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CD56-Expression on IFNα-generated DC

- A New Biphenotypic Cell Type? -

Dissertation

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A. Abkürzungen

APC	Antigen-Presenting Cells
CD	Cluster of Differentiation
CTL	Cytotoxic T-Lymphocytes
DC	Dendritic Cells
DEC205	Dendritic and Epithelial Cells 205
Fc-Receptor	Antibodies Binding Receptor
FKS	Fetales Kälberserum
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HSP	Heat Shock Protein
IFN	Interferon
IKDC	IFN-Producing Killer Dendritic Cells
IL	Interleukin
KIR	Killer Immunoglobulin-like Receptor
LK	Lymphknoten
MHC	Major Histocampatibility Complex
MIC	MHC-I Related Chain
MLR	Mixed Leukocyte Reaction
MTC	Medullary Thyroid Carcinoma
NCAM	Neural Cell Adhesion Molecule
NCR	Natural Cytotoxicity Receptor
NK	Natural Killer Cells
NKT	Natural Killer T-Cells
pDC	Plasmacytoid Dendritic Cells
RAE-1	Retinoic Acid Early Inducible Transcript
TH	T-Helper Cells
TLR	Toll-like Receptor
TLSP	Thymic Stromal Lymphopoetin
TNF	Tumor Necrosis Factor
TRAIL	TNF-related Apoptosis Inducing Ligand

B. Einleitung

1. Dendritische Zellen

Die körpereigene Tumorabwehr wird sowohl durch das angeborene als auch durch die erworbene Immunität reguliert, wobei sie durch zytotoxische T Zellen (CTL), natürliche Killerzellen (NK) und natürliche Killer T-Zellen (NKT) ausgeführt wird.
Einer der Hauptakteure in diesem System sind die dendritischen Zellen (DC), indem sie das Immunsystem aktivieren und regulieren[1-3].

1.1 Eigenschaften von Dendritischen Zellen

DC wurden erstmals im Jahre 1973 von Steinman und Cohn an Hand von zytologischen Merkmalen beschrieben[4].
Die ersten funktionellen Eigenschaften wurden wenige Jahre später ebenfalls durch Steinman und Cohn charakterisiert.
Sie fanden heraus, dass DC als Zielzellen in einer so genannten *„Mixed Leukocyte Reaction (MLR)“* eine 100-300fach höhere Stimulation von allogenen T-Zellen erzeugten als unselektierte Leukozyten[5-8].

Mit diesen initialen Versuchen begann die Charakterisierung der DC.
Sie zeigte, dass DC hochpotente Antigen-präsentierende Zellen (APC) mit der einzigartigen Eigenschaft sind, sowohl endogene als auch exogene Antigene aufzunehmen und zu verarbeiten, um diese anschließend auf ihrer Oberfläche sowohl $CD4^+$ als auch $CD8^+$T-Zellen zu präsentieren[2].
Zu diesem Zweck wandern DC nach der Antigenaufnahme in regionale Lymphknoten (LK), um dort mit den verschiedenen T-Zell-Subpopulationen zu interagieren.
Zusätzlich kann es im LK zu Interaktionen sowohl mit B-Zellen (humorale Immunantwort) [9] als auch mit NK- und NKT-Zellen [10;11] kommen.

1.2. Ex vivo Generierung von Dendritischen Zellen

Auf Grund ihrer einzigartigen Rolle als Koordinator der Immunantwort sind in den letzten beiden Jahrzehnten viele Anstrengungen unternommen worden, DC *ex vivo* herzustellen, um sie in der Immuntherapie einzusetzen, z.B. gegen solide Tumore. Diese *ex vivo* generierten DC wurden mit Zytokinen und mit spezifischen Antigenen stimuliert (z.B. ein spezifisches Tumorantigen), um sie letztlich Patienten zu re-injizieren. Dadurch sollte erreicht werden, dass eine spezifische Immunreaktion gegen das verwendete Antigen generiert wird.

Bereits 1994 konnten die Arbeitsgruppen Romani und Sallusto zeigen, dass man mit aus peripherem Blut isolierten Monozyten unter Verwendung von Interleukin 4 (IL-4) und Granulozyten Makrophagen Kolonie-Stimulierendem Faktor (GM-CSF) unreife DC herstellen kann[12;13].
Diese wurden anschließend mit Tumorantigenen inkubiert und durch verschiedene Kombinationen von Zytokinen zur Ausreifung gebracht [14;15].

Im Laufe der Jahre wurden immer wieder neue Methoden zur Herstellung von DC entwickelt.
So wurden DC z.B. aus $CD34^+$ Stammzellen[16;17] oder Monozyten, die man direkt aus dem peripheren Blut gewinnen kann, generiert.
Dabei wurden verschiedene Arten von Stimulantien verwendet, wie z.B. Interleukin 15 (IL-15) plus GM-CSF[18], Tumor-Nekrose-Faktor alpha (TNFα) plus GM-CSF [19], Thymic Stromal Lymphopoetin (TLSP) [20-22] oder Toll-like-Rezeptor Liganden (TLR-Ligands)[23].

Die Gruppe Santini benutzte Anfang 2000 Interferon alpha (IFN-α) an Stelle von IL-4 zusammen mit GM-CSF.
Hierdurch entwickelten sich nach drei Tagen Stimulation „halb reife (semi-mature) DC“ [24].
IFN-α gehört zur Gruppe der Typ I IFN und besitzt starke antivirale, antitumor und immunregulatorische Aktivität[25;26] . Hauptsächlich wird es von pDC sezerniert[27;28].

2. NK-Zellen

Ein anderer wichtiger Teilnehmer an der immunologischen Tumorabwehr sind die natürlichen Killerzellen (NK-Zellen).
Sie haben die Eigenschaft, entartete oder virus-infizierte Zellen direkt zu lysieren[29;30].
Zusätzlich aktivieren sie das Immunsystem durch Sekretion bestimmter Zytokine und Chemokine[31].

2.1 NK-Zellen und ihre Rezeptoren

NK-Zellen verfügen über aktivierende und hemmende Rezeptoren auf ihren Oberflächen.

Ein sehr gut charakterisiertes System von hemmenden Rezeptoren ist das *Killer Immunoglobin-Like Rezeptor* (KIR)-System.
Als Liganden dienen dabei verschiedene MHC-Moleküle.
Kommt es nun z.B. bei einer Virusinfektion zur Verminderung von MHC-Molekülen auf der Oberfläche der infizierten Zellen, so sind die hemmenden Signale vermindert und es überwiegen aktivierende Signale; die Zelle wird lysiert[32;33].
Eine andere Gruppe mit hemmender Signalfunktion ist die Gruppe um die CD94/ NKG2-Rezeptoren[34;35].

Ein gut untersuchter aktivierender Rezeptor ist der NKG2D-Rezeptor, der diverse Stressmoleküle auf Tumorzellen oder Virus-infizierten Zellen erkennt[36-38].
Dazu gehören z.B. das Heat Shock Protein (HSP) und das Retinoic Acid Early Inducible Transcript (Rae-1) bei der Maus sowie die MHC-I Related Chain A/ B (MICA/ B) beim Menschen.
Eine weitere Gruppe aktivierender Rezeptoren sind die Natürlichen Zytotoxischen Rezeptoren (NCR) wie NKp30, 44 oder 46, die ihrerseits unterschiedliche Liganden besitzen[39].

2.2. NK-Zellen und ihre Effektoreigenschaften

Nach aktivierender Bindung werden von den NK-Zellen Moleküle freigesetzt, die eine direkte zytotoxische Aktivität haben.
Dazu gehören der FasLigand[40;41] und der TRAIL[42], die beide sowohl gebunden auf der Oberfläche als auch in löslicher Form vorkommen. Dagegen kommen das Granzym B und das Perforin nur in löslicher Form vor[43].

Zudem setzen NK-Zellen verschiedene Zytokine und Chemokine frei.
Hierzu zählt insbesondere das Interferon gamma (IFNγ), das eine direkte zytotoxische Aktivität auf Tumorzellen und Virus-infizierte Zellen besitzt[44]. Zusätzlich reguliert IFNγ das Immunsystem und stimuliert es in Richtung einer zytotoxischen Immunantwort über zytotoxische $CD8^+$ T-Zellen und TH1-Zellen.

C. Aufgabenstellung

Um eine Möglichkeit zu finden, das Immunsystem bei Patienten mit stark metastasiertem medullärem Schilddrüsenkarzinom (MTC) auf das Malignom hin zu sensibilisieren und zu stimulieren, sollte ich im Zuge meiner Doktorarbeit die ex vivo Herstellung von DC aus IFN-α und GM-CSF-stimulierten peripheren Monozyten etablieren, die Zellen charakterisieren und analysieren.
Dabei entdeckte ich eine Zellpopulation, die sowohl phänotypische als auch funktionelle Eigenschaften von DC aufwies.
Zusätzlich befand sich jedoch der NK-Zell-Marker CD56 auf ihrer Oberfläche. Darüber hinaus zeigte die Population eine lytische Aktivität gegenüber Tumorzellen. Daher stellte sich die Frage, um was für eine Art von Zelle es sich hierbei handelte.

Im Weiteren sollte diese DC-Population von unserer Arbeitsgruppe verwendet werden, um sie mit Calcitonin als Antigen zu inkubieren.
Calcitonin wird typischerweise von medullären Schilddrüsenkarzinomzellen überexprimiert und sollte in diesem Fall als Zielantigen für das Immunsystem dienen.

D. Originalarbeiten (siehe Anhang)

1. IFN-α Skews Monocytes into $CD56^+$-Expressing Dendritic Cells with Potent Functional Activities In Vitro and In Vivo

2. Characterization of Monocyte-derived IFNα-generated Dendritic Cells

E. Diskussion

Der ursprüngliche Plan meiner Doktorarbeit war die Etablierung des Verfahrens zur Herstellung von DC aus Monozyten aus dem peripheren Blut nach Santini[45].
Im weiteren Verlauf sollten diese hergestellten DC analysiert, charakterisiert und daraufhin untersucht werden, in wieweit sie zur Verwendung für eine zelluläre Immuntherapie bei Patienten mit medullärem Schilddrüsenkarzinom genutzt werden können.

Um die generierten DC auch Menschen verabreichen zu können, wurden die Monozyten ohne den Zusatz von fetalem Kälberserum (FKS) stimuliert, da dessen Anwendung am Menschen als kritisch angesehen wird.
Es zeigte sich, dass sich trotz dieser Umstellung nach 3 Tagen DC aus den Monozyten entwickelten.
Dieses wurde einmal durch den Verlust des CD14-Markers deutlich, zum anderen aber auch durch die vermehrte Expression von typischen DC-Markern. Hierzu gehören MHC-Moleküle sowie CD40-, CD80- und CD86-Moleküle, die für die funktionellen Eigenschaften der DC entscheidend sind.
MHC-Moleküle präsentieren Antigene auf den Zelloberflächen und stellen somit das „erste Signal“ der T-Zell-Aktivierung dar. Exogene Antigene werden dabei über MHC-Klasse II und endogene über MHC-Klasse I Moleküle präsentiert. DC können dabei exogene Antigene sowohl $CD4^+$ T-Zellen über MHC II als auch $CD8^+$ T-Zellen über MHC I Moleküle präsentieren. Diese Eigenschaft kommt nur bei DC vor, nicht aber bei anderen APC[46].
Für eine potente T-Zell-Stimulation wird zusätzlich auch noch ein „zweites Signal“ benötigt, das über kostimulatorische Moleküle vermittelt wird. Dieses führt sowohl zu einer stärkeren Bindung als auch zu einer zusätzlichen Signalübertragung zwischen T-Zellen und DC.
Allerdings waren die von uns hergestellten DC noch nicht vollständig ausgereift. Dieses wurde durch das Fehlen des Ausreifungsmarker CD83 deutlich. Aus diesem Grund würden wir die Population als „halb reifen (semi mature) DC-Typ“[47;48] bezeichnen.

Bei der weiteren Untersuchung der DC-Population fiel auf, dass einige der DC zusätzlich auf ihrer Oberfläche das CD56-Molekül besaßen.
Dieses Molekül kommt typischerweise auf NK- und NKT-Zellen vor und war bis dahin noch nie auf DC beschrieben worden.
CD56 (auch NCAM genannt) ist für den Zell-Zell-Kontakt wichtig[49] und es gibt Hinweise für eine direkte lytische Aktivität[50;51] . Ebenfalls spielt es eine Rolle bei der Entwicklung der NK-Zellen, da es mit Molekülen auf der Oberfläche von Fibroblasten interagiert.

Es stellte sich nun die Frage, um was für einen Zelltyp es sich bei der $CD56^+$ Population handelte.
Waren es DC mit einem NK-Zell-Marker, waren es NK-Zellen mit DC-Oberflächenmolekülen oder war es sogar eine neue Zellpopulation, die sowohl NK-Zelle als auch DC war?

In diesem Zusammenhang sei erwähnt, dass Anfang 2006 die so genannten IKDC (IFN-produzierende Killer DC) von zwei unabhängigen Laboren beschrieben wurden [52;53]. Diese Zellpopulation zeigte ebenfalls sowohl das phänotypische Muster einer NK-Zelle als auch das von DC, wobei sie große Mengen IFNγ und IL-12 produzierte. IFNγ wird typischerweise von NK-Zellen produziert und trägt sowohl zur Antitumor Immunität als auch zur T-Zell-Differenzierung bei[54].
IL-12 hingegen wird von DC als „drittes Signal“ freigesetzt und dient der Differenzierung von naiven CD4-Helfer-Zellen. Sie entwickeln sich unter dem Einfluss von IL-12 in TH1-Zellen weiter, die wiederum IFNγ freisetzen und dadurch zur Antitumor Immunität beitragen[55].
Des Weiteren konnten sowohl DC-typische Eigenschaften als auch die Lyse von Tumoren über den TRAIL-Mechanismus gezeigt werden. Dabei handelt es sich um einen Liganden aus der TNF-Familie, der als lösliche oder gebundene Form vorkommt. Er ist in der Lage Apoptose in TRAIL-Rezeptor tragenden Zellen (z.B. Tumorzellen) zu induzieren [56].
Im Verlauf konnte jedoch gezeigt werden, dass es sich bei den IKDC um stark aktivierte NK-Zellen handelte, die unter bestimmten Umständen in der Lage waren, T-Zellen zu stimulieren[57-59].

Bei der korrekten Einordnung der von uns beschriebenen Zell-Population wurde schnell klar, dass es sich bei unserer Population um eine DC-Population handeln musste.
Die Analyse zeigte zahlreiche DC-Marker auf der Zelloberfläche, wohingegen keine weiteren NK-Zell-Marker, außer CD56, exprimiert wurden.
Eine durchgeführte Elektronenmikroskopie demonstrierte ebenfalls einen DC-Charakter.
Hierdurch grenzte sich unsere Population ebenfalls von den Populationen ab, die als HLA-exprimierende NK-Zellen bezeichnet werden. Verschiedene Gruppen konnten zeigen, dass NK-Zellen durch Rezeptor-Aktivierung in der Lage sind HLA-Moleküle auf ihrer Oberfläche zu exprimieren und naive T-Zellen zu stimulieren[69].

Zur weiteren Untersuchungen wurden die DC in eine $CD56^+$ Zell-Population und eine $CD56^-$ Zell-Population aufgetrennt, um funktionelle DC- und NK-Zell-Tests durchzuführen.

Beide Populationen unterschieden sich nur in vier verschiedenen Oberflächen-molekülen voneinander.
Die stärkere CD14-Reduktion und höhere Expression von CD40 auf $CD56^+$ DC deutete zunächst auf einen mehr ausgereiften DC-Typ hin. Dieses sollte sich auch noch bei den T-Zell-Stimulationstests zeigen.
Jedoch wiesen die $CD56^-$ DC eine gering höhere Expression von CD83 auf.

Funktionell zeigte sich, dass beide, sowohl die $CD56^+$ Zell-Population als auch die $CD56^-$ Population, in der Lage waren, exogene Antigene mittels Phagozytose aufzunehmen. Allerdings war die $CD56^-$ Population minimal geringer effektiv.
Die exogene Aufnahme von Antigenen ist eine typische DC-Eigenschaft. Sie kann sowohl per Phagozytose[60-62] als auch durch Macropinocytose erfolgen. Zusätzlich kann sie auch durch bestimmte Rezeptoren (z.B. Macrophage Mannose Receptor, DEC-205, Fc-Rezeptor), die AK-markierte Zellen oder spezielle Zuckermoleküle auf Zelloberflächen registrieren, vermittelt werden.

Als weiteres Kernmerkmal der DC gilt die Stimulation von naiven T-Zellen, entweder als allogene Antigenquelle (MLR) oder durch die Präsentation eines exogen aufgenommenen Peptids (autologe Reaktion).
Beide Zell-Populationen zeigten einen potenten allogenen MLR. Dieser war stärker im Vergleich zu MLRs, bei denen frisch isolierte Monozyten oder IL-4 generierten DC aus Monozyten verwendet wurden. Das gleiche galt auch für die autologe T-Zell Stimulation mittels Tetanus Toxoid.
In beiden Versuchsanordnungen zeigte sich nur ein geringer Unterschied zwischen der $CD56^+$ und $CD56^-$ Zell-Population, wobei die $CD56^+$ DC immer ein kleines Stück potenter waren.
Zusätzlich konnte gezeigt werden, dass die unselektionierten DC auch in der Lage waren, eine antigenspezifische Immunantwort in vivo hervorzurufen. Ein erneuter Beweis für ihren DC-Charakter.

Neben diesen typischen DC-Eigenschaften ergab sich ebenfalls eine typische NK-Zell-Eigenschaft.
Hierzu gehörte die Lyse von Tumorzellen, die jedoch auf die $CD56^+$ Population beschränkt und im Vergleich zu klassischen NK-Zellen auch nur sehr gering ausgeprägt war.
Dass der TRAIL-Mechanismus hierbei die Hauptrolle spielte ist nicht verwunderlich, da verschiedene Arbeiten gezeigt haben, dass IFN-α die TRAIL-Expression auf Monozyten und DC steigern kann[63;64].
Andere Lyse-Mechanismen spielten dabei keine Rolle. Dazu gehört der FasLigand-Mechanismus, der jedoch bei DC vorkommen kann, die aus dem Knochenmark hergestellt wurden[65;66]. Ebenfalls existieren Berichte über einen Lyse-Mechanismus bei DC mittels Granzym B/ Perforin, wenn frisch isolierte DC mit TLR7/ 8-Agonisten stimuliert wurden[67].
Es lässt sich daher vermuten, dass es auf die Art der Stimulation der Zellen ankommt, welchen Lyse-Mechanismus sie benutzen.

Die Zytokinproduktion der beiden Zell-Populationen hingegen deutet wiederum auf einen DC-Charakter hin, da sie beide große Mengen an IL-12 freisetzten, ein typisches Merkmal von DC [68].
Hingegen setzten sie nicht wie für NK-Zellen üblich IFNγ frei, obwohl sie intrazytoplasmatisch positiv für IFNγ waren. Dieses lässt vermuten, dass das produzierte IFNγ keine entscheidende Funktion hat.

F. Zusammenfassung

Zusammenfassend lässt sich aufzeigen, dass es sich bei der $CD56^+$ Zell-Population um eine DC-Population handelt, die auf Grund der Stimulation mit IFN-α TRAIL produziert. Dadurch ist sie in der Lage, TRAIL-sensitive Tumorzelllinien abzutöten. Ansonsten weist die $CD56^+$ Zell-Population typische DC-Eigenschaften auf und ist dabei sogar potenter als mit IL4 hergestellte DC.

Die Tatsache, dass sich die $CD56^+$ Population sowohl bzgl. der Oberflächenmarker als auch in Hinblick auf ihre Funktionalität nur gering von der $CD56^-$ Zell-Population unterscheidet (Ausnahme: Tumorlyse) mag daran liegen, dass es sich hierbei nicht um zwei verschiedene Populationen, sondern um eine Population von DC, die sich in unterschiedlichen Reifestadien befindet, handelt.
Dies verdeutlich auch der Versuch, bei dem isolierte $CD56^-$ DC nach 2 zusätzlichen Tagen ohne weitere Stimulation ebenfalls einen $CD56^+$ Charakter entwickelten[70].
Ein ungenügender Reifezustand könnte darüber hinaus erklären, warum $CD56^-$ DC trotz intrazytoplasmatischer TRAIL-Expression nicht in der Lage sind, TRAIL-sensitive Tumorzellen zu lysieren. Vermutlich können sie das TRAIL noch nicht sezernieren oder sie können die Tumorzellen nicht identifizieren.

Neuere Veröffentlichungen, die sich auf meine Arbeit beziehen, unterstützen unsere These.
So konnte Milush et. al. im Jahr 2009 zeigen, dass CD7 als Oberflächenmarker dazu dient zu unterscheiden, ob es sich bei CD56-exprimierenden Zellen um Monozyten/ DC oder NK-Zellen[71] handelt.
Im gleichen Jahr demonstrierten Gruenbacher et. al., dass $CD56^+$ DC zur Zolebromid-vermittelten Proliferation von $\delta\gamma$ –T-Zellen beitragen[72].
Beide Arbeiten bestätigen, dass es sich bei unserer Population um CD56-exprimierende DC handelte.

Abschließend konnte durch unsere Arbeitsgruppe gezeigt werden, dass diese DC-Population für den Einsatz am Patienten sicher anwendbar ist. Zusätzlich zeigte sich, dass DC ebenfalls *in vivo* in der Lage sind, eine AG-spezifische Immunantwort zu induzieren.
Inwieweit unsere DC-Population allerdings effektiver ist als anders generierte DC-Populationen und welche Rolle ihre Lyse von Tumorzellen *in vivo* tatsächlich spielt, muss noch in weiteren Versuchen und in klinischen Studien etabliert werden.

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I. Anhang

The Journal of Immunology

IFN-{alpha} Skews Monocytes into CD56+-Expressing Dendritic Cells with Potent Functional Activities In Vitro and In Vivo

Claudia Papewalis, Benedikt Jacobs, Margret Wuttke, Evelyn Ullrich, Thomas Baehring, Roland Fenk, Holger S. Willenberg, Sven Schinner, Mathias Cohnen, Jochen Seissler, Kai Zacharowski, Werner A. Scherbaum and Matthias Schott

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IFN-α Skews Monocytes into CD56$^+$-Expressing Dendritic Cells with Potent Functional Activities In Vitro and In Vivo[1]

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The antitumor effect of IFN-α is mediated by the activation of CTLs, NK cells, and the generation of highly potent Ag-presenting dendritic cells (IFN-DCs). In this study, 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 coincubation 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 with CD56$^-$ IFN-DC. Based on these results, five patients with metastasized medullary thyroid carcinoma were treated for the first time with monocyte-derived tumor Ag-pulsed IFN-DCs. After a long term follow-up (in mean 37 mo) all patients are alive. Immunohistochemical analyses of delayed-type hypersensitivity skin reaction showed a strong infiltration with CD8$^+$ cells. In two patients no substantial change in tumor morphology was detected. Importantly, by analyzing PBMCs, these patients also showed an increase of Ag-specific IFN-γ-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 antitumor effect induced by IFN-α. *The Journal of Immunology,* **2008, 180: 1462–1470.**

Interferon-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, 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 occurring IFN-α is secreted from IFN-producing cells, now known as plasmacytoid dendritic cells (pDCs)[4] (7). Therefore, DC may directly trigger cytolytic activity of NK cells. In contrast, 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 IFN-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 plasmacytoid DCs as they show the molecular expression profile of both NK cells and DCs. They produce substantial amounts of IL-12 and IFN-γ, depending on activation stimuli. Most interestingly, IKDCs directly kill typical NK targets mediated by the 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

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[4] Abbreviations used in this paper: pDC, plasmacytoid dendritic cell; IKDC, IFN-producing killer DC; MTC, medullary thyroid carcinoma; CT, computed tomography; DTH, delayed-type hypersensitivity; IFN-DC, IFN-α generated DC.

reveal direct cytolytic activity (18, 19), however, without identifying a certain DC subset. In addition, Banchereau et al. (20) already reported on the in vivo use of $CD34^+$ progenitors which were activated with type I IFN and which were used for the treatment of stage IV melanoma patients leading to the induction of tumor Ag-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 TRAIL. Intracytoplasmatic cytokine staining revealed that most of $CD56^+$ IFN-DCs as well as $CD56^-$ IFN-DCs are IFN-γ producing as well. Mixed leukocyte reactions, however, revealed a slightly stronger capacity in stimulating allogenic T cells compared with $CD56^-$ 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-aa-long tumor-specific peptide. In the past, we already described that calcitonin may serve as specific tumor Ag 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.

Materials and Methods

Cell separation and DC 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). Untouched $CD14^+$ peripheral blood monocytes were immunomagnetically purified by using a depletion mixture of biotinylated Abs 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 (Invitrogen Life Technologies) at 2.5×10^6/ml in the presence of GM-CSF (1000 U/ml; Leukine) and IFN-α (1000 U/ml; Roferon-A, Roche) without FCS. 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, IL-4-DCs were generated as formerly described (22).

For in vivo use in cancer patients, DCs were generated in a Good Manufacturing Practice facility (permission received from the Regional Council Düsseldorf: No. 24.30-04.01-001) from monocytes from each patient from an initial leukapheresis (≥4.5×10^9 nucleated cells) followed by Ficoll density gradient centrifugation and an adherens step (1–2.5×10^8 DCs/15 ml RPMI 1640 for 2 h). Adherent cells were cultured for 3 days with GM-CSF and IFN-α as described above. After 72 h, DCs were pulsed with full-length human calcitonin (100 μg/ml; cibacalcin; Novartis), known to be a MTC cell-specific Ag 21. After 2 h, cells were harvested, washed four times with isotonic NaCl, and resuspended in 100 μl of 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 mAbs used for flow cytometry were purchased from BD Pharmingen if not otherwise indicated. All Abs were FITC-, PE-, PerCP Cy5.5-, or allophycocyanin-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 toward CD56, CD16, CD94, CD161, and CD337 (both from BD Pharmingen) and anti-NKG2A, NKG2D, NKp44, and NKp46 (purchased from Beckman Coulter) were used. Annexin V (Abcam) was used for the detection of apoptotic cells. A positive control for annexin V staining was implanted by incubation cell samples with camptothecin (6 μM, 4 h at 37°C).

For detection of intracellular Ags, cells were treated with brefeldin A (Golgi plug) for 3 h 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. Samples were analyzed using a FACSComp device (BD Biosciences). 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 (1×10^6) were labeled with 100 μCi of ^{51}Cr for 1 h at 37°C. Cells were washed three times, resuspended 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 h). In some experiments, anti-TRAIL RIK-2 mAb (10 μg/ml) or TRAIL ligand (eBioscience), respectively, were added. Supernatants were collected and measured by a Wizard Automatic Gamma Counter (Wallac). Data were expressed as the mean ± SD of triplicate wells. The percentage of cytotoxicity was calculated as: cytotoxicity (percent) = ((experimental group cpm − spontaneous cpm)/(total cpm − spontaneous cpm)) × 100.

Mixed lymphocyte reaction

Allogeneic $CD3^+$ T lymphocytes were purified using anti-CD3-conjugated magnetic microbeads and seeded into 96-wells plates at 5×10^5 cells/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) was added to each well and incubation was continued for additional 18 h. Cells were finally collected by a Packard Instrument Filtermate Harvester onto Unifilter-96 (PerkinElmer) and thymidine uptake was quantified by scintillation counting using a TriLux MicroBeta (Wallac).

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 allophycocyanin-labeled mouse CD56 Abs, followed by cell permeabilization and staining with FITC-labeled anti-IFN-γ Abs (BD 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). Supernatants were collected from a 1-day culture of pure $CD56^-$ and $CD56^+$ IFN-DC, respectively, as well as of cocultures 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 kV under a Hitachi electron microscope H-600.

Patients

Table I depicts a summary of patient's characteristics. Patients with histological proven 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 s.c. injections with a median of 1.9×10^8 IFN-DCs ($\pm 0.8 \times 10^8$ DCs, range 0.7–4.0×10^8 DCs) per vaccine. Patients were followed up for 18–46 mo (mean 37 mo). Metastatic lesions were evaluated by computed tomography (CT) scans and ultrasonic examinations. World Health Organization definitions were used for stable disease with a change of <25% in tumor size and tumor markers without occurrence of new lesions for a minimum of six weeks. Measurements of CEA (Roche) and calcitonin (Roche Diagnostics) were performed by commercial kits according to the manufacturer's instructions. Because the cut-off for calcitonin detection is ~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

Table I. *Patient's characteristics and status before DC vaccination*

Patient No.	Age, Sex	Tumor Type	HLA Type	Status before Therapy		
				Calcitonin (pg/ml)	CEA (μg/L)	Metastases
1	42, M	Sporadic	DRB1 0301, 1501	229,833	2989	Liver, lung, LN, bone
2	30, M	Sporadic	DRB1 0401, 0701	77,430	65	Lung, LN bone
3	64, M	Sporadic	DRB1 0701, 1501	2,240	43	Lung, LN
4	42, M	Sporadic	DRB1 0801, –	12,450	377	Lung, LN liver (suspected)
5	61, M	Hereditary	DRB1 0401, 1301	69,426	124	Liver, lung, LN

Tumor type: sporadic/hereditary medullary thyroid carcinoma. Metastases: LN: lymph node.

change of tumor markers of >25% difference for at least 3 mo was considered as progression (23).

Intracellular cytokine staining

Autologous, cryopreserved PBMCs (1×10^6 cells/ml) of treated patients were cultured in RPMI 1640 in the presence of full-length calcitonin (32 aa; cibacalcin; Novartis) 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 Abs, intracellular staining was performed with PE-labeled anti-IL-4 or FITC-labeled anti-IFN-γ Abs (BD Pharmingen), respectively.

Delayed-type hypersensitivity (DTH) and immunohistochemistry

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 h after intradermal injection. In one patient (no. 1), we additionally tested DTH reactivity by intradermal injection of pure calcitonin (10 μg in 100 μl of isotonic NaCl) followed by skin biopsy. In this patient, a biopsy (diameter 5 mm) of the DTH site was taken 24 h after injection. Serial paraffin-embedded sections were stained with a mAb against human CD8 (concentration: 1:200; clone C8/144B; DakoCytomation) in a moist chamber at 37°C for 60 min. Bound Ab was detected using avidin-biotin complex (ABC) peroxidase method (ABC Elite kit; Vector Laboratories). 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 unpaired *t* tests, respectively, depending on the data used for calculation using GraphPad Prism 4.0 computer software (GraphPad Software).

Results

Generation of DCs 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 hereafter. 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), costimulatory 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 interestingly, 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 APCs 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 costimulatory

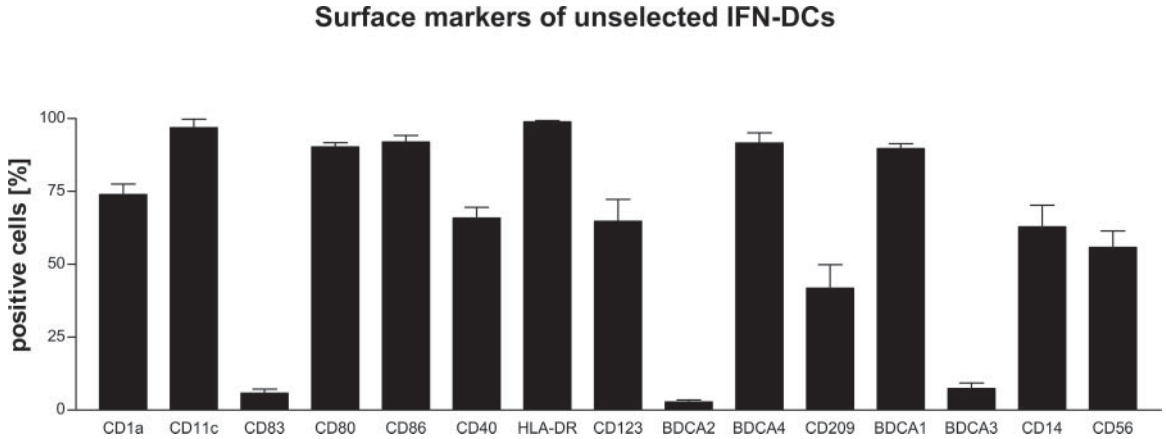

FIGURE 1. Immunophenotypic pattern of IFN-DCs. Isolated monocytes (purity >98%) were cultured over 3 days with GM-CSF and IFN-α. Resulting IFN-DCs were stained with a panel of Abs and analyzed by flow cytometry. As demonstrated in previous studies, IFN-DCs reveal a semimature phenotype with a moderate expression of CD83 whereas costimulatory 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 plasmacytoid 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.

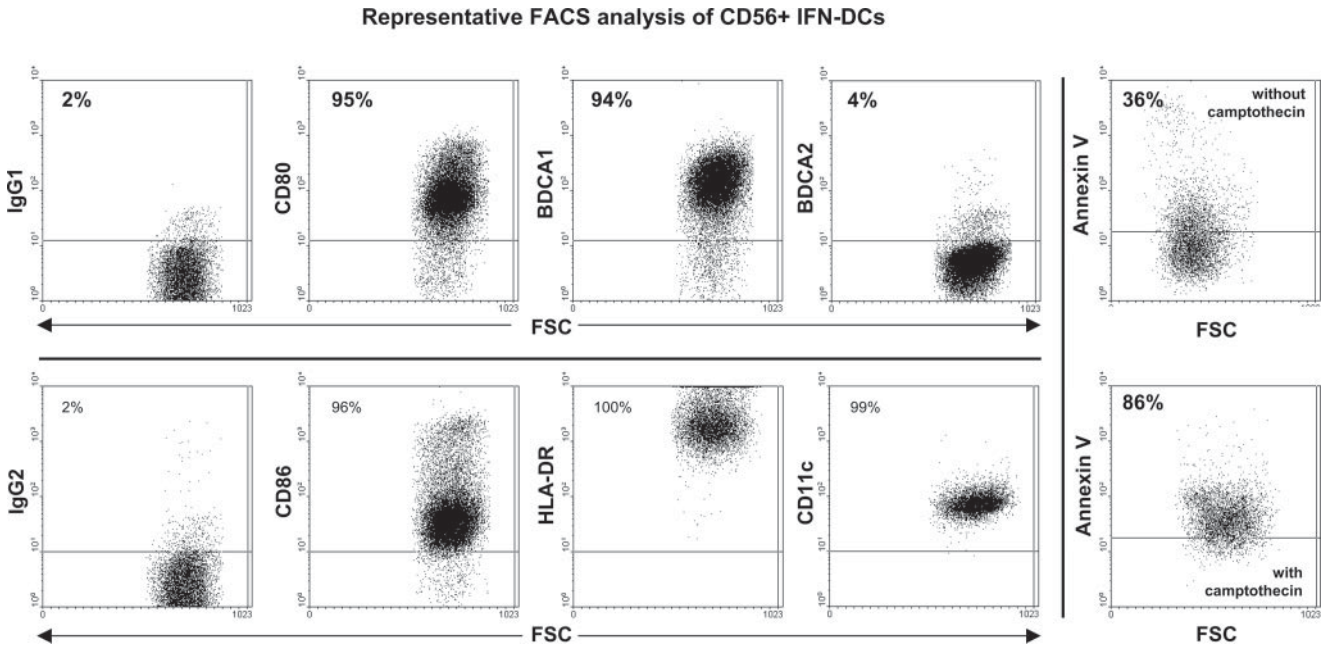

FIGURE 2. Representative FACS analysis of CD56$^+$ IFN-DCs. After incubation with GM-CSF and IFN-α, CD56$^+$ cells were isolated by positive selection and stained with anti-CD11c, -CD80, -CD86, -HLA-DR, -CD40, -BDCA-1, and -BDCA-2 mAbs. Appropriate isotype controls performed in parallel were nearly identical, 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.

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 with 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 with 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 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,

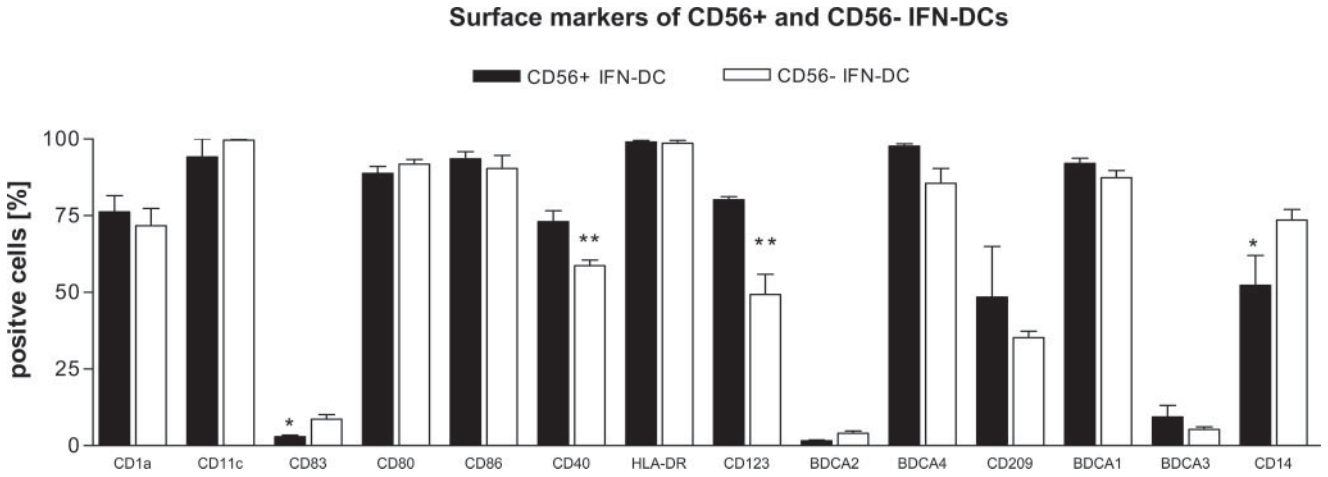

FIGURE 3. Immunophenotypic pattern of CD56$^-$/CD56$^+$ IFN-DCs. CD56$^+$ IFN-DCs were purified with CD56 mAb-conjugated microbeads leading to a purity of the CD56$^+$ cells of ≥98%. Thereafter, CD56$^+$ and CD56$^-$ IFN-DCs were stained with a panel of Abs and were analyzed by flow cytometry. CD56$^+$ 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 with CD56$^-$ IFN-DCs as well as CD40 which was up-regulated on CD56$^+$ IFN-DCs. Marker expression is given as mean (±SD) of at least five DC preparations.

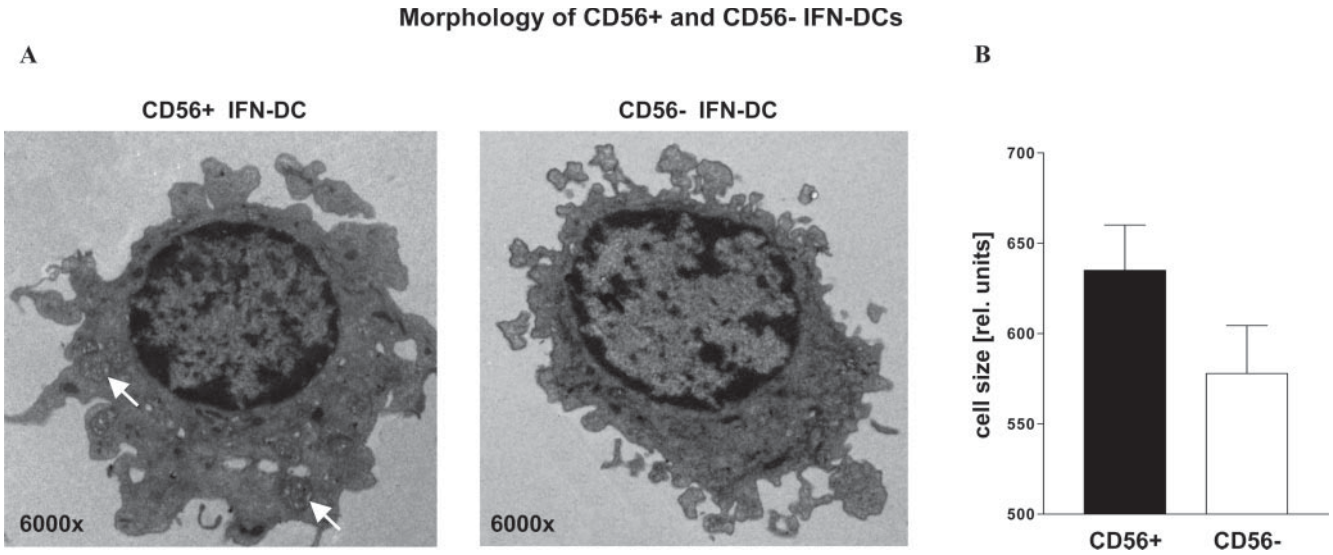

FIGURE 4. Morphology of CD56$^+$ and CD56$^-$ IFN-DCs. CD56$^+$ and CD56$^-$ cells were purified with CD56 mAb-conjugated microbeads. Thereafter, both cell fractions were analyzed by electron microscopy (*A*). Both cell types showed similar phenotypes with numerous pseudopodia. The cytoplasm of CD56$^+$ 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 with CD56$^-$ IFN-DCs (577.9).

these cells were cocultured for 18 h 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. 5*A*).

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. 5*B*). Intracytoplasmatic cytokine analyses of CD56$^+$ IFN-DCs revealed that in mean 95.2 ± 2.7% of all CD56$^+$ IFN-DCs were positive for TRAIL (representative FACS analysis is given in Fig. 5*C*). CD56$^-$ IFN-DCs also showed a TRAIL expression. This was, however, less pronounced compared with 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.

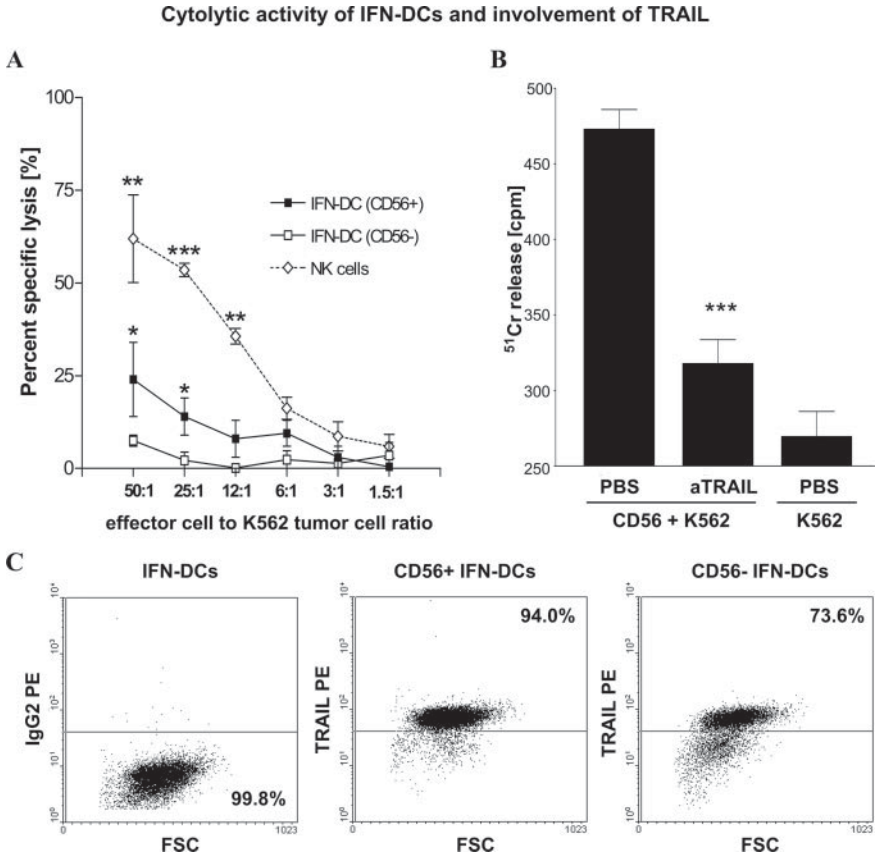

FIGURE 5. Cytolytic activity of IFN-DCs and involvement of TRAIL. After generation of IFN-DCs CD56$^+$ and CD56$^-$ cells were separated. *A*, Both cell fractions were cocultured with ^{51}Cr-labeled K562 tumor cells for 18 h at different cell/K562 tumor cell ratios. Supernatants were collected and analyzed by gamma-radiation analysis (CD56$^+$ IFN-DCs, ■; CD56$^-$ IFN-DCs, □). For internal control, K562 tumor cells were cultured alone and in the presence of a detergent for direct lysis. NK cells were used as positive control (◇). Results of three independent experiments are shown. *B*, CD56$^+$ IFN-DCs were cocultured in a ratio of 1:50 with or without anti-TRAIL (10 μg/ml). The lysis activity could be partially blocked (−81 ± 2%) after coculturing 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 with CD56$^+$ IFN-DCs. Isotype control for whole IFN-DCs is given in the *left panel.*

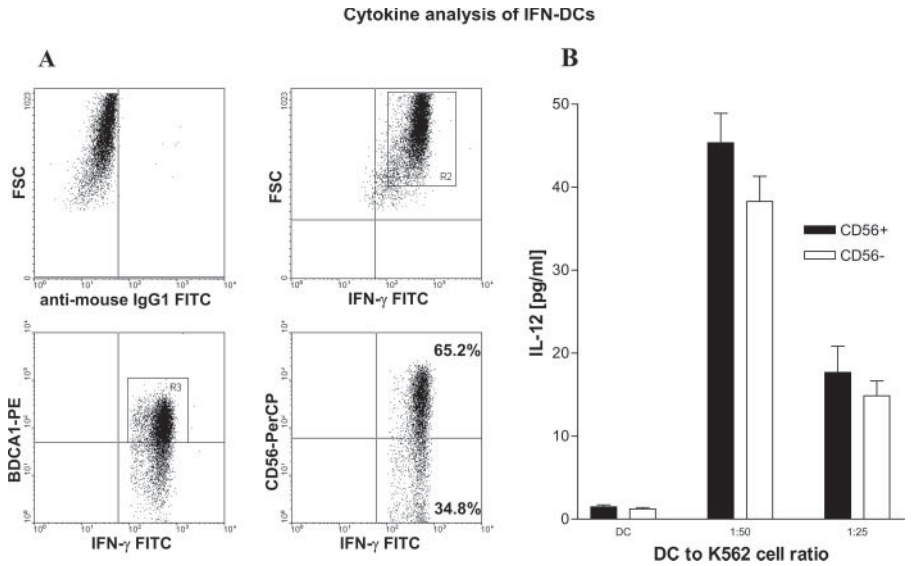

FIGURE 6. Cytokine analysis of IFN-DCs. Representative intracellular cytokine staining (*A*) shows that the majority of IFN-DCs are IFN-γ-producing (*upper right panel*; isotype control is shown on the *upper left panel*). Detailed analyses revealed that the majority of cells are BDCA1 positive (gated 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 *B*. $CD56^+$ and $CD56^-$ cells were separated after generation of IFN-DCs. Both cell fractions were cultured alone or cocultured with K562 tumor cells in two different ratios, respectively. Supernatants were collected and frozen until analysis. Coculture with K562 tumor cells, resulted in a slight increase of IL-12 production in $CD56^+$ IFN-DCs (up to 49 pg/ml ± 6%) compared with $CD56^-$ 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.

Cytokine production by IFN-DC

For specific determination of IFN-γ-secreting cells intracellular cytokine staining was performed. After 3 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 Fig. 6*A*). 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 3 days of culture with IFN-α, neither $CD56^-$ nor $CD56^+$ IFN-DCs produced any significant amounts of IL-12 measured within the supernatants of cells (Fig. 6*B*). Importantly, however, after additional 24 h of coculture 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 APCs

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, IL-4-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 with immature monocytes ($p < 0.0001$) and, most importantly, compared with IL-4-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 with $CD56^-$ 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 MTC. The patient's characteristics are given in Table I. 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 DTH skin reactivity. After the second 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

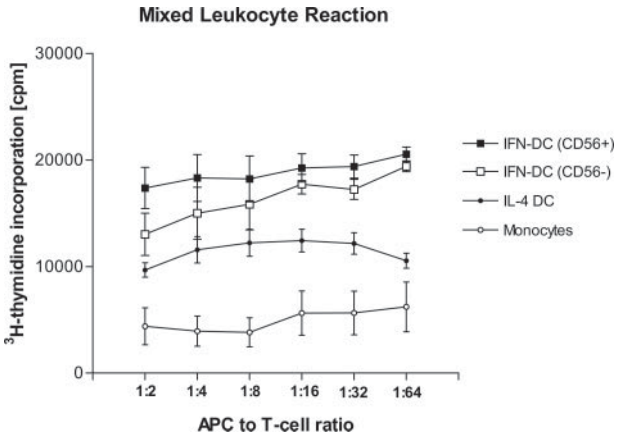

FIGURE 7. MLR of APCs. $CD56^+$ IFN-DCs and $CD56^-$ IFN-DCs, respectively, were cocultured with allogeneic $CD3^+$ T lymphocytes at different stimulator-to-responder ratios for 6 days. [^{3}H]Thymidine (1 μCi) was added for 18 h, and thymidine uptake was quantified by scintillation counting. At all stimulator cell:T cell ratios, $CD56^+$ IFN-DCs (■) revealed a slightly stronger stimulating capacity compared with $CD56^-$ IFN-DCs (□; $p = 0.036$). Freshly isolated monocytes (○) as well as IL4-generated DCs (●) were used as controls. At all stimulator-to-responder ratios IFN-DCs were more potent in stimulating allogenic T cells compared with immature monocytes ($p < 0.0001$) and compared with IL4-DCs ($p < 0.0001$). Results are shown from three independent experiments.

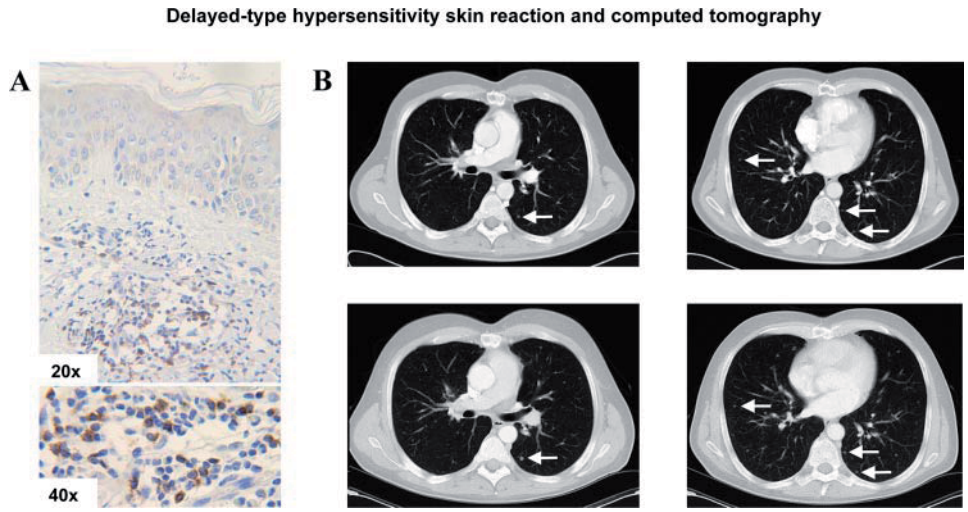

FIGURE 8. Delayed-type hypersensitivity skin reaction and CT. *A*, Skin biopsy of patient 1 demonstrates strong epidermal infiltration with $CD8^+$ CTL 48 h after s.c. administration of calcitonin (10 μg in 100 μl of isotonic NaCl). Paraffin sections were incubated with mAbs against CD8 and stained with the avidin-biotin complex peroxidase method. *B*, CT of the chest of patient 4. Pretherapeutic scan (*upper row*) and follow-up examination 44 mo later (*bottom row*). After a long-term follow-up, there is a tiny increase of small pulmonary metastases (arrows).

hours after Ag injection there was a strong perivascular and epidermal infiltration with $CD8^+$ T lymphocytes (Fig. 8*A*). This clearly indicates the ability of IFN-DCs to induce a Th1-like Ag-specific immune response in vivo.

During follow-up of (in mean) 37 mo, there were three patients (nos. 1, 2, and 5) who experienced a progression determined by CT 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 mo, serum carcinoembryonic Ag in patient 4 showed a slight decrease (332 μg/L after treatment compared with 377 μg/L before treatment), whereas in the CT scan of the lung only a tiny increase was detected (Fig. 8*B*). Pulmonary CT scan of patient 3 showed a stable disease whereas the tumor markers CEA and calcitonin showed a small increase of <25% (52 μg/L after treatment vs 43 μg/L before treatment for CEA and 2090 vs 2,240 pg/ml for calcitonin, respectively). Importantly, by analyzing PBMC these patients also showed an increase of calcitonin-specific IFN-γ-secreting T cells (Fig. 9).

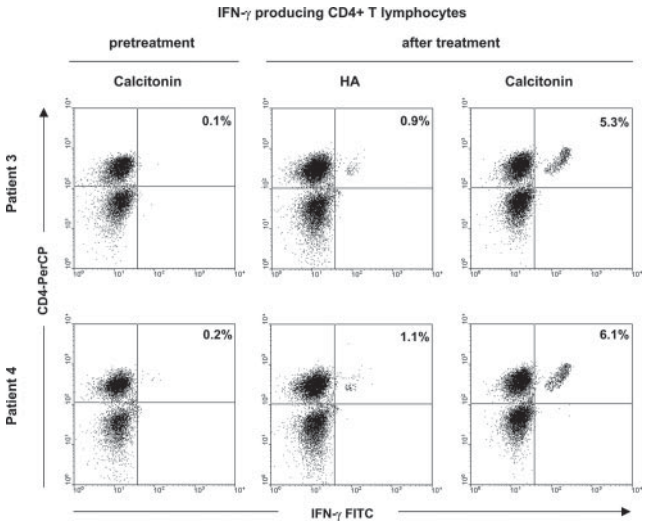

FIGURE 9. Percent IFN-γ-producing $CD4^+$ T lymphocytes in response to calcitonin determined by intracytoplasmatic cytokine staining. PBMC from patients 3 and 4 were isolated from blood samples collected monthly. Patient's PBMC were exposed for 16 h to 100 μg/ml calcitonin (*left* and *right panels*) and to a control protein (human albumin, *middle panels*). For the last 3 h, brefeldin A was added to inhibit protein secretion, cells were stained for extracellular CD4 and intracellular 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 with pretreatment (*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*).

Discussion

Type I 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 CTLs (4) and the activation of NK cells (6). In the last couple of years, another mechanism, namely the differentiation of monocytes into DCs by IFN-α (IFN-DCs) have been proposed to be responsible as well (30, 31). When compared with the classical IL 4-generated DCs (IL-4-DCs), IFN-DCs exhibit a typical DC morphology and express a similar level of costimulatory 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-1β, 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 Ags compared with classical DCs (36). Most important, in the context of cytotoxicity, these cells also reveal direct tumoricidal activity via the 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 APCs 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 cocultured 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 coincubation 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. (45) discovered tumor-infiltrating myeloid DCs and pDCs that gained lytic functions and exogenous supply of TLR7 agonists. 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.

It is important to note that the initial intent of this study was to identify the human counterpart of a new cell population recently identified in mice termed IKDCs (11, 12). These cells are distinct from conventional DCs and pDCs as they show the molecular expression profile of both NK cells and DCs. Moreover, they produce high amounts of IL-12 and IFN-γ. Most importantly, 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 Ags under certain circumstances, especially in tumoral context (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 NK cell receptor-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 with 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 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 supernatant 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 IL-4-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 cannot be termed as human IKDCs as other classical NK cell markers are still missing and cytolytic activity is much lower compared with NK cells.

To the best of our knowledge, this is the first report demonstrating monocyte-derived IFN-DC treatment in cancer patients. As demonstrated by DTH skin reaction, our results suggest that IFN-DCs induce Ag-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 et al. (20) reporting on the in vivo use of CD34$^+$ progenitors which were activated by type I IFN and which were used for the treatment of patients with stage IV melanoma. The authors reported on no statistically significant survival advantage in these patients. Importantly, however, in six of seven patients tumor Ag-specific recall memory CD8$^+$ T cells able to secret IFN-γ and to proliferate could be detected. More recently, another study from Di Pucchio et al. (49) reported on a pilot study to determine the effects of IFN-α administered as adjuvant of tumor-specific Ags in stage IV melanoma patients. The authors detected an enhancement of CD8$^+$ T cells recognizing native and modified tumor Ags and most important a significant increase of DC precursors. These cells had an enhanced APC function in some patients with stable disease (49). These data are in line with another report from Yamamoto et al. (28) reporting on markedly induced TRAIL expression on CD14$^+$ monocytes and enhanced cytotoxic activity toward hepatocellular carcinoma cells after coincubation with IFN-α.

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 APCs. 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 to improve clinical efficacy.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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Characterization of Monocyte-derived IFNα-generated Dendritic Cells

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Key words
- dendritic cells
- natural killer cells
- interferon alpha
- CD56
- cytotoxicity
- vaccination

Abstract

The antitumor effects of IFNα is mainly mediated by the activation of cytotoxic T lymphocytes (CTLs), the activation of natural killer (NK) cells, and the generation of highly potent antigen-presenting dendritic cells (IFN-DCs). Recently, we demonstrated that these cells partially express the NK cell marker CD56 and reveal a direct cytotoxic immunity towards tumor cells. The aim of the present study was to explore these cells in more detail with respect to their phenotypical and functional characteristics. Flowcytometric analyses revealed that a 5-day incubation time of CD14+ monocytes with IFNα results in a steady increase of CD56 surface expression of these cells from 25% (±2%) on day 1 up to 68% (±11%) on day 5. Interestingly, additional culturing of negatively selected CD56– IFN-DCs also resulted in a partial CD56 surface expression. By comparing both cell types in more detail we found a significant decrease of CD14 expression on CD56+ IFN-DCs (66±6%) compared to CD56- IFN-DCs (76±6%). On the basis of functional tests, CD56+ IFN-DCs revealed a slightly increased phagocytosis capacity compared to CD56– IFN-DCs as only 82% of CD56- IFN-DCs showed a positive intracytoplasmatic signal after 60 minutes coculturing with FITC-labeled albumin, whereas 91% of CD56+ IFN-DCs were positive. Moreover, CD56+ IFN-DCs revealed a stronger T cell stimulation capacity compared to CD56- IFN-DCs. These results together with our previously described data suggest that CD56+ IFN-DCs and CD56– IFN-DCs may represent one identical cell population with different maturation status rather than two separate cell entities. Because of their high stimulating capacity and their direct cytolytic effects these cells represent a new promising tool for cellular anticancer therapy.

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Abbreviations

DC	Dendritic cell
FACS	Fluorescent-activated cell storing
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN-DC	Interferon-α generated DC
IKDC	Interferon-producing killer DCs

Introduction

Interferon alpha (IFN-α) belongs to the group of type I IFNs endowed with potent antiviral, antitumor and immunoregulatory activities [1,2]. It is mainly produced by the so-called natural IFN-producing cells (IPCs) – now known as plasmacytoid dendritic cells (PDCs) – after exposure to viruses or other stimuli, respectively [3,4]. The antitumor and antiviral effects of IFNα is mediated by the differentiation of the Th1 subset, the generation of CTLs and by the promotion of the *in vivo* proliferation and survival of T cells [2,5–7]. 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 [8].

In the past, another alternative mechanism has been proposed by demonstrating that monocyte-derived IFNα-generated DCs (IFN-DCs) also reveal a direct tumoricidal activity via the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [12]. In these studies TRAIL expression have been demonstrated on a molecular [9,10] and protein level [9,11]. Most recently, we confirmed 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 crucial, as up to now CD56 expres-

sion has only been described to be present on NK cells and NKT cells, however not on antigen-presenting cells such as DC.
The aim of the present study was to explore the *in vitro* generation pathway of IFN-DCs in more detail. Especially, we aimed to investigate whether CD56- IFN-DCs and CD56+ IFN-DCs represent two distinct cell populations or rather one identical subtype with different maturation status. To do this phenotypical analyses as well as functional tests including analyses of phagocytosis capacity and T cell stimulation tests were performed.

Material and Methods

Cell separation and dendritic cell generation

For *in vitro* experiments, peripheral blood mononuclear cells (PBMCs) from healthy volunteers were used (study number of the local ethical review board: 2608/05). All magnetic separations were performed on the basis of microbead technology (Miltenyi Biotec, Bergisch Gladbach, Germany). Negatively selected CD14+ peripheral blood monocytes were immunomagnetically purified by using a depletion cocktail of biotinylated antibodies and antibiotin mAb-conjugated microbeads. As verified by flow cytometry analysis, a purity of >98% of CD14+ cells was obtained by a secondary purification step using anti-CD3 and anti-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 IFNα (1000 U/ml; Roferon-A®, Roche, Mannheim, Germany) without fetal calf serum. After culturing for 3 days, CD56+ IFN-DCs were purified with CD56 mAb-conjugated microbeads leading to a purity of ≥98%. CD56+ and CD56- IFN-DCs were then used for further analyses.

Phenotypic analysis of DCs by flow cytometry analysis

All monoclonal antibodies (mAbs) used for flow cytometry were purchased from BD PharMingen (Heidelberg, Germany), unless otherwise indicated. All antibodies were FITC-, PE-, PerCP Cy5.5-, or APC-conjugated, respectively, and measured in parallel to appropriate isotype controls. Several DC markers were characterized by using anti-CD80, -CD83, -CD86, -CD40, -CD11a, -CD11c, -CD54, -CD208, -CD123, -CD209, -CD14 and -HLA-DR mAbs (BD Biosciences). Additionally, for characterization of NK cell surface markers mAbs towards CD56 and CD337 (both from BD PharMingen) and anti-NKG2A, NKG2D, NKp40, and NKp46 (purchased from Beckman Coulter, Krefeld, Germany) as well as CD16 and CD49b were used. Samples were analyzed using a FACSComp device (BD Biosciences, Heidelberg, Germany). Data were analyzed using CellQuest^{PRO} software (BD Biosciences). A minimum of 10000 events were measured from each DC preparation before administration.

Phagocytosis capacity

Phagocytosis capacity of IFN-DCs was determined by coincubation of cells with FITC-labeled albumin (BD Biosciences) in the presence of RPMI-1640 plus 10% fetal calf serum (Invitrogen) for 5 minutes or 60 minutes, respectively. Thereafter, cells were harvested and labeled with anti-CD56 APC, and measured by FACS as described above.

T cell stimulation tests

T cell stimulatory capacity of CD56+ IFN-DCs and CD56– IFN-DCs in comparison to monocytes were performed by coincubation of these cells with autologous T cells at different stimulator-effector-ratios. Autologous CD3+ T lymphocytes were purified using anti-CD3 conjugated magnetic microbeads and seeded into 96-wells plates at 2×10^5 cells per well. Monocyte-derived cells were added to each well in triplicate at different stimulator-to-responder ratios together with 8 LFU/ml tetanus toxoid (Bilthoven, NL). After 5 days, 1 μCi of [3H]-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 (PerkinElmer, Boston, MA, USA) and thymidine uptake was quantified by scintillation counting using a Trilux Mikro Beta (Wallac, Turku, Finland).

Statistical analysis

The results were analyzed for statistical significance by paired t-test using GraphPad Prism 4.0. computer software (GraphPad Software Inc., San Diego, CA).

Results

Generation of IFN-DCs and phenotypical analyses

Dendritic cell marker

IFN-DCs were generated as recently described [12] by culturing positively selected CD14+ monocytes with clinical grade GM-CSF and IFNα. Thereafter, CD56+ and CD56– IFN-DCs were selected resulting in 98.7±2.1% purity of CD56+ cells and an 80.7±3.7% purity of CD56- cells, respectively. As recently demonstrated [12], both cell populations did not show any marked differences for most surface markers. By comparing both cell types in more detail, however, we found a significant decrease of CD14 expression on CD56+ IFN-DCs (66±6%) compared to CD56- IFN-DCs (76±6%; **Fig. 1**). In addition, the maturation marker CD83 was less pronouncedly expressed on CD56+ IFN-DCs compared to CD56– IFN-DCs (6±2% vs. 11±1%, respectively). The two DC markers CD123 and CD209 were expressed as following: CD123 88.3±2.9% on CD56+ cells vs. 65±8% on CD56- IFN-DCs; and CD209 45±9% on CD56+ cells vs. 33.5 ± 12% on CD56- IFN-DCs.
Costimulatory molecules including CD80 (92±1.5%) and CD86 (85±2%), CD40 (99.5±1%) and HLA-DR-molecules (100±1%) were upregulated on both cells. Moreover, different adhesion molecules such as CD11c (98±0.2%), CD11a (95±5%) and CD54 (99.5±1%) are expressed on both cells as well. By contrast, the maturation marker DC-LAMP (CD208) is only marginally expressed (1.2 ± 0.5%).

NK cell marker

Because CD56 is mainly expressed by natural killer (NK) cells and natural killer T (NKT) cells, we also looked for additional natural cytotoxic receptors (NCR) specific for NK cells including NKp30 (4±1%), NKp40 (1±0.2%), or NKp46 (5±0.6%). However, none of them were found with a remarkable level on these cells. Furthermore, other NK cell markers like CD16 (1±1%) or CD49b (1±0.4%) as well as the two other NK cell-specific markers NKG2A (1±0.2%) and NKG2D (2.5±2%) were neither found (**Fig. 2**).
Importantly, we also looked on the acquisition of CD56 during development of IFN-DCs from monocytes. As shown in **Fig. 3** there is a steady aquisition of CD56 on these cells while

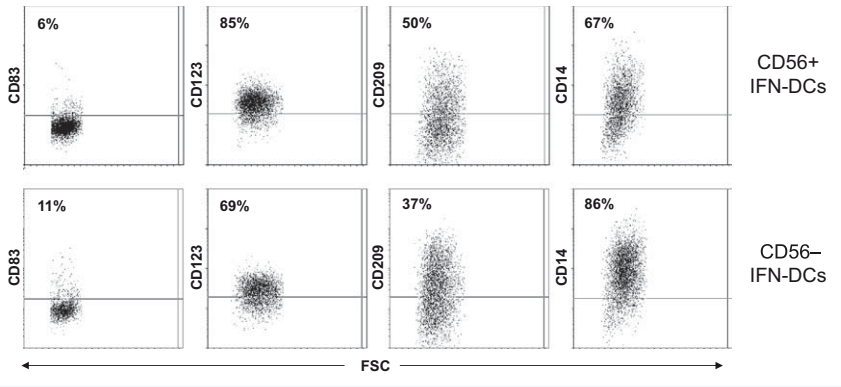

Fig. 1 Representative FACS analysis of CD56+ IFN-DCs and CD56– IFN-DCs. After incubation of monocytes with GM-CSF and IFNα cells were analyzed for different markers. IFN-DCs showed surface markers typically for common DCs (CD209) as well as for plasmocytoid DCs (CD123). Detailed analyses, however, revealed that CD56+ IFN-DCs show a stronger expression of CD123 and CD209 compared to CD56- IFN-DCs. In parallel, the monocytes marker CD14 is more suppressed on CD56+ IFN-DCs compared to CD56– IFN-DCs.

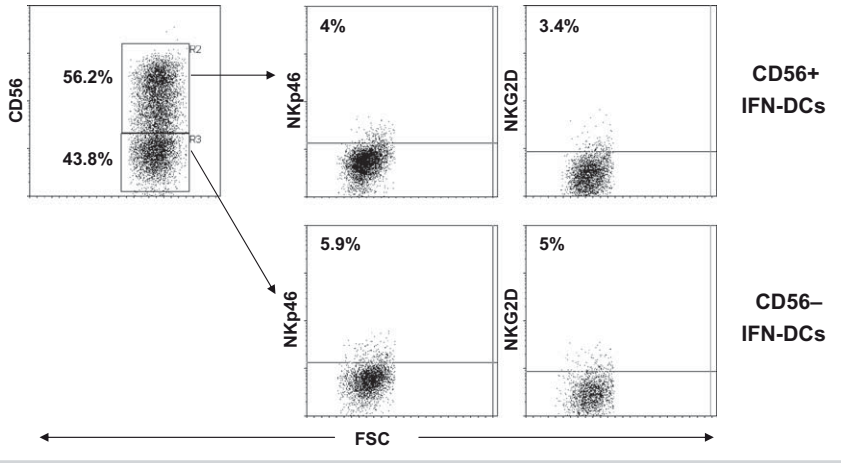

Fig. 2 Immunophenotypic analysis of different NK cell marker. IFN-DCs were gated for CD56 expression. Subsequently, different NK cell markers were analyzed. Independent of the CD56 expression all other NK cell marker tested, including NKp46 and NKG2D were only marginally expressed.

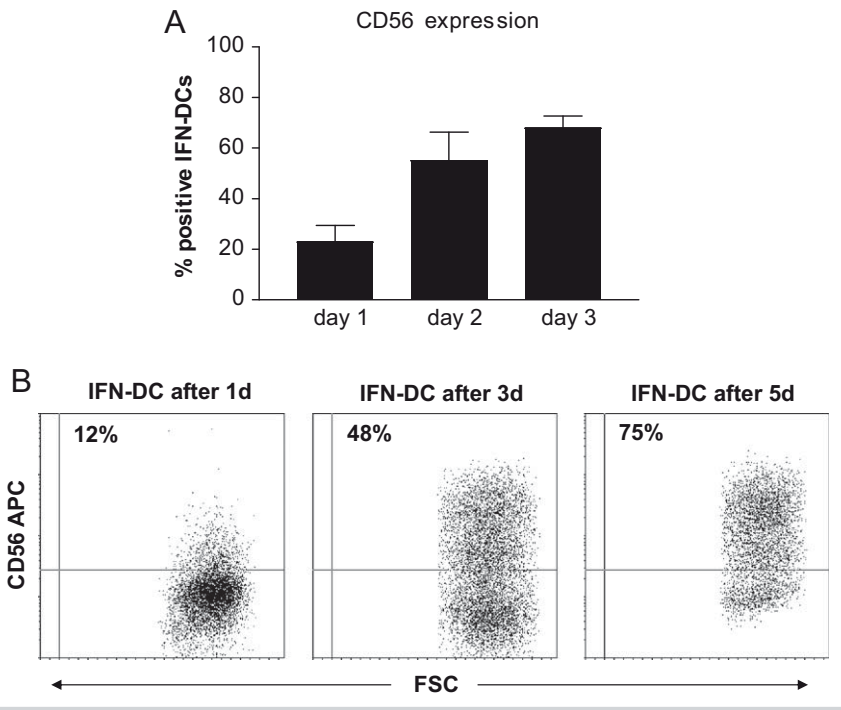

Fig. 3 Time course of CD56 expression of IFN-DCs while culturing with IFN-α. IFN-DCs were cultured from CD14+ monocytes over 5 days with GM-CSF and IFNα. While culturing CD56 expression was measured at different time points. Mean values (**A**) and representative FACS analysis (**B**) clearly demonstrates a steady increase of CD56 expression of these cells.

culturing with IFNα. The maximum was seen after 5 days of culturing as 68 ± 11 % of IFN-DCs were CD56 positive. Moreover, we also cultured negatively selected CD56– IFN-DCs for additional 36 hours without substitution of any cytokines. Since a negative selection kit was used for purification in mean 19 ± 4 % of IFN-DCs already expressed CD56 on their surface. Interestingly, the majority of negatively selected cells also acquired CD56 on their surface indicating that

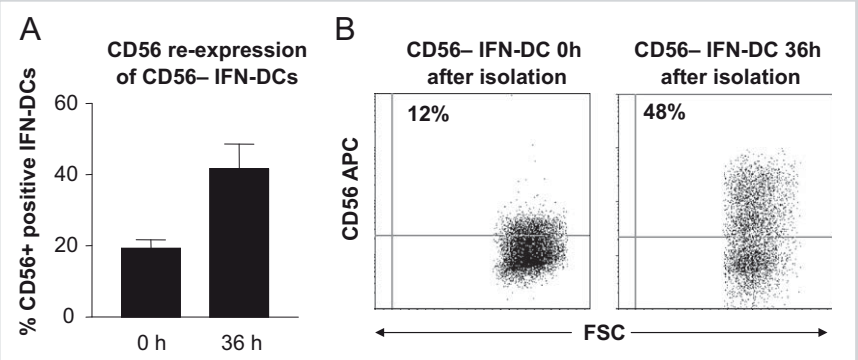

Fig. 4 Time-dependent CD56 expression of CD56- IFN-DCs. IFN-DCs were cultured from CD14+ monocytes over 3 days with GM-CSF and IFNα. Thereafter, CD56- IFN-DCs were negatively selected resulting in purity of in mean 81%. These cells were cultured for additional 36 hours without any cytokines resulting in a steady increase of CD56 expression on these cells.

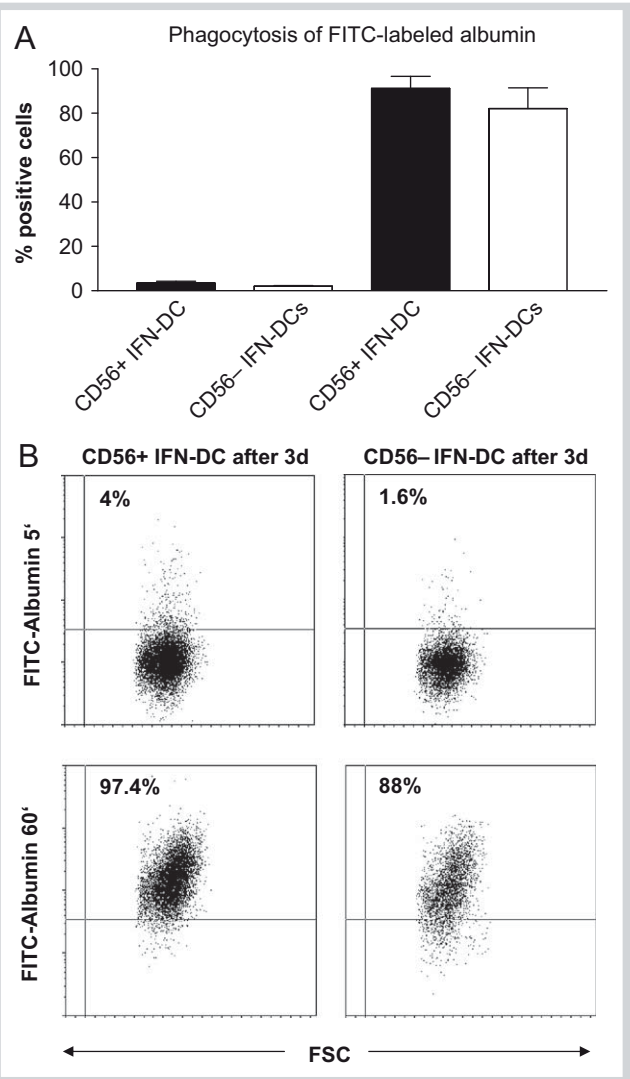

Fig. 5 Phagocytosis capacity of CD56+ and CD56– IFN-DCs. (**A**) IFN-DCs were incubated with FITC-labeled albumin. After a 5 minutes incubation time (left bars) only a tiny fraction (between 2–3%) of IFN-DCs were positive. After 60 minutes, however, the majority of IFN-DCs showed a positive signal by FACS analysis. Detailed analyses revealed that CD56+ IFN-DCs showed an even stronger phagocytotic capacity compared to CD56- cells (91 vs. 82% positivity). (**B**) Representative FACS analysis of FITC-labeled albumin-treated CD56+ and CD56- IFN-DCs.

CD56+ and CD56– IFN-DCs do not represent two distinct populations rather than one cell population with different maturation status (◉ **Fig. 4**).

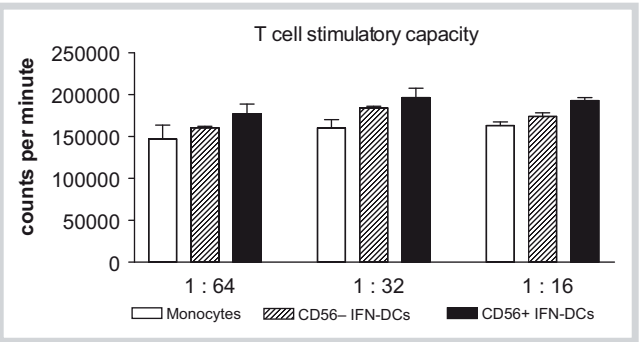

Fig. 6 T cell stimulating capacity of IFN-DCs. At all stimulator/responder ratios IFN-DCs were found to be more potent for stimulating T cells compared to immature monocytes ($p = 0.004$). Detailed analyses of CD56+ and CD56– IFN-DC revealed that CD56+ IFN-DC showed a moderate stronger capacity in stimulating autologous T cells compared to CD56- IFN-DC at all DC/T cell ratios ($p = 0.01$).

Functional analyses of CD56+ IFN-DCs

Recently, we have demonstrated that CD56+ IFN-DCs show an increased direct cytotoxic capability compared to CD56– IFN-DCs as well as an increased expression of IFNγ [12]. We have now investigated the phagocytotic capacity of both cell types using FITC-labeled albumin (◉ **Fig. 5**). After a 5 minutes incubation time, only a tiny fraction (between 2–3%) of IFN-DCs or monocytes, respectively, was stained positive (data not shown). Differences between both groups were not significant. Detailed analyses of CD56+ and CD56– IFN-DCs did neither reveal any significant differences. After 60 minutes, however, more than 80% of all IFN-DCs showed a positive signal, indicating a strong phagocytotic capacity of these cells. Detailed analyses of CD56+ and CD56– IFN-DCs revealed an even higher phagocytotic capacity of CD56+ cells (91.2±9%) compared to CD56- cells (82.1±6%). These differences, however, did not reach statistical significancy.

T cell stimulating capacity of IFN-DCs

To compare the antigen-specific stimulatory capacity of CD56+ IFN-DCs and CD56– IFN-DCs, T cell assays with naive CD3+ T cells were performed. Antigen-presenting CD14+ monocytes were used as additional controls. As formerly described [13], IFN-DCs were found to be more potent for stimulating T cells compared to immature monocytes at all stimulator/responder ratios ($p = 0.004$; ◉ **Fig. 6**). Detailed analyses of CD56+ and CD56– IFN-DC revealed that CD56+ IFN-DC showed a moderate stronger capacity in stimulating autologous T cells compared to CD56– IFN-DC at all DC/T cell ratios ($p = 0.01$).

Discussion

Recently, we have demonstrated that *in vitro* culturing of CD14+ monocytes with IFNα results in the generation of highly potent antigen-presenting dendritic cells (IFN-DCs) with direct cytotoxic activity towards tumor cells [12]. Tumoricidal activity of these cells is mainly mediated by a subtype of IFN-DCs expressing the natural killer (NK) cell marker CD56. This is important, as up to now CD56 expression was thought to be specific for NK cells and NKT cells, respectively. Lysis activity of CD56+ IFN-DCs was identified to be mediated by the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway which was shown to be strongly upregulated in these cells. These results were in line with previous data also showing TRAIL expression of these cells on a molecular [9, 10] as well as on protein level [9, 11]. The aim of the present study was to specify differences between CD56+ and CD56– IFN-DCs in more detail. Especially, we aimed to investigate a detailed CD56 expression profile at different stages of IFN-DC development, the phagocytosis capacity of these cells and the T cell stimulatory capability, respectively.

Based on our phenotypical analyses we were able to demonstrate that CD14+ monocytes steadily increase their CD56 expression profile from 25% on day 1 up to 68% on day 5 while culturing with IFNα and GM-CSF. Interestingly, CD56– IFN-DCs which have been cultured from monocytes over 3 days and which were then negatively selected and cultured for additional two days also express CD56 on a remarkable level (mean 42%). Because of that and because of the steady increase of CD56 expression from monocytes over 3 days, we might postulate that CD56+ and CD56– IFN-DCs do not represent two distinct cell populations, but rather one identical cell type with different maturation status. This concept is supported by the fact that phagocytosis and T cell stimulation capacity between both cell types only differs marginally. In addition, both cell types reveal a nearly identical size which has been determined by flow cytometry and by electron microscopy [12].

The *in vivo* relevance of monocyte differentiation into DCs mediated by IL-4 remains unclear since the exposure of monocytes to IL-4 can hardly mimic the cytokine milieu during infection [14]. In contrast, exposure of monocyte to IFNα represents a more physiological way since IFNα is secreted in large amounts by plasmocytoid DCs during viral or bacterial infection, respectively, and therefore reveal a critical role for linking innate and adaptive immunity [13]. These cells may, therefore, represent an ideal tool for inducing cytotoxic immunity in humans [15–17]. Banchereau and co-workers already reported on the *in vivo* use of CD34+ progenitors which were activated with type I interferon and used for the treatment of stage IV melanoma patients leading to the induction of tumor antigen-specific recall memory CD8+ T cells in the majority of patients [18]. In our own study [12] we demonstrated a stable disease in two out of five patients with medullary thyroid carcinoma. In both patients a large increase of calcitonin-specific IFNγ-secreting CD4+ T cells could be detected.

In summary, our present data clearly demonstrate that IFN-DCs express the surface marker CD56 and that CD56+ IFN-DCs have the ability of taking up antigens and to stimulate autologous T lymphocytes. These cells may also have the ability to directly lyse tumor cells. These cells may, therefore, represent a promising tool for immunization strategies against cancer including endocrine malignancies [15–17].

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