulated on a molecular [18,31] and protein level [18,19] and which explains the direct cytolytic activity of IFN-DCs [18,19]. In our present study, we confirm the cytolytic activity of IFN-DC caused by (soluble) TRAIL. Importantly, however, we were able to specify a subpopulation of IFN-DCs with cytolytic activity expressing the surface marker CD56. This is absolutely crucial, as up to now CD56 expression has only been described to be present on NK cells and NKT cells, however not on antigen-presenting cells such as DC. CD56 itself (also known as NCAM) is responsible for cell to cell contact [37,38]. In addition, there is some evidence of a direct CD56-mediated cytolytic activity [39,40]. According to our data, CD56+ IFN-DCs exhibited cytolytic activity up to 24% whereas CD56- IFN-DCs did not. Tumoricidal activity could almost completely be blocked when cells were co-cultured with anti-TRAIL indicating that lysis mechanism of CD56+ IFN-DC is mediated by TRAIL. Importantly, however, on the basis of intracellular staining a significant number of CD56- IFN-DCs were TRAIL positive as well. The differences in cytolytic activity between both cell populations could be explained by different amounts of secreted TRAIL reaching target cells because of a closer cell / cell contact. This concept is supported by the fact that cytolytic activity of CD56+ IFN-DCs as well as the minimal cytolytic activity of CD56- IFN-DCs could only incompletely be blocked after conincubation with anti-TRAIL.

For detailed analysis, we also examined other lysis mechanisms including granzyme / perforin and CD95 ligand (FasL). In rodents a Fas-dependent direct lysis activity of bone marrow-derived DCs has already been described [41,42]. In humans HLA-DR$^+$, lineage$^-$ DC which were freshly isolated from peripheral blood have also been shown to reveal a high tumoricidal activity against various tumor cell lines depending on the engagement of TNF, TRAIL, Lymphotoxin-$\alpha_1\beta_4$ and FasL [43,44]. Most recently, Stary et al. discovered tumor infiltrating myeloid DCs and plasmocytoid DCs that gained lytic functions and exogenous supply of TLR7 agonists [45]. Furthermore, the authors demonstrated that HLA-DR$^+$ CD11c$^+$ blood-derived myeloid DCs could express perforin and granzyme B in the presence of TLR7/8 agonists, and serve as potent killer DCs against the chronic myelogenous leukemia cell line K562, but not against the Jurkat T cell line (known to be refractory to perforin/granzyme mediated death) [45]. With the exception of TRAIL, all aforementioned lysis mechanisms could not be detected on a phenotypical level on IFNα-generated DCs. This might be explained by the different cell subpopulations investigated, different agonists used for stimulation and possibly by lysis mechanisms not known so far, respectively.

Importantly to note, our initial intend of this study was to identify the human counterpart of a new cell population recently identified in mice termed interferon-producing killer dendritic cells (IKDCs) [11,12] . These cells are distinct from conventional DCs and plasmocytoid DCs as they show the molecular expression profile of both NK cells and DCs. Moreover, they produce high amounts of interleukin-12 (IL-12) and IFN-γ. Most important, IKDCs directly kill typical NK target cells mediated by the TRAIL pathway. The cytolytic capacity of IKDCs subsequently diminishes, associated with the loss of NKG2D receptor on these cells. Most recently it has been proposed that IKDCs could rather represent a subtype of activated NK cells that gain the ability to present antigens under certain circumstances, especially in tumoral context (personally communicated) [13-15]. As mentioned above, human IFN-DCs generated from monocytes over 3 days of culturing with IFN-α revealed NK cell markers namely CD56 and TRAIL. In contrast, however, we could not identify a significant amount of other NK cell surface markers such as NKp46, NKG2A/CD94, NKG2D, Nkp30 (CD337), Nkp44 and NKR-P1 (CD161). These markers were only detected on a marginal level (data not shown). The near absence of these receptors e.g. of the stimulating receptor NKG2D are potentially responsible for the weaker cytolytic activity of CD56+ IFN-DCs compared to classical NK cells. In contrast to NK cells [46], even additional culturing of CD56+ IFN-DCs with K562 tumor cells did not significantly change the quantitative expression profile of any of these NK cell receptors (NKRs) on CD56+ IFN-DCs. We also investigated the ability of IFN-DCs to

produce IFN-γ. Intracellular cytokine staining revealed that the majority of IFN-DCs were IFN-γ positive, independent of the expression of CD56, indicating that CD56+ and CD56- IFN-DCs are not two distinct cell populations rather than cells at different stages of development. The amount of secreted IFN-γ in the supernatent was, however, too low to be detectable by ELISA (data not shown). The positive IFN-γ cytokine staining is in line with recent data showing a stimulation-dependent secretion of high amounts of IFN-γ of human cord blood monocyte-derived DCs [47] and IL4-DCs [48], respectively. So, monocyte-derived IFN-DCs partially reveal phenotypical and functional characteristics of NK cells including CD56 and TRAIL and the production of IFN-γ, however, they can not be termed as human IKDCs as other classical NK cell marker are still missing and cytolytic activity is much lower compared to NK cells.

To the best of our knowledge, this is the first report demonstrating monocyte-derived IFN-DCs treatment in cancer patients. As demonstrated by delayed-type hypersensitivity skin reaction, our results suggest that IFN-DCs induce antigen-specific CD8+ T cells as an indicator of a potential CTL response in humans. These findings were flanked by a Th1-cytokine pattern which is crucial for the induction and maintenance of an adequate CTL immunity. Our data are in line with a study by Banchereau and coworkers reporting on the *in vivo* use of CD34+ progenitors which were activated by type I interferon and which were used for the treatment of patients with stage IV melanoma [20]. The authors reported on no statistically significant survival advantage in these patients. Importantly, however, in six of seven patients tumor antigen-specific recall memory CD8+ T cells able to secret IFN-γ and to proliferate could be detected. More recently, another study from Di Pucchio and coworkers reported on a pilot study to determine the effects of IFN-α administered as adjuvant of tumor-specific antigens in stage IV melanoma patients [49]. The authors detected an enhancement of CD8+ T cells recognizing native and modified tumor antigens and most important a significant increase of DC precursors. These cells had an enhanced antigen-presenting cell function in some patients with stable disease [49]. These data are in line with another report from Yamamoto and coworkers reporting on a markedly induced TRAIL expression on $CD14^+$ monocytes and enhanced cytotoxic activity towards hepatocellular carcinoma cells after co-incubation with IFN-α [28].

In summary, our results show that cytotoxic activity of IFN-α-generated monocyte-derived DCs is mainly mediated by a subset of cells expressing CD56. Up to now it has been thought that this marker is specific for NK and NKT cells but is not expressed on antigen-presenting cells. We also demonstrate that CD56+ IFN-DCs express TRAIL, a major lysis mechanism in

cytotoxic immunity. We were, therefore, being able to specify one tumor lysis mechanism induced by IFN-α. Based on our data, it could be postulated that future cancer immunization trials in should be performed with CD56+ IFN-DCs in order to improve clinical efficacy.

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Figure 1: Immunophenotypic pattern of IFN-DCs

Isolated monocytes (purity >98%) were cultured over 3 days with GM-CSF and IFN-α. Resulting IFN-DCs were stained with a panel of antibodies and analyzed by flow cytometry. As demonstrated in previous studies IFN-DCs reveal a semimature phenotype with a moderate expression of CD83 whereas co-stimulatory molecules CD80 and CD86 as well as HLA class II molecules were strongly expressed. Additionally, IFN-DCs showed surface markers typically for common DCs (CD209, BDCA 1) as well as for plasmocytoid DCs (CD123, CD11c). Contamination with CD3+ and CD19+ cells was low (<1%), whereas a significant number of CD56+ cells were detected. Marker expression is given as mean (± SD) of at least five DC preparations.

Figure 2: Representative FACS analysis of CD56+ IFN-DCs

After incubation with GM-CSF and IFN-α CD56+ cells were isolated by positive selection and stained with anti-CD11c, -CD80, -CD86, -HLA-DR, -CD40, -BDCA-1 and –BDCA-2 monoclonal antibodies. Appropriate isotype controls performed in parallel were nearly identical, therefore only one control is shown (mouse anti-IgG2a). To verify the viability of CD56+ IFN-DCs Annexin-V staining was also performed. Camptothecin-treated cells were used as positive control.

Figure 3: Immunophenotypic pattern of CD56- / CD56+ IFN-DCs

CD56+ IFN-DCs were purified with CD56 mAb-conjugated microbeads leading to a purity of the $CD56^+$ cells of ≥98%. Thereafter, CD56+ and CD56- IFN-DCs were stained with a panel of antibodies and were analyzed by flow cytometry. CD56+ IFN-DCs showed slightly increased marker expression typical for DCs including CD209, BDCA1 and BDCA4. In addition, CD14 was less frequent seen on CD56+ IFN-DCs compared to CD56- IFN-DCs as well as CD40 which was upregulated on CD56+ IFN-DCs. Marker expression is given as mean (± SD) of at least five DC preparations.

Figure 4: Morphology of CD56+ and CD56- IFN-DCs

CD56+ and CD56- cells were purified with CD56 mAb-conjugated microbeads. Thereafter, both cell fractions were analyzed by electron microscopy (A). Both cell types showed similar

phenotypes with numerous pseudopodia. The cytoplasm of CD56+ IFN-DCs contained few organelles (mitochondria), the shape of the nucleus of both cell populations did not show marked differences including a same pattern of chromatin. (B) Forward sideward scatter generated by flow cytometry analysis, revealed that CD56+ IFN-DC are bigger in size (mean 635.0) compared to CD56- IFN-DCs (577.9).

Figure 5: Cytolytic activity of IFN-DCs and involvment of TRAIL

After generation of IFN-DCs CD56+ and CD56- cells were separated. (A) Both cell fractions were co-cultured with ^{51}Cr labelled K562 tumor cells for 18 hours at different cell / K562 tumor cell ratios. Supernatants were collected and analyzed by gamma radiation analysis (CD56+ IFN-DCs black box, CD56- IFN-DCs white box). For internal control, K562 tumor cells were cultured alone and in the presence of a detergent for direct lysis. Natural killer (NK) cells were used as positive control (white rhomb). Results of three independent experiments are shown. (B) CD56+ IFN-DCs were co-cultured in a ratio of 1:50 with or without anti-TRAIL (10 µg/ml). The lysis activity could be partially blocked (-81% ± 2%) after co-cultering with anti-TRAIL indicating that the majority of lysis activity is mediated by TRAIL. For internal controls, K562 cells were cultured alone (C) Representative FACS analysis for TRAIL of CD56+ IFN-DCs (middle panel) and CD56- IFN-DCs (right panel) showing a weaker TRAIL expression in CD56- IFN-DCs compared to CD56+ IFN-DCs. Isotype control for whole IFN-DCs is given in the left panel.

Figure 6: Cytokine analysis of IFN-DCs

Representative intracellular cytokine staining (A) shows that the majority of IFN-DCs are IFN-γ-producing (upper right panel; isotype control is shown on the upper left panel). Detailled analyses revealed that the majority of cells are BDCA1 positive (gated for R2). Importantly, IFN-γ-producing IFN-DCs consisted of CD56+ as well as CD56- IFN-DCs (gated for R3).

Detection of IL-12 is shown in Figure 6B. CD56+ and CD56- cells were separated after generation of IFN-DCs. Both cell fractions were cultured alone or co-cultured with K562 tumor cells in two different ratios, respectively. Supernatants were collected and freezed until analysis. Co-culture with K562 tumor cells, resulted in a slight increase of IL-12 production in CD56+ IFN-DCs (up to 49 pg/ml ± 6%) compared to CD56- IFN-DCs (up to 41 pg/ml ± 5%).

Results from three independent experiments are shown. Differences between CD56+ IFN-DCs and CD56- IFN-DCs were not significant.

Figure 7: Mixed lymphocyte reaction of antigen-presenting cells (APC)

CD56+ IFN-DCs and CD56- IFN-DCs, respectively, were co-cultured with allogeneic $CD3^+$ T lymphocytes at different stimulator-to-responder ratios for 6 days. [^{3}H]-thymidine (1 µCi) was added for 18 hours, and thymidine uptake was quantified by scintillation counting. At all stimulator cell / T cell ratios, CD56+ IFN-DCs (black boxes) revealed a slightly stronger stimulating capacity compared to CD56- IFN-DCs (white boxes; $p = 0.036$). Freshly isolated monocytes as well as IL4-generated DCs were used as controls (white dot). At all stimulator-to-responder ratios IFN-DCs were more potent in stimulating allogenic T cells compared to immature monocytes ($p < 0.0001$) and compared to IL4-DCs ($p < 0.0001$). Results are shown from three independent experiments.

Figure 8: Delayed-type hypersensitivity skin reaction and computed tomography

(A) Skin biopsy of patient 1 demonstrates strong epidermal infiltration with CD8+ cytotoxic T lymphocytes 48 hours after subcutaneous administration of calcitonin (10 µg in 100 µl isotonic NaCl). Paraffin sections were incubated with monoclonal antibodies against CD8 and stained with avidin-biotin complex peroxidase method. (B) Computed tomography of the chest of patient 4. Pretherapeutic scan (upper row) and follow-up examination 44 months later (bottom row). After a long term follow-up there is a tiny increase of small pulmonary metastases (arrows).

Figure 9: Percent IFN-γ producing CD4+ T lymphocytes in response to calcitonin determined by intracytoplasmatic cytokine staining

Peripheral blood mononuclear cells (PBMC) from patient 3 and 4 were isolated from blood samples collected monthly. Patient's PBMC were exposed for 16 hours to 100 µg/ml calcitonin (left and right panels) and to a control protein (human albumin, middle panels). For the last 3 hours brefeldin A was added to inhibit protein secretion, cells were stained for extra-cellular CD4 and intra-cellular IFN-γ. In two patients, which responded to immunotherapy (patients 3 and 4), there was a significant increase of calcitonin-specific IFN-γ-secreting T

lymphocytes after the second vaccination (right panels) compared to pre-treatment (left panels). For control, after stimulation of T cells which were collected after therapy and which were stimulated with human serum albumin only a small number of IFN-γ-secreting T cells could be detected (middle panels).

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