**Faculteit Farmaceutische, Biomedische
en Diergeneeskundige Wetenschappen**

Departement Farmaceutische Wetenschappen

Interleukin-15 dendritic cells induce potent activation of natural killer cells:

towards the design of an improved dendritic cell vaccine to treat acute myeloid leukemia

Laboratorium voor Experimentele Hematologie
Promotor : Dr. Eva Lion
Co-Promotor : Prof. dr. Ingrid De Meester
Begeleider : Dr. Sébastien Anguille

Eindverhandeling ingediend
tot het behalen van het diploma van
Master in de Geneesmiddelenontwikkeling
door VAN ACKER Heleen

Antwerpen 2013

DANKWOORD

Het schrijven van deze thesis vormt een perfect sluitstuk van vijf mooie en leerrijke jaren aan de universiteit van Antwerpen en het behalen van mijn diploma in de geneesmiddelenontwikkeling-apotheker. Bovendien hebben deze laatste maanden in het Laboratorium voor Experimentele Hematologie mijn fascinatie voor wetenschappelijk onderzoek alleen maar versterkt, alsook mijn belangstelling voor immunologie en oncologie. In deze context zou ik graag verschillende mensen bedanken.

In eerste plaats wens ik mijn promotor dr. Eva Lion te bedanken om mij de kans te geven onderzoek te verrichten binnen mijn interesseveld en een student te willen begeleiden met een beperkte voorkennis van het onderzoeksdomein en -methoden. Ik ben haar zeer erkentelijk voor het opvolgen van mijn werk, het stimuleren van mijn probleemoplossend vermogen, alsook voor haar kritische evaluatie en ondersteuning. Ook voor de tijd en moeite die zij gestoken heeft in het nalezen van mijn thesis en de hulp bij het tot stand brengen van het eigenlijke resultaat gaat mijn dankbaarheid uit.

Dr. Sébastien Anguille wil ik bedanken voor het delen van zijn kennis, zijn geduld en om mij te betrekken bij alle aspecten van wetenschappelijk onderzoek. Onder zijn leiding heb ik zeer veel bijgeleerd en mij een aantal vaardigheden eigen gemaakt, die een perfecte basis vormen voor mijn eigen doctoraat. Aan zijn gestructureerde en gedreven manier van werken kan ik alleen maar een voorbeeld nemen. Bedankt voor alle hulp wanneer ik deze nodig had en voor de assistentie bij het schrijven van mijn thesis.

Eva en Sébastien, ik had me geen twee betere begeleiders kunnen wensen.

Daarnaast ook een gemeend woord van dank aan mijn co-promotor Prof. dr. Ingrid De Meester voor haar interesse in mijn werk en om een link te voorzien met het departement Farmaceutische Wetenschappen. Prof. dr. Dirk Hendriks en Prof. dr. Luc Pieters, mijn medelezers, voor het vrijmaken van hun tijd om mijn masterproef te beoordelen.

Prof. dr. Viggo Van Tendeloo en Prof. dr. Zwi Berneman voor het ter beschikking stellen van hun laboratorium.

Verder wil ik ook nog Johan, Yannick, Evelien, Rachid, Nadia, Tine en alle anderen bedanken voor het creëren van een productieve en bovendien zeer aangename labosfeer.

Tenslotte wil ik een dankwoord uitbrengen aan mijn vrienden om er vijf fijne jaren van te maken en steeds in mij te geloven. Mijn ouders en zussen, Zoë en Sara, wil ik specifiek bedanken voor hun niet aflatende morele steun en Zoë voor haar hulp en aanmoediging.

TABEL OF CONTENTS

LIST OF USED ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
AML	acute myeloid leukemia
AF647	Alexa Fluor 647
APC	allophycocyanin
APC:Cy7	allophycocyanin:cyanine dye 7
CD	cluster of differentiation
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
CR	complete remission
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNAM-1	DNAX accessory molecule-1
EDTA	ethylenediaminetetra-acetic acid
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	human
HRP	horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
IFN	interferon
IMDM	Iscove's modified Dulbecco's medium
IL	interleukin
LPS	lipopolysaccharides
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIC	MHC class I polypeptide-related sequence
MIP1	macrophage inflammatory protein-1
MRD	minimal residual disease
mRNA	messenger RNA
NCR	natural cytotoxicity receptor
NK	natural killer
PE	phycoerythrin
PBL	peripheral blood lymphocytes

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PI	propidium iodide
RPMI	Roswell Park Memorial Institute medium
SD	standard deviation
SEM	standard error of the mean
SSC	side scatter
TCR	T-cell receptor
Th1	T helper 1
TNF	tumor necrosis factor
TLR	Toll-like receptor
WT1	Wilms' tumor protein 1

1. INTRODUCTION

1.1. ACUTE MYELOID LEUKEMIA

Data from the World Health Organization confirm that cancer is a leading cause of death, being responsible for 7.6 million deaths per year worldwide. This makes cancer one of the major public health issues of the 21st century (2). Although overall the leukemias are a relatively rare group of malignancies, they account for about 1.2% of all deaths worldwide, thereby being in the top 20 of leading causes of mortality. Acute myeloid leukemia (AML) is the most common type (25%) of leukemia among adults, characterized by the rapid malignant proliferation of myeloid white blood cell precursors in the bone marrow, compromising normal hematopoiesis and thereby causing anemia, infections and/or bleeding (3, 4). AML is a highly lethal disease; even with treatment, the 5-year overall survival rate is less than 25%. This figure is even worse in AML patients older than 65 years, who face a 5-year overall survival of 5.1%. The particularly unfavorable prognosis of older AML patients further strengthens the health impact of this disease. Especially in the context of an ever-aging population, this causes the expected incidence of AML to steadily increase in the near future (5-7).

1.2. STANDARD TREATMENTS OF AML

AML is generally treated with chemotherapy, which consists of two sequential phases: the induction phase (aimed at achieving a status of complete remission [CR], i.e. <5% leukemic cells in the bone marrow) and the consolidation phase (aimed at maintaining CR) (8). The gold standard chemotherapy regimen for AML consists of a combination of cytarabine and anthracyclin (or a related agent) (3, 7), which allows for achieving a CR in 60-80% of young adults and 40-60% of older patients (9, 10).

Despite this high CR rate, the majority of patients with AML will eventually relapse. Relapse is caused by the persistence of a small reservoir of chemotherapy-resistant AML cells, a condition known as “minimal residual disease” (MRD). Outgrowth of these persistent AML (stem) cells often leads to full clinical relapse (7, 11, 12). The prognosis of AML relapse is extremely poor and treatment options are unsatisfactory, explaining the overall grim survival picture (13). The only treatment with a demonstrable impact on MRD and relapse rate is allogeneic hematopoietic stem cell transplantation (HSCT). The rationale behind this therapy is the so-called “graft-versus-leukemia” effect, which is mediated by donor T-lymphocytes and natural killer (NK) cells that are capable of recognizing and eliminating residual AML cells (14-16). Allogeneic HSCT, however, causes significant

morbidity and mortality, mainly because of "graft-versus-host-disease" (a condition that is characterized by donor-mediated immune attack of normal cells/tissues). This makes older patients, which represent the majority of the AML patients, generally unsuited for allogeneic HSCT (7, 14). It also underscores the need for more effective and less aggressive treatment strategies, especially for older AML patients (17).

1.3. EMERGING TREATMENTS OF AML: IMMUNOTHERAPY AND DENDRITIC CELL-BASED VACCINATION

1.3.1 The field of immunotherapy

Recently, immunotherapy became a prominent research topic because of insights in the role of the immune system in suppressing and eliminating tumor cells, including leukemic cells (17). It has been proven that natural immune responses of both the innate and adaptive immune system can occur against AML cells (16, 18). On the other hand, AML cells can develop different mechanisms to escape immune surveillance. With immunotherapy, the aim is to enhance the strength of the anti-tumor immune response and to overcome these escape mechanisms (18, 19).

There are different approaches of immunotherapy, distinguishing between passive and active methodologies. Passive immunotherapy comprises the transfer of active humoral (antibodies) or cell-mediated (adoptive T cells) immunity. This approach does not rely on the patient's immune system and is therefore not capable of eliciting a memory immune response. For AML, passive immunotherapeutic approaches include monoclonal antibodies directed towards cluster of differentiation (CD)33 or CD52 expressed on the AML surface (20) and adoptive transfer of T cells and NK cells (4, 21). With active immunotherapy, the patient's own immune system is addressed aiming at the induction of adaptive immunity and long-term protection. Active vaccination strategies include cytokine, peptide and whole tumor cell administration (4) and dendritic cell (DC)-based vaccination. For AML, e.g. interleukin (IL)-2 (18), IL-15 (22) and type I interferons (IFN) (23) have been used. The most studied peptide for vaccination is Wilms' tumor protein 1 (WT1), wherein the efficacy already has been confirmed in a considerable number of patients (24). Moving to the forefront of immunity, however, is DC-based immunotherapy (25).

1.3.2. Immunologic principles of dendritic cell vaccination

Dentritic cells, whose primary function is to capture, process and present antigens to T cells, play a critical role in stimulating adaptive (antigen-specific) immunity. They are the only

antigen-presenting cells of the human immune system endowed with the potential to prime naïve T cells into antigen-specific CD8+ cytotoxic T lymphocytes (CTL) (26). In the context of tumor immunity, such CTL are capable of recognizing and mediating lysis of tumor cells that express tumor-derived antigen(s) to which these CTL are directed. This recognition occurs through the interaction of the T-cell receptor (TCR) of the CTL with antigenic peptides displayed on the tumor cell surface via major histocompatibility complex (MHC) class I molecules. In addition to their potent capacity to stimulate adaptive immunity, DC are also central to controlling the innate (non-antigen-specific) arm of the immune system (25, 27), of which NK cells are important constituents (*vide infra*). NK cells can mediate tumor cell lysis in a non-MHC-restricted fashion and thus also play an important role in tumor immunosurveillance. The ability to stimulate both adaptive and innate anti-tumor immunity makes DC attractive tools for cancer immunotherapy, including AML which is known to be susceptible to CTL- as well as NK cell-mediated killing (7).

Although the nomenclature of the human DC system is complex, DC are generally subdivided into plasmacytoid DC, which produce type 1 IFN (e.g. IFN-α) in reaction to viral antigens, and myeloid DC (28-30). The latter can produce large quantities of IL-12, underlying their capacity to induce a $CD4^+$ T-helper 1 (Th1) immune response resulting in the generation of antigen-specific $CD8^+$ CTL responses (30, 31).

Under basal circumstances, DC occur in an immature state and patrol continuously all over the body to sense danger (e.g. invading pathogens, malignant cells) (32, 33). As professional antigen-presenting cells, DC are equipped with a sophisticated endocytic system that allows them to effectively take up antigenic material and to process it for presentation to T cells (33). Upon antigen encounter, DC mature and start up-regulating MHC class I and class II molecules, T-cell co-stimulatory molecules such as CD80 and CD86 and the chemokine receptor CCR7, which directs their migration towards the T cell-rich areas of the lymph nodes (26, 34). Once arrived in the lymph node, an immunological synapse will be established between the DC and T-lymphocytes.

Three signals are required for effective T cell activation. The first signal is provided when the TCR recognizes a MHC-bound antigen on the cell surface of the DC (Fig 1.1, ❶) (17, 27, 35). Endogenous antigens (i.e. antigens that are generated inside the DC) are generally processed through the MHC class I pathway to activate $CD8^+$ T cells. Exogenous antigens, on the other hand, will be presented via MHC class II molecules to $CD4^+$ T cells (26, 36).

The second signal is provided by the interaction of CD28 on the T cells with CD80 or CD86 on the DC surface (Fig 1.1, ❷). Other important co-stimulatory molecules expressed on the DC surface are: CD70, which binds to CD27, ICOSL, interconnecting with ICOS, TL1A, OX40L and 4-1BBL which bind respectively DR3, OX40 and 4-1BB. Co-inhibition is effected by PDL-1 and PDL-2, who bind their receptor PD-1 on the T-cell surface and CD80 or CD86 binding CTLA-4 (17, 27, 36-39). The third signal involves the secretion of IL-12p70 by the DC as a result of the interaction between CD40 (expressed on the DC) and CD40 ligand (expressed on the T cell; Fig 1.1, ❸) (17, 27, 36).

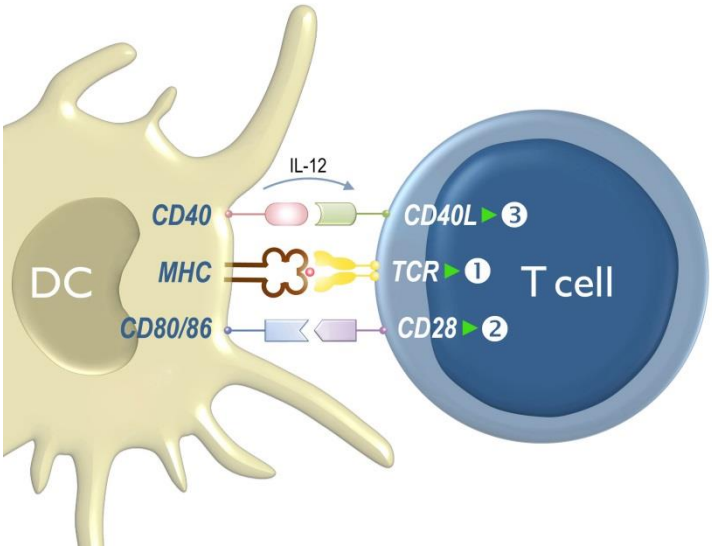

Figure 1.1. Three-signal requisite for T-cell activation (from Anguille *et al*, Cytotherapy 2012 (17)).

1.3.3. Generation of dendritic cells for clinical immunotherapy in AML

Since DC are a rare population of blood cells (< 1% of the peripheral blood mononuclear cell [PBMC] fraction), most of the DC preparations used for clinical vaccination purposes are generated *ex vivo* from precursor cells. Among the most popular precursor cells used for DC vaccine manufacturing are $CD14^+$ peripheral blood monocytes (32, 40). Monocytes represent around 10-20% of the PBMC fraction and can easily be obtained in sufficient numbers by means of a leukapheresis (i.e. procedure by which white blood cells are collected from the patient; Fig 1.2, ❶) (7). After leukapheresis, monocytes are differentiated into immature DC by culturing them *in vitro* in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (Fig 1.2, ❷) (17, 41). The resultant DC are commonly called "IL-4 DC". The differentiation from monocytes to immature IL-4 DC lasts about 5-6 days, after which the DC are matured/activated. This is usually achieved by adding a mixture of pro-inflammatory cytokines, commonly referred to as the Jonuleit

maturation cocktail, which is composed of tumor necrosis factor (TNF)-α, IL-1β, IL-6, and prostaglandin E2 (PGE_2; Fig 1.2, ❸) (42, 43).

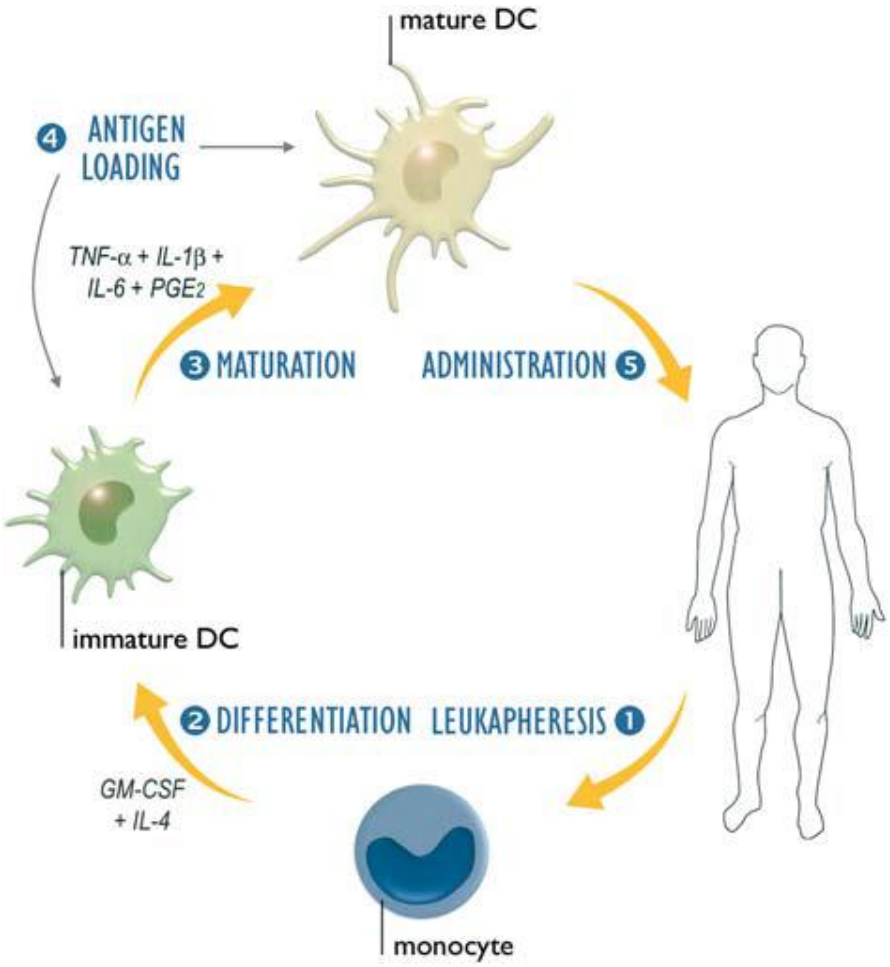

Figure 1.2. Schematic overview of monocyte-derived DC-based vaccination (from Anguille *et al*, Cytotherapy 2012 (17))**.** Autologous peripheral blood monocytes are obtained after leukapheresis ❶ and differentiated *in vitro* into immature DC ❷. This is followed by a maturation step ❸, after which the DC are loaded with a tumor antigen ❹ and subsequently re-administered to the patient ❺.

As the primary aim of DC-based immunotherapy is to exploit the unique antigen-presenting capacities of DC to induce protective tumor antigen-specific CTL immunity, DC should undergo an antigen-loading step prior to administration to the patient (Fig 1.2, ❹) (17). A large number of tumor antigens have been identified in AML (24). One example is WT1, which has been proposed as a highly suitable candidate target antigen for inclusion in DC vaccine formulations because of the overexpression in the majority of AML patients, and perhaps more important on their hematopoietic stem cells. In addition, WT1 interferes with normal differentiation of myeloid cells and prevents indirectly the apoptosis of AML cells. In the context of immunogenicity, WT1 is a convenient antigen because of its length and the number of different epitopes (17, 24, 44).

DC can be loaded with antigen using a variety of techniques, with exogenous pulsing of tumor antigen-derived peptides being the most commonly used one. Electroporation of antigen-encoding messenger RNA (mRNA) is a relatively new antigen-loading strategy that is gaining increasing popularity (45). It involves a non-viral gene transfer method based on a transitory electric pulse creating short-term pores in the cell membranes allowing molecules such as mRNA to enter the cell. Resealing of the cell membrane is ensured by transferring the transfected cells in serum-supplied medium (7, 24, 45). DC process the mRNA after which they are able to present multiple epitopes to other cells of the immune system (15). After antigen loading, the *ex vivo* generated DC are re-administered to the patient (Fig 1.2, ❺), preferentially via intradermal injection as this route of administration provides the best chance of an effective migration of the DC to the lymph nodes where they will encounter T cells and, hopefully, induce antigen-specific CTL (36, 40).

1.3.4. Clinical studies of DC vaccination in AML patients

DC vaccine strategies have been tested in patients with AML in the framework of phase I and II clinical trials. An overview of these clinical DC vaccine trials in AML is given in Anguille *et al.* Cytotherapy 2012 (17). These studies were set up in order to determine the safety, toxicity and feasibility of this new treatment approach (17). The first randomized, multi-center phase II clinical study of DC vaccination in AML patients will soon be initiated and coordinated by the Center for Cell Therapy and Regenerative Medicine of the Antwerp University Hospital and the Laboratory of Experimental Hematology of the University of Antwerp (46).

Previous trials have been performed in three clinical situations (17): to treat post-transplant relapse of AML (47), in AML patients with advanced disease (48) and in the post-remission setting as a less toxic alternative to allogeneic HSCT to eradicate MRD and prevent AML relapse (49, 50). From these trials, it can be concluded that DC vaccination in AML is feasible and safe (51). DC vaccination is well-tolerated by patients, with local side effects at the injections sites being common and systemic side effects (e.g. thrombocytopenia, auto-immunity) being extremely rare. Importantly, it was demonstrated that DC vaccination is capable of generating a leukemia antigen-specific T cell response. The immunogenicity is generally demonstrated through delayed-type hypersensitivity skin testing or by immune effector cell detection assays. Altogether it can be concluded that DC vaccination in AML patients holds clinical potential (17).

Clinical responses have, however, been rather heterogeneous. Best results have been obtained in the post-remission setting, in which the capacity of DC vaccination to control MRD and prevent relapse has been clearly established (17). In a considerable number of patients DC vaccination does not appear to result in overt clinical benefit, underscoring the need to optimize the current DC-based vaccine strategies (15, 47, 49). It is within this context that much current research effort is being devoted to redesign the protocol for *ex vivo* DC vaccine manufacturing in order to allow for the generation of DC with "optimal" immunostimulatory characteristics.

1.4. OPTIMIZATION OF DC VACCINE STRATEGIES FOR AML

1.4.1 Generation of monocyte-derived DC using IL-15 instead of IL-4

In an attempt to increase the immunostimulatory and anti-leukemic capacity of DC vaccination, the Laboratory of Experimental Hematology recently designed a novel protocol for the *ex vivo* generation of monocyte-derived DC. Three major changes were made to the currently used IL-4 DC protocol (40):

(1) the length of the differentiation culture was reduced to 24-48 hours

(2) IL-4 was replaced by IL-15 during monocyte-derived DC differentiation

(3) the gold standard Jonuleit maturation cocktail (43) was replaced by a maturation mixture that triggers DC activation/maturation through stimulation of a Toll-like receptor (TLR). Toll-like receptors are a family of cell surface and intracellular receptors that sense "danger" signals, as will be discussed in further detail below.

DC generated according to this alternative culture protocol are hereafter called "IL-15 DC" because of the use of IL-15 instead of IL-4 during monocyte-derived DC differentiation.

The pleiotropic cytokine IL-15 was chosen because of its distinctive immunostimulatory properties and its critical role in homeostasis and activation of the innate and adaptive immune system (22, 40, 52). Concerning the adaptive immune system, IL-15 is indeed important for the generation of antigen-specific CTL (33), CTL memory homeostasis and memory T-cell expansion (22) and it renders Th1 cells and CTL resistant to the suppressive functions of regulatory T cells (53). Concerning innate immunity, IL-15 is a known promotor of NK cell proliferation and activation and is critically involved (via transpresentation) in NK-DC crosstalk (54, 55). With this knowledge, we anticipate significant IL-15 DC-mediated NK cell activation and induction of anti-leukemic T cell responses (54).

As concerns the maturation procedure, we observed that IL-15 DC are rather refractory to the maturation-inducing effects of the classic Jonuleit maturation cocktail, but that a powerful activation/maturation of IL-15 DC could be achieved by using resiquimod, (i.e. R848) an agonist for TLR7 and TLR8 (40). Both TLR are intracellulary located and at least TLR8 is known to be expressed by IL-15 DC (56). TLR7 and TLR8 are involved in the recognition of pathogen-associated molecular patterns, more specifically single-stranded RNA sequences derived from viruses such as influenza virus or human immunodeficiency virus (57). It is, therefore, not surprising that TLR7/8 signaling provides a potent maturation stimulus to IL-15 DC. In addition to this, a main advantage of triggering DC maturation through the TLR pathways is that it facilitates the capacity of the DC to produce IL-12p70, which, as described above, is critical for the induction of Th1 cellular immune response (30, 40, 42, 58-60).

1.4.2 Effect of IL-15 DC on the adaptive immune system

To be considered as a real alternative for IL-4 DC, IL-15 DC must prove that they possess an enhanced ability to trigger the adaptive immune system, more specific to have the capacity to present AML-associated tumor antigens and to induce an AML-antigen-specific immune response. In our research group, electroporation of IL-15 DC with WT1 mRNA is the preferred transfection method. However, the application of a more intense electrical field is required for the electroporation of the IL-15 DC in comparison with the IL-4 DC. This is a consequence of their smaller size, due to the short-term culture (15, 61). Taking this into account, IL-15 DC can be successfully transfected. The protein expression of WT1 can be demonstrated 24 hours post-electroporation based on immunochemistry. In addition, it was confirmed that this expression leads to the stimulation of $CD8^+$ T cells in a WT1-specific fashion (62). Experiments are ongoing to examine if IL-15 DC are correspondingly able to induce a WT1-specific Th1 immune response as well, important in the context of long-lived tumor immunity.

1.4.3 Effect of IL-15 DC on the innate immune system

1.4.3.1 NK cells

The aim of this thesis is to determine whether IL-15 DC, in addition to their potent capacity to stimulate adaptive immunity, are also able to activate the innate immune system. As mentioned above, NK cells are the primary effector cells of the innate immune system. Human NK cells are characterized by a $CD56^+$ $CD3^-$ $NKp46^+$ phenotype and can be further subdivided into two subpopulations, namely the predominantly cytotoxic $CD56^{dim}$

$CD16^{bright}$ subset (~90% of circulating peripheral blood lymphocytes) and the $CD56^{bright}$ $CD16^{dim}$ NK cells, whose primary function is to produce immunoregulatory cytokines (63-65). Both subsets also express different chemokine receptors and adhesion molecules, which explains that $CD56^{bright}$ NK cells preferentially migrate to the secondary lymphoid organs, while $CD56^{dim}$ NK cells traffic to acute inflammatory sites (66-68). Importantly, depending on the stimuli they receive, both NK cell subtypes can exert a killer activity or secrete cytokines and change their migratory profile (63, 64, 69).

A variety of cytotoxic effector molecules, including TNF-α, TNF-α-related apoptosis-inducing ligand, FAS ligand and the perforin/granzyme cell death pathway, are involved in the cytotoxic function of NK cells (70-72), which allow NK cells to kill "foreign" cells such as virus-infected cells or tumor cells. In contrast to CTL, NK cells recognize and kill their targets in a non-MHC-dependent fashion. In addition to cell-mediated cytotoxicity, NK cells also exert an immunoregulatory function, influencing other cells of the immune system. In this way, NK cells are capable to mature and activate DC and help T cells by activating or suppressing them. The immunoregulatory function of NK cells is characterized by the secretion of high levels of IFN-γ and TNF-α and includes a range of other cytokines and chemokines such as IL-5, IL-10, IL-13, GM-CSF, CCL2 (monocyte chemotactic protein-1), CCL3 [Macrophage inflammatory protein-1 (MIP1)-α)] and CCL4 [MIP1-β] (67, 71, 73).

Activation of NK cells is controlled by an interplay between activating and inhibitory receptors (73). This must ensure that NK cells only kill "foreign" cells and maintain tolerance towards healthy self-cells. The presence of MHC class I on the cell surface of a target cell results in the principal inhibitory signal, due to the expression of MHC class I-specific inhibitory receptors on NK cells (i.e. the killer immunoglobulin-like receptors, the leukocyte immunoglobulin-like receptors and the CD94-NKG2 heterodimers) (74, 75). The main activating NK cell receptors are the natural cytotoxicity receptors (NCR) NKp30, NKp44, NKp46 and NKp80, the Fcγ receptor III (CD16), DNAX accessory molecule-1 (DNAM-1) and NKG2D (34, 64, 76-79). NK cell activation can also be induced by cytokines, including IL-2, IL-12, IL-15, IL-18, IL-21, type I IFN and by TLR ligands (34, 64, 73, 76).

1.4.3.2. NK-DC crosstalk

In addition to T cell stimulation, DC can also mediate NK cell activation, promoting both cytotoxic and immunoregulatory NK cell functions (65, 80). Interaction between DC and NK cells is not unidirectional, as NK cells have been shown to promote DC maturation and improve their capacity to stimulate T-cell responses (63, 65).

Both cell-to-cell contact mechanisms and soluble factors have been implied in NK-DC crosstalk. For example, like T cells, NK cells can express costimulatory ligands such as CD28 and CD40L, both of which have been shown to play an important role in establishing NK-DC contact (65). Contact between DC and NK cells has also been described to be mediated through the interaction of MHC class I polypeptide-related sequence (MIC)A and MICB on the DC surface with the NKG2D receptor on NK cells (81, 82), through the NCR NKp30 and NKp46 on NK cells and their ligands expressed by DC (82) or through CX3CR1-CX3CL1 NK-DC interaction leading to the polarized secretion of preassembled stores of IL-12 by DC towards NK cells (65). Apart from these cell-to-cell contact mechanisms, DC also exert their activating effects on NK cells through secretion of a range of cytokines. Among the most important NK cell-stimulatory cytokines released by DC are IL-2, IL-12, IL-15, IL-18 and type I IFN (34, 73). IL-15 is usually not secreted by DC, but transpresented by IL-15Rα on the DC to NK cells. IL-15 transpresentation is confirmed to be a primary mechanism of DC-mediated NK cell activation (54, 55).

NK-DC interaction can take place in the T cell areas of lymph nodes, especially with the $CD56^{bright}$ $CD16^{-}$ NK cell subset (83) and in inflamed and malignant tissue (84). In order that cell-to-cell contact can take place, it is convenient that NK cells and DC traffic towards each other. Migration of NK cells can be regulated by DC through the secretion of chemokines, resulting in the recruitment of NK cells in the lymph nodes as well as at the tumor or inflammation site (63, 68, 84).

1.4.3.3 The role of NK cells in anti-leukemia immunity

In AML patients, significantly impaired NK cell functions, including NK cell defects and AML evasion mechanisms, can facilitate escape from immune surveillance and affect patient outcome (19). Importantly, NK cells have the ability to eliminate autologous and allogeneic leukemic cells through their cytotoxic and immunoregulatory capacities (19, 85). *In vitro*, their cytotoxic potential is reflected by the killing of several hematological cell lines, including the NK-sensitive leukemic cell line K562 (standard assay) (63). *In vivo*, this

is evidenced by *(1)* the positive impact on the prognosis of cancer patients with high NK cell infiltration at the tumor site, a large amount of circulating NK cells and high cytotoxic and IFN-γ-secreting capacity, and *(2)* the effect of donor-versus-recipient NK cell alloreactivity (85-88). Regaining full innate immune cell activity in AML with (immuno)therapy is therefore an attractive approach.

1.4.3.4. DC vaccination and NK cells

Since DC are known to be capable of inducing NK cell activation, DC vaccine strategies offer a unique opportunity to simultaneously stimulate both the adaptive and innate arms of the anti-tumor immune response. Given the prime role of NK cells in anti-leukemia immunity and the demonstration of a positive correlation between NK cell immune responses (19) and clinical outcome in DC vaccine trials (63), one of the keys to successful DC vaccination in AML could be to generate a DC vaccine that, in addition to its capacity to induce leukemia antigen-specific CTL, is equipped with a capacity to harness the NK cell cytotoxic and immunoregulatory functions against AML cells (Fig 1.3.). In this thesis, therefore, we aim to investigate whether our novel IL-15 DC vaccine system is endowed with a capacity to induce innate anti-leukemia immunity.

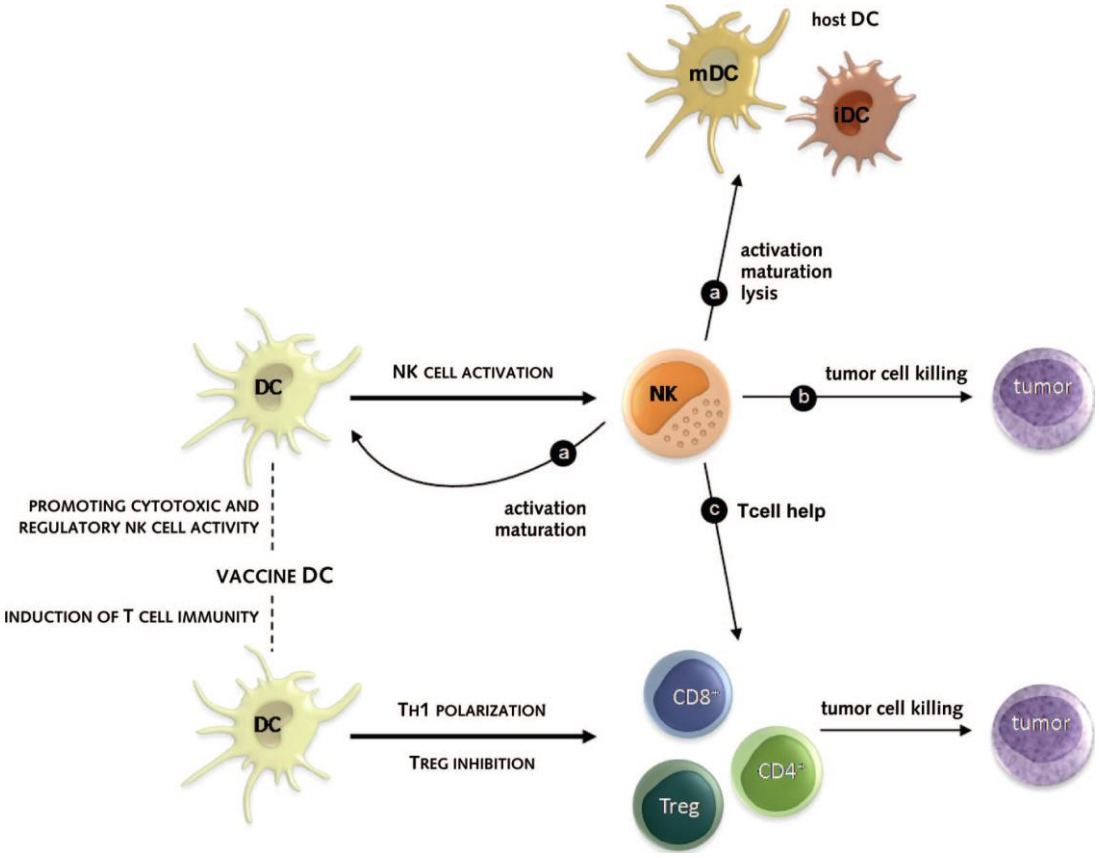

Figure 1.3. How NK cells can contribute to the antitumor efficacy of DC-based vaccination (from Lion *et al*, The Oncologist 2012 (63)).

2. OBJECTIVES

The overall objective of this research is to elucidate whether and how IL-15 DC can activate NK cell functions in a human in vitro model of AML.

With the challenge to improve the clinical efficacy of DC vaccine protocols, we believe that IL-15 and TLR agonists make attractive candidate molecules to harness DC for NK cell activation and to overcome tumor escape mechanisms. Both IL-15 and TLR agonists have been described to directly and indirectly activate NK cells and DC. It has been demonstrated that DC generation with the inclusion of IL-15 in differentiation cocktails (40, 89, 90) and/or the use of TLR agonists in maturation cocktails (40, 60, 91, 92), enhanced the NK cell-activating capacity of the DC and their reciprocal crosstalk with NK cells.

It has been demonstrated that IL-15 DC have superior immune-activating properties as compared to conventional IL-4 DC, evidenced by a *(1)* higher migratory potential, *(2)* advantageous cytokine secretion profile (IFN-γ, IL-12p70), *(3)* superior capacity to stimulate antigen-specific T cell responses (40) and *(4)* unique NK-like killing properties (62). Here, we compared the NK cell-activating capacity of conventional IL-4 DC with our novel IL-15 DC and aimed to uncover their mechanisms of action. Four objectives were premised:

(1) Determine whether IL-15 DC are able to attract NK cells
(2) Evaluate phenotypic activation of NK cells after contact with IL-15 DC
(3) Examine if the two main functions of NK cells, i.e. IFN-γ secretion and cytotoxicity, are activated after IL-15 DC stimulation
(4) Search for underlying mechanisms of IL-15 DC-mediated NK cell activation

3. MATERIALS AND METHODS

3.1. PURIFICATION OF HUMAN PBMC: FICOLL DENSITY GRADIENT CENTRIFUGATION

PBMC were isolated from healthy donor buffy coat preparations (provided by the Antwerp Blood Transfusion Centre) or fresh heparinised (BD Biosciences, Erembodegem, Belgium) whole blood from lab donors. To isolate PBMC, fresh heparinised blood or buffy coats are diluted up to 1/3 with PBS/EDTA consisting of phosphate buffered saline (PBS; Gibco, Paisley, UK) supplemented with 1% ethylenediaminetetra-acetic acid (EDTA; Merck, Darmstadt, Germany) and 200 µL/L gentamycin (10 mg/mL; Gibco). Next, the blood is brought on top of ficoll (Ficoll-Paque TM PLUS; GE Healthcare, Diegem, Belgium) and carefully centrifuged (740 g, 30 min, without brakes) leading to segregation of different layers i.e. plasma, PBMC, ficoll and red blood cells. The PBMC fraction is collected and washed three times with PBS/EDTA (310 g, 10 min). The obtained cell pellet is resuspended in appropriate medium for further use.

3.2. MAGNETIC-ACTIVATED CELL SORTING (MACS)

Magnetic-activated cell sorting is applied in order to separate cells based on the expression of (several) surface antigens. First, specific antibodies, bound to magnetic beads, react with their corresponding cell surface marker, capturing the cells. Next, the cell suspension is transferred on a magnetic column which is placed in a magnetic field (MACS separator; Miltenyi Biotec, Bergisch Gladbach, Germany). Two types of sorting are distinguished i.e. negative cell selection and positive cell selection. With positive selection, the cells of interest are labeled and thus retained on the magnetic column, while with negative selection the cells that need to be depleted are labeled and the cells of interest pass through the magnetic column.

3.2.1. Positive cell selection: CD14 and CD56 purification

Positive magnetic cell selection was used for purification of $CD14^+$ monocytes from PBMC and of $CD56^+$ cells from DC cultures, according to the manufacturer's instructions (Miltenyi Biotec). For both selection protocols, cells were washed and resuspended in 80 µL MACS buffer (i.e. PBS/EDTA supplemented with 0.5% bovine serum albumin [Sigma-Aldrich, Bornem, Belgium]) per 10^7 cells. Per 10^7 cells, 20 µL of MACS MicroBeads (CD14 or CD56) were added and incubated for 15 minutes at 4 °C. After washing (453 g, 5 min) magnetic bead-labeled cells were applied onto a pre-rinsed column of the type LS+ (for up to 10^8 positive cells) placed in the magnetic field of a MACS separator. The column was

washed 3 times with MACS buffer. Magnetic bead-labeled ($CD14^+$ or $CD56^+$) cells are retained in the magnetic field of the column, whereas unlabeled cells pass through. Finally, the column is removed from the separator and $CD14^+$ or $CD56^+$ cells are flushed out using a plunger.

3.2.2. Negative cell selection: NK cell isolation

Untouched NK cells were isolated from monocyte-depleted peripheral blood lymphocytes (PBL) or PBMC using a human NK cell isolation kit (Miltenyi Biotec). In brief, cells were resuspended in 40 µL of cold MACS buffer per 10^7 cells. Non-NK cells were indirectly magnetically labeled with 10 µL NK cell biotin-antibody cocktail per 10^7 cells for 20 minutes at 4 °C. Next, 20 µL of microbead cocktail per 10^7 cells was added for 30 minutes at 4 °C. Magnetic bead-labeled cells were washed (300 g, 10 min), resuspended in 500 µL of MACS buffer and brought onto a pre-rinsed LS+ column. Untouched NK cells pass through the column and are collected, while the magnetically labeled non-NK cells are retained in the column.

3.3.GENERATION OF MONOCYTE-DERIVED DC

Differentiation - Monocytes were resuspended into Roswell Park Memorial Institute medium (RPMI; BioWhittaker; Verviers, Belgium) with 2.5% heat-inactivated human (h)AB serum (Sigma-Aldrich, Bornem, Belgium) and seeded in a 6-well culture plate (Corning Life Sciences; Schiphol-Rijk, The Netherlands) at a final concentration of 1,2 x 10^6 cells/mL. 800 IU/mL GM-CSF (Gentaur; Brussels, Belgium) and 200 ng/mL IL-15 (Immunotools; Friesoythe, Germany) or 20 ng/mL IL-4 (Invitrogen, Camarillo, CA, USA) were added to obtain respectively IL-15 DC after 24 hours and IL-4 DC after 5 days incubation in a 37 °C / 5% CO_2 incubator (Table 3.1).

Maturation - The desired maturation cocktail (Table 3.1) was added to the DC cultures; a mixture of proinflammatory cytokines and PGE_2 (a.k.a. Jonuleit cocktail) for the IL-4 DC and a TLR7/8 agonist-based maturation cocktail for the IL-15 DC. The resultant mature DC were harvested 24-48 hours after addition of the maturation agents. All components of the maturation cocktails are purchased from Invitrogen, with the exception of PGE_2 (Pfizer, Puurs, Belgium), IFN-γ (Immunotools) and R848 (Resiquimod; Alexis Biochemicals, San Diego, USA).

Table 3.1. Differentiation and maturation procedures used in the present study

	IL-4 DC			IL-15 DC		
differentiation		concentration	time		concentration	time
	GM-CSF	800 IU/mL	5 days	GM-CSF	800 IU/mL	24 h
	IL-4	20 ng/mL	5 days	IL-15	200 ng/mL	24 h
maturation		concentration	time		concentration	time
	TNF-α	10 ng/mL	48 h	R848	3 μg/mL	24 h
	IL-1β	10 ng/mL	48 h	TNF-α	2.5 ng/mL	24 h
	IL-6	1000 IU/mL	48 h	IFN-γ	5000 IU/mL	24 h
	PGE_2	1 μg/mL	48 h	PGE_2	1 μg/mL	24 h

DC harvest - IL-15 DC and IL-4 DC are harvested after 24-48 hours of maturation using a Pasteur pipette. The wells are rinsed with PBS/EDTA and placed for 10 minutes at 4 °C. This ensures that adherent cells detach and all cells can be collected. The total cell suspension is washed (453 g, 5 min), after which the cell pellet can be resuspended in the preferred medium at the desired concentration.

3.4. CELL FREEZING PROTOCOL

Cells are cryopreserved in freezing solution consisting of 90% fetal bovine serum (FBS; Perbio Science; Erembodegem, Belgium) and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich; Bornem, Belgium). The cell pellet is gently resuspended in half of the total volume of cold 100% FBS. Next, the cell suspension is diluted with the double volume of cold 80% FBS / 20% DMSO. The vials are placed into a cryocontainer to obtain controlled freezing (decrease in temperature of 1 °C/min) and stored at -80 °C. For long-term freezing, cells are transferred to liquid nitrogen (-196 °C).

3.5. CELL THAWING PROTOCOL

Frozen cells are quickly thawed in a warm water bath (37 °C) and subsequently transferred to pre-heated (37 °C) Iscove's modified Dulbecco's medium (IMDM; Invitrogen) with 10% FBS and 100 μL DNase (Roche; Vilvoorde, Belgium). After 15 minutes incubation on rollers, cells are washed (300 g, 10 min) and resuspended in pre-warmed medium (IMDM / 10% FBS) at a density of maximum 1.10^7 cells/mL to allow thawed cells to rest at 37 °C in a 5% CO_2 incubator for 1-3 hours or overnight.

After the resting phase, viability of the cells is verified flow cytometrically by adding propidium iodide (PI; Sigma-Aldrich) to the samples. Data acquisition is performed on a FACScan™ multiparametric flow cytometer (BD Biosciences).

3.6. NK CELL MIGRATION

To investigate the NK cell-attracting capacity of IL-15 DC, a transwell chemotaxis assay was performed using 24-well transwells with 5 μm pore size polycarbonate membrane inserts (Corning). The lower wells were filled with 600 μL of 24 hours wash-out supernatant of matured IL-15 DC or IL-4 DC. The wash-out supernatant was obtained by washing mature DC thoroughly and culturing them at 1 x 10^6 cells/mL in RPMI / 2,5% hAB medium without differentiation or maturation factors. After 24 hours, cell-free supernatant was collected and frozen at -20 °C. In the upper wells, 1 x 10^5 NK cells in 100 μL culture medium (RPMI / 2,5% hAB) were inserted. 600 μL of RPMI / 2.5% hAB serum in the lower compartment served as a negative control representing the random migration of NK cells. As a positive control, 1 x 10^5 NK cells were seeded in the lower compartment in 700 μL RPMI / 2.5% hAB. NK cell migration was allowed for 3 hours at 37 °C / 5% CO_2. Inserts were removed carefully and migrated NK cells were collected from the lower compartment. After washing, cells were resuspended in a fixed volume (200 μL) and counted flow cytometrically (60 seconds acquisition at a continuous flow rate of 10 events/sec) on a FacsAria II (BD Biosciences). Migration was calculated as % migrated NK cells using the following formula: (number of migrated NK cells in the condition - number of migrated NK cells in the negative control) / number of NK cells in the positive control.

3.7. FLOW CYTOMETRY

Flow cytometry is a technique which permits phenotyping at a cellular level and sorting of individual particles based on their measured properties. For this purpose, a single cell suspension is brought in a fluid stream by means of hydrodynamic focusing with sheath fluid. The cells are irradiated with a laser, resulting in scattering of light. The forward scatter (FSC) correlates with the size and the side scatter (SSC) correlates with the complexity of the cell. Labeling of cells with fluorochrome-labeled monoclonal antibodies offers the possibility to detect fluorescence per cell. After excitation by the lasers, each fluorochrome gives light emission per cell at a particular wavelength. The difference in wavelength between excitation and emission is called the Stokes shift. To overcome spectral overlap, fluorescence compensation must be carried out (93, 94).

3.7.1. Membrane phenotyping

Each cell expresses a variety of surface markers (e.g. CD antigens) of which the combination depends on the cell-type and the degree of cell maturation. These molecules can be targeted by fluorochrome-labeled antibodies. In general, 1 x 10^6 cells are stained with 10 µL of fluorochrome-labeled antibody, followed by an incubation step of 15 minutes at room temperature. The excess of monoclonal antibodies is washed away (453 g, 5 min) after which cells are ready for flow cytometric acquisition.

3.7.1.1. DC-mediated phenotypic NK cell activation

To see whether IL-15 DC-stimulated NK cells have an activated phenotype as compared to IL-4 DC-stimulated NK cells, membrane staining was performed on co-cultures of NK cells with IL-15 DC or with IL-4 DC. NK cells and DC were cultured at a 1:1 ratio (0.1 x 10^6 cells / cell type) for 24-48 hours in 300 µL IMDM / 10% FBS in a round bottom 96-well plate (Corning).

A panel of fluorochrome-conjugated antibodies was used to determine NK cell surface expression after 24 hours and 48 hours of co-culture: CD56-fluorescein isothiocyanate (FITC), NKG2D-phycoerythrin (PE), CD16-V450, NKp30-Alexa Fluor 647 (AF647), CD69-allophycocyanin:cyanine dye 7 (APC:Cy7), CD25-PE, CCR7-V450, NKp46-APC and HLA-DR-APC:H7. Corresponding isotype controls, originating from the same species and isotype as the antigen-specific antibodies, were performed to rule out non-specific antibody binding to membrane-bound Fc receptors. All monoclonal antibodies were purchased from BD Biosciences. Ten minutes prior to acquisition, the viability marker 7-aminoactinomycin D (7-AAD) was added to the samples. Data were collected on FACSAria II flow cytometer.

3.7.1.2. IL-15/IL-15Rα detection on IL-15 DC and IL-4 DC

Prior to surface staining of CD56-FITC and IL-15-PE or IL-15Rα-PE (R&D systems; Abingdon, United Kingdom) or corresponding isotype controls (IgG1-PE and IgG2b-PE (BD biosciences), respectively), cell non-specific binding sites were saturated with mouse gamma globulines (11.1 mg/mL; Jackson ImmunoResearch; West Grove, USA) for 10 minutes. All samples were acquired on a FACScan™ flow cytometer.

3.7.2. Cytotoxicity assay

A flow cytometry-based lysis assay was performed in order to determine the killing activity of NK cells stimulated with IL-15 DC or with IL-4 DC. To this end, NK cells and autologous IL-15 DC or IL-4 DC were co-cultured in triplicate in flat bottom 96-well plates (Corning) for 48 hours at a 1:1 ratio (0.1 x 10^6 cells/cell type) in 300 µL IMDM / 10% FBS. Three different target cells were used, i.e. K562 (NK cell-sensitive), Daudi (NK cell-resistant) and autologous CD56-depleted PBL.

PKH67 labeling - Prior to co-culture, target cells were labeled with the PKH67 Fluorescent Cell Linker according the protocol provided by the manufacturer (Sigma-Aldrich). In brief, 10 x 10^6 target cells are washed three times (400 g, 5 min) in serum-free medium and resuspended in 0.5 mL diluent C (Catalog Number G8278). Next, 0.5 mL PKH67 dye solution is added in a final concentration of 2 x 10^{-6} M. The staining is stopped by adding an equal volume of 100% FBS after 4 minutes. The PKH67-labeled cells are washed (400 g, 5 min) three times with IMDM / 10% FBS to remove excess dye and then ready for further use or kept overnight at 0.5-1 x 10^6 cells/mL IMDM / 10% FBS at 37 °C / 5% CO_2. PKH67 dye intensity stays high and homogeneous up to 3 days following staining.

4h cytotoxicity assay - $PKH67^+$ target cells (K562, Daudi or PBL) were added to 48 hours co-cultures of NK cells and IL-15 DC or NK cells and IL-4 DC at a 5:5:1 ratio (0.1:0.1:0.02 x 10^6 cells/well) for 4 hours. Target cells alone were used as negative controls and conditions with the effector NK cell line NK92 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were used as positive controls. After 4 hours incubation, triplicates of each condition were pooled and stained with Annexin-V-APC (BD Biosciences) and PI. Annexin-V binds to phosphatidylserin, which is exposed during apoptosis on the surface of the cell in a calcium-dependent manner. In combination with PI, distinction can be made between viable (Annexin-V^-PI^-), early apoptotic (Annexin-V^+PI^-), late apoptotic (Annexin-V^+PI^+) and necrotic cells (Annexin-V^-PI^+) (95). Percentage killing was calculated using the following equasion: 100 – [(% annexin V^-PI^- target cells in the presence of NK cells / % annexin V^-PI^- target cells without NK cells) x 100]. Where indicated, the effect of cell-to-cell contact in NK-DC co-cultures was evaluated using 96-well Transwell 0.4 µm pore size polycarbonate membranes (Corning), with 0.1x10^6 NK cells cultured in the lower wells and 0.1x10^6 DC seeded on the transwell insert. All data were collected on a FACSAria II flow cytometer.

3.8. CYTOKINE PRODUCTION

IFN-γ and IFN-α secretion was determined by means of enzyme-linked immunosorbent assays (ELISA). In general, 96-well plates are coated with capture antibody and incubated overnight at room temperature. The wells are washed where after block buffer is added for one hour prior to the affixing of the standard and samples. The cytokines are detected by antibodies directly or indirectly bound to horseradish peroxidase (HRP). A color reaction is measured based on the conversion of a substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, IFN-γ ELISA; tetramethylbenzidin; IFN-α ELISA) by HRP.

IFN-γ ELISA (Peprotech, Rocky Hill, USA) was performed on cell-free supernatant of 48 hours co-cultures of NK cells with IL-15 DC or with IL-4 DC in presence or absence of K562 or Daudi target cells. NK cells stimulated for 12 hours with PMA/ionomycin served as positive control. Non-stimulated cells were considered as negative controls. Transwell conditions (see 3.7.2. Cytotoxicity assay) were included to assess contact dependency. Each sample was measured in triplicate. IFN-α ELISA (eBioscience, Vienna, Austria) was performed on 24 hours wash-out supernatant of IL-15 DC.

3.9. DATA MINING AND STATISTICAL ANALYSIS

FlowJo version 10.0.6 (Tree Star; Ashland, USA) was used to carry out flow cytometric data analysis. Statistical analysis and graphing were performed using GraphPad Prism software (v5.0; San Diego, USA). The Kolmogorov-Smirnov test was executed in order to determine if the data follow a Gaussian distribution. Statistical analysis was performed using Paired t-test (cytotoxicity; K562 and Daudi), the Wilcoxon matched-pairs signed rank test ($CD56^{bright/dim}$ distribution, cytotoxicity assay; PBL, migration assay), repeated-measures ANOVA with Bonferroni's posthoc testing (phenotyping assay, transwell cytotoxicity, IFN-γ ELISA) and Friedmann test with Dunn's multiple comparison test (phenotyping assay). P-values < 0,05 were considered statistically significant.

4. RESULTS

4.1. IL-15 DC ARE ABLE TO RECRUIT NK CELLS

To determine the NK cell-recruiting capacity of DC, a three-hour chemotaxis assay towards 24 hours wash-out supernatant of matured autologous IL-15 DC or IL-4 DC was performed (Fig 4.1). Our results show that NK cells are attracted to soluble factors produced by IL-15 DC (12.91 ± 2.02 % migration), but not by IL-4 DC (1.69 ± 3.01 % migration; n=3, p=0.25).

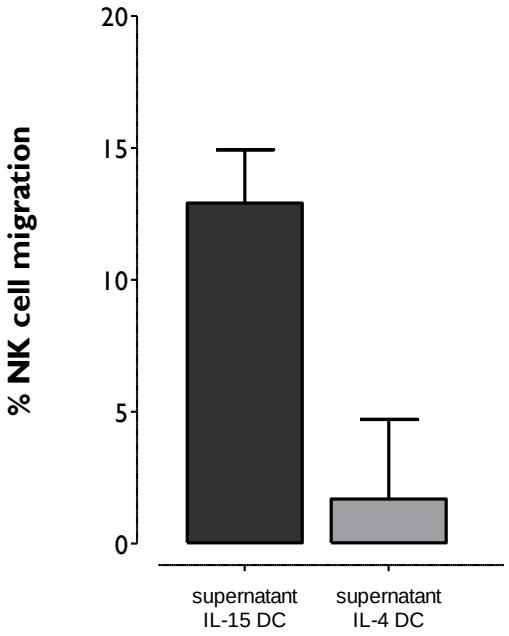

Figure 4.1. NK cells are recruited by IL-15 DC. The % NK cell migration (±SEM) towards 24 hours wash-out supernatant of matured IL-15 DC or IL-4 DC is plotted (n = 3).

4.2. IL-15 DC PHENOTYPICALLY ACTIVATE NK CELLS

With this experiment, DC-mediated NK cell activation was evaluated in terms of phenotypic activation. The NK cell surface expression of CD56, CD16, NKG2D, NKp30, NKp46, CD25, CD69, CCR7 and HLA-DR was determined on viable (7-AAD$^-$) NK cells (scatter profile) after 24 hours and 48 hours co-culture with DC. Figure 4.2 illustrates the mean fluorescence intensities (MFI) of the different NK cell membrane markers on all NK cells (CD56$^+$) and in more detail on the CD56^{bright} and CD56^{dim} NK cell subsets, 48 hours after co-culture with IL-15 DC or with IL-4 DC as compared to their unstimulated counterparts. Table 4.1 depicts the percentages difference between DC-stimulated NK cells and unstimulated NK cells for the total NK cell fraction (CD56$^+$) and for the CD56$^{bright/dim}$ NK cell subsets (mean ± SD; n=6; Overton cumulative histogram subtraction algorithm (96)). All effects occur after 24 hours co-culture (data not shown) and become more pronounced after 48 hours (Fig 4.2 and Table 4.1).

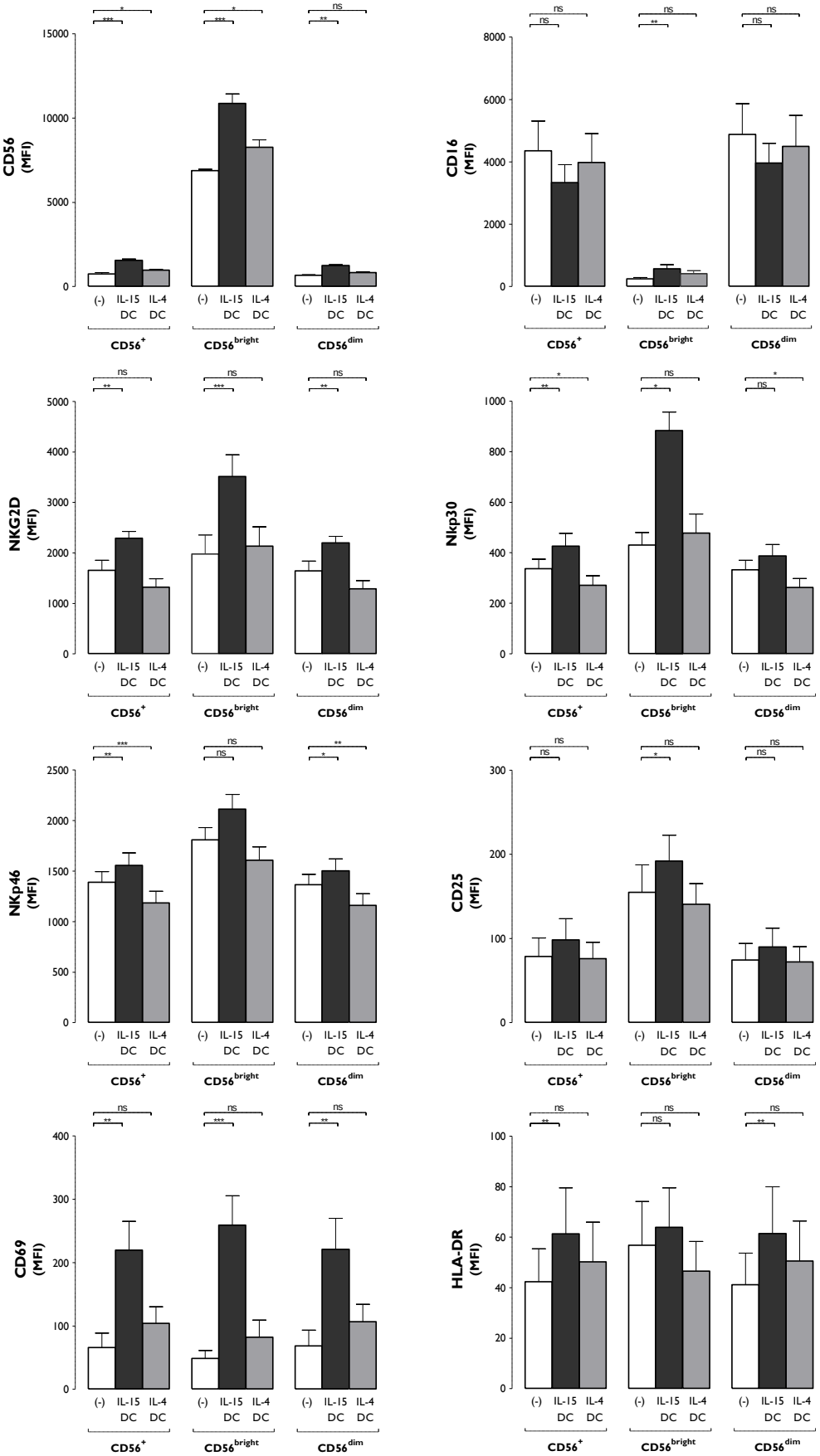

Figure 4.2. Phenotypic NK cell activation by IL-15 DC and IL-4 DC. Data show the MFI ± SEM of membrane markers on all ($CD56^{+}$), $CD56^{bright}$ and $CD56^{dim}$ NK cells after 48 hours stimulation (n = 6). 7-AAD negative cells were assessed within the NK cell population based on FSC-SSC and further subdivided based on CD56 expression. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

Table 4.1. Overton (%) of IL-15 DC- and IL-4 DC-stimulated NK cells (±SD).

	$CD56^{+}$ NK cells		$CD56^{bright}$ NK cells		$CD56^{dim}$ NK cells	
	IL-15 DC	IL-4 DC	IL-15 DC	IL-4 DC	IL-15 DC	IL-4 DC
CD56	42,8 ± 9,9	20,1 ± 9,5	51,7 ± 14,3	26,2 ± 15,0	42,2 ± 12,3	19,8 ± 10,3
CD16	1,2 ± 1,1	0,7 ± 0,6	23,2 ± 4,5	15,2 ± 6	1,5 ± 1,1	0,6 ± 0,4
NKG2D	25,7 ± 15,2	2,0 ± 5,0	38,2 ± 14,2	11,4 ± 8,6	24,1 ± 14,4	1,8 ± 4,5
NKp30	13,3 ± 8,4	0,5 ± 0,3	42,0 ± 12,4	10,5 ± 9,5	9,8 ± 8,5	0,3 ± 0,2
NKp46	11,1 ± 6,3	0,1 ± 0,2	16,2 ± 12,1	3,8 ± 5,2	10,3 ± 6,5	0,1 ± 0,2
CD25	11,0 ± 5,2	4,5 ± 3,8	16,8 ± 10,5	5,3 ± 5,5	9,8 ± 5,4	4,3 ± 3,6
CD69	33,6 ± 11,1	13,4 ± 12,3	47,6 ± 15,4	15,3 ± 7,8	32,6 ± 11,0	13,6 ± 12,8
CCR7	2,6 ± 2,1	3,4 ± 2,0	4,5 ± 4,2	7,2 ± 7,3	2,4 ± 1,8	3,2 ± 1,9
HLA-DR	7,9 ± 3,1	3,7 ± 1,3	7,9 ± 6,0	2,3 ± 2,7	7,9 ± 3,0	3,9 ± 1,4

The prototypic NK cell marker CD56 is significantly upregulated after stimulation with either DC type, but most pronounced upon IL-15 DC stimulation. The upregulation of CD56 is the highest on the $CD56^{bright}$ NK cell fraction (Fig 4.2). This result can be associated with the statistically significant increase in the $CD56^{bright}$ subset after co-culture with IL-15 DC (Fig 4.3). The $CD56^{bright}$ NK cell fraction accounts for 5.2 ± 2.0 % of the unstimulated NK cells compared with 10.6 ± 4.0 % after 48 hours IL-15 DC stimulation (Fig 4.3).

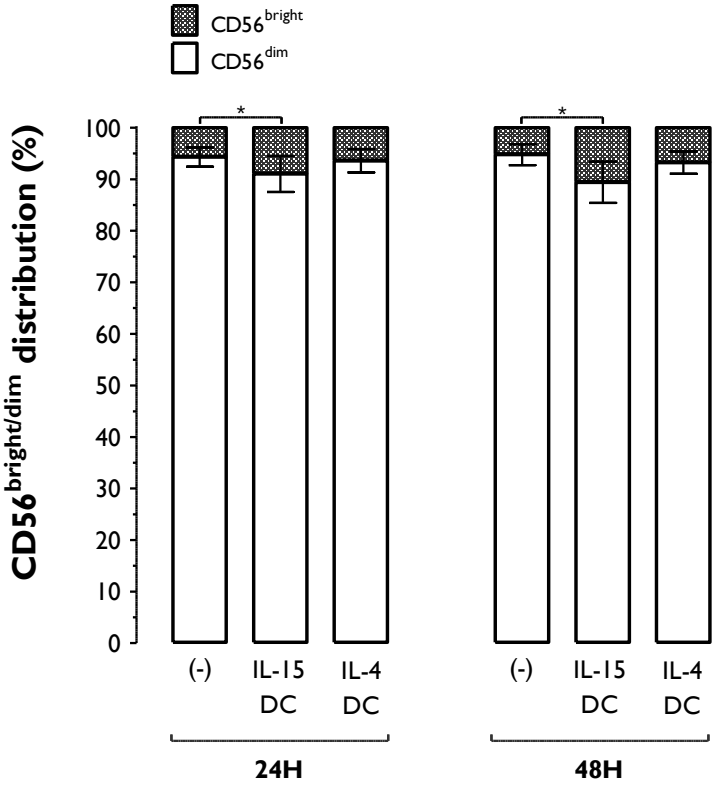

Figure 4.3. $CD56^{bright/dim}$ distribution on NK cells following co-culture with mature DC. The percentage $CD56^{bright}$ and $CD56^{dim}$ expression ± SEM on unstimulated (-), IL-15 DC-stimulated and IL-4 DC-stimulated NK cells after 24 hours and 48 hours co-culture is shown (n=6; *, $p < 0.05$).

Highly expressed on CD56^{dim} NK cells and lowly expressed on CD56^{bright} NK cells, the low affinity Fc receptor CD16 is a key molecule in mediating antibody-dependent cell-mediated cytotoxicity by NK cells (97, 98). Following DC stimulation, CD16 is significantly increased on the CD56^{bright} NK cell subset and this only after stimulation with IL-15 DC. Similarly, the IL-2 receptor alpha chain CD25, on resting NK cells only expressed on the CD56^{bright} fraction, is also significantly upregulated on CD56^{bright} NK cells by means of MFI following IL-15 DC stimulation but not upon IL-4 DC stimulation.

The activating receptor NKG2D is only affected after IL-15 DC stimulation. Both CD56^{bright} and CD56^{dim} NK cells show a significant enhancement of NKG2D expression. The NCR NKp30 and NKp46 are differentially affected by DC stimulation. IL-15 DC significantly upregulate the NKp30 expression on the CD56^{bright} NK cells, whereas IL-4 DC significantly downregulate NKp30 on the CD56^{dim} NK cells. Similar for NKp46, IL-15 DC upregulate and IL-4 DC downregulate the receptor expression significantly, but only affect the CD56^{dim} NK cells.

The activation marker CD69 is significantly upregulated on all NK cells only upon stimulation with IL-15 DC. A 3-fold increase in MFI is observed in the total NK cell population (219.7 ± 45.93, n=6) and the highest CD69 upregulation occurs in the CD56^{bright} NK cell subset with a 5-fold increase in MFI, both in relation to unstimulated NK cells (Fig 4.2). This corresponds with a mean CD69 expression of 8.13 ± 7.82 % for unstimulated NK cells, 35.44 ± 10.29 % for IL-15 DC-stimulated NK cells and 16.32 ± 7.82 % for IL-4 DC-stimulated NK cells. Figure 4.4. shows a representative example of the shift in CD69 expression by NK cells after DC stimulation.

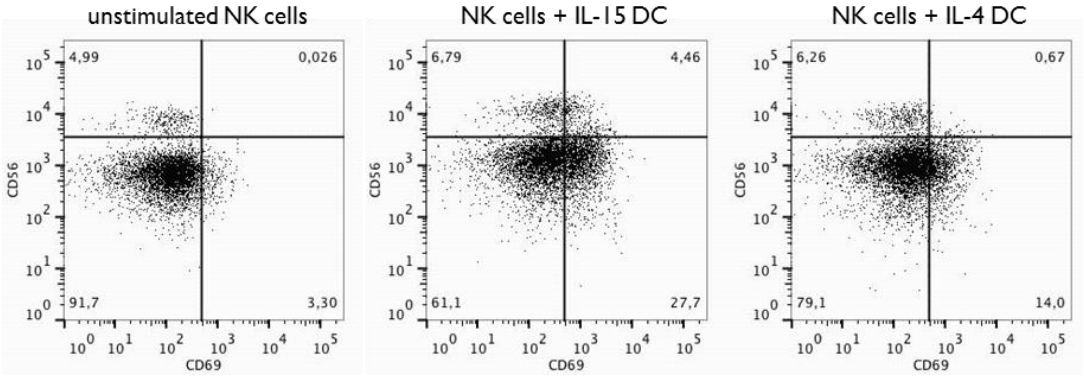

Figure 4.4. CD69 expression on NK cells after interaction with IL-15 DC and IL-4 DC. The CD69 surface expression of viable (7-AAD^{-}) lymphocytes (based on FSC-SSC) is plotted against CD56. Percentages in the quadrant show basal (unstimulated NK cells) and IL-15 DC- and IL-4 DC-induced CD69 expression on CD56$^{bright/dim}$ NK cell subsets. A representative example of one donor is shown (n=6).

The MHC class II receptor HLA-DR, weakly expressed on NK cells, is significantly upregulated on CD56^{dim} NK cells following stimulation with IL-15 DC. The expression of the chemokine receptor CCR7 was not significantly affected after interaction with IL-15 DC nor IL-4 DC (data not shown). Altogether, NK cell stimulation with IL-15 DC results in compelling phenotypic activation as compared to stimulation with IL-4 DC. Interestingly, the activation is most prominent in the CD56^{bright} NK cell fraction (Fig 4.2 and Table 4.1).

4.3. IL-15 DC DO NOT STIMULATE NK CELL IFN-γ SECRETION

In addition to phenotypic activation, we wanted to assess if IL-15 DC are capable of promoting the IFN-γ-secreting capacity of NK cells. No significant amount of IFN-γ was found in the supernatant of NK cell monocultures, nor when cultured together with K562 (Fig 4.5) or Daudi (data not shown) target cells.

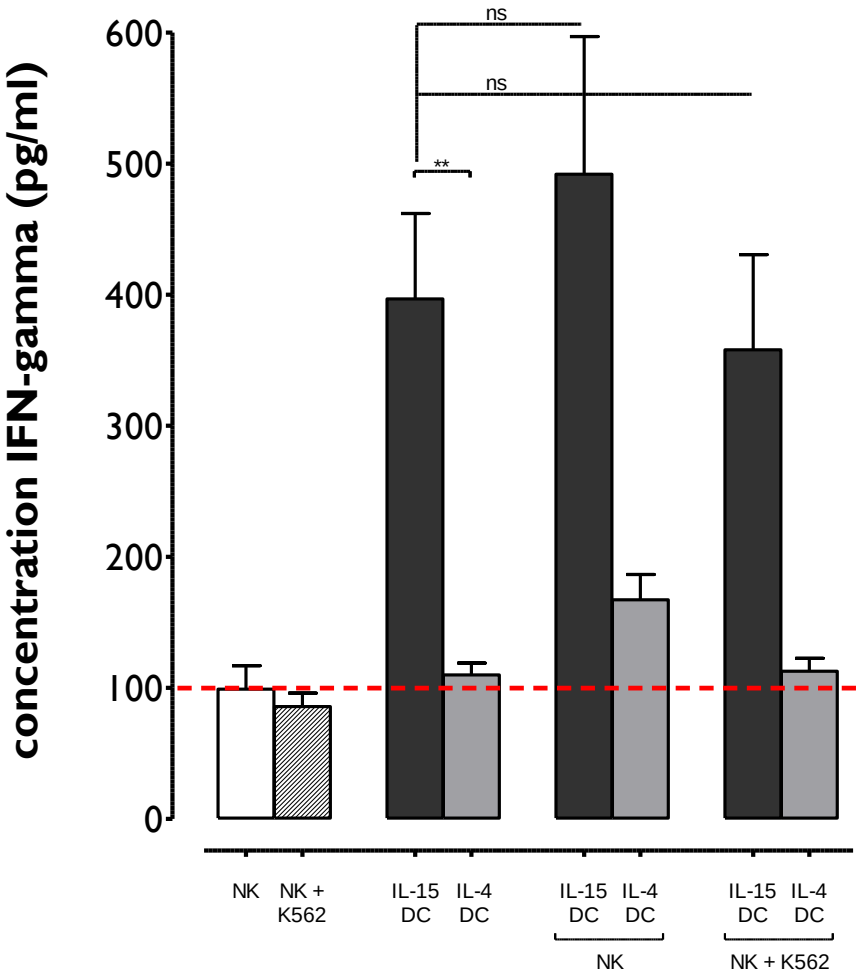

Figure 4.5. IFN-γ secretion by co-cultures of NK cells and/or IL-15 DC or IL-4 DC and/or K562 target cells. Supernatant of 48h co-cultures of NK-DC (1:1 ratio) and 4h target cell-stimulated NK-DC co-cultures (5:5:1 ratio) was collected for IFN-γ analysis with ELISA. The mean IFN-γ concentration of triplicate conditions for 6 donors is shown (±SEM). The red dotted line indicates the background detection limit. (**; $p < 0.01$; ns, not significant)

Importantly, IL-15 DC secrete a significant amount of IFN-γ as compared with IL-4 DC ($p < 0.01$). No significant difference in IFN-γ concentration was found in NK-DC co-cultures in comparison with DC alone, for both IL-15 DC and IL-4 DC. Addition of target cells (K562 or Daudi) to the NK-DC co-cultures, also did not result in an increased IFN-γ secretion (Fig 4.5). Blocking of NK-DC contact had no effect on the IFN-γ concentration in two- and three-party co-cultures (data not shown).

4.4. IL-15 DC PROMOTE NK CELL-MEDIATED KILLING OF NK-SENSITIVE K562 CELLS AND INDUCE KILLING OF NK-RESISTANT DAUDI CELLS BUT NOT AUTOLOGOUS PBL

To examine if IL-15 DC can activate the cytotoxic capacity of NK cells, co-cultures of DC and NK cells were setup for 48 hours at a 1:1 ratio. After 48 hours, PKH67-labeled target cells (K562, Daudi or autologous CD56-depleted PBL) were added to the NK-DC co-cultures for an additional 4 hours, followed by a PI/Annexin-V-based flow cytometric lysis assay. The % cytotoxicity is defined as 100 – [(% annexin V^-PI^- target cells in the presence of NK cells / % annexin V^-PI^- target cells without NK cells) x 100]. Figure 4.6. depicts an example of the gating strategy.

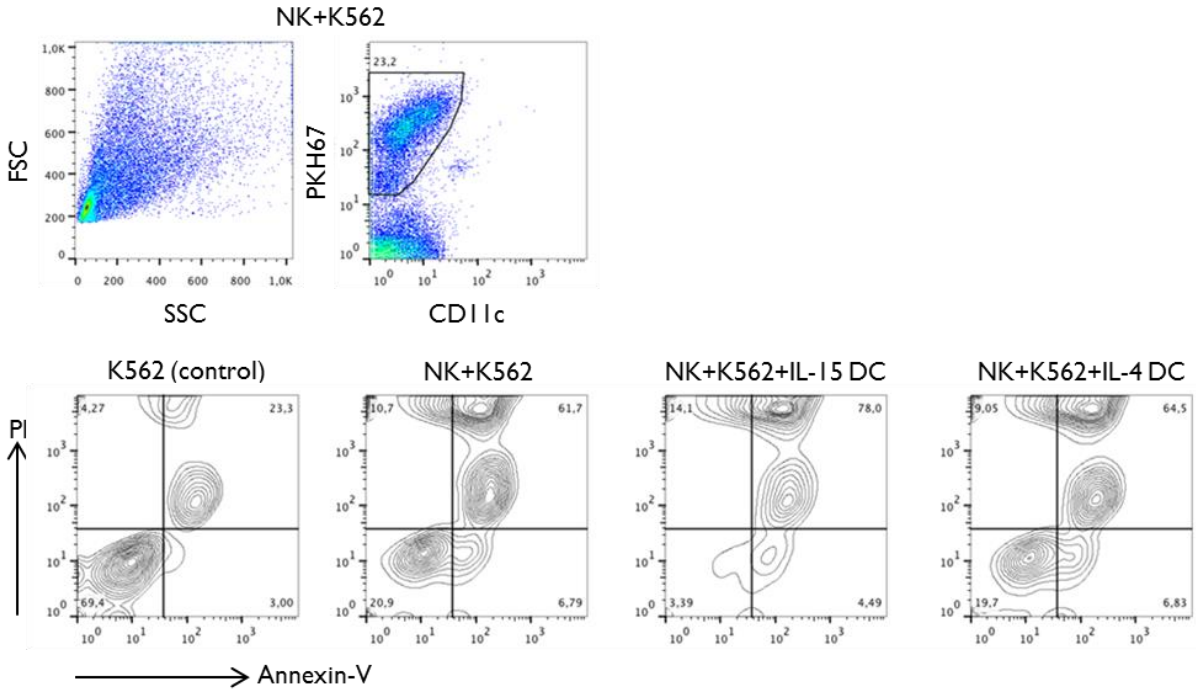

Figure 4.6. Gating strategy for the analysis of NK cell-mediated target cell killing. For the analysis of the annexin-V/PI-based flow cytometric cytotoxicity assay, $PKH67^+CD11c^-$ target cells were selected (upper right graph) to determine their viability profile (lower row). The % killing was calculated based on the percentage of the lower left of the quadrant (i.e. viable annexin-V^-PI^- cells). In the top left graph, the FSC is displayed

against the side scatter. A representative example of K562 target cell viability for all conditions from one out of 10 donors is shown.

As shown in figure 4.7, IL-15 DC but not IL-4 DC, significantly enhanced the NK cell-mediated killing of the NK-sensitive K562 cell line as compared to the killing capacity of resting peripheral blood NK cells (n=10; $p<0.001$). A mean increase of 22% killing after co-culture with IL-15 DC was achieved, with a maximum of 97% killing of K562 cells. In addition, IL-15 DC-stimulated NK cells show vigorous cytotoxic activity against the NK cell insensitive cell line Daudi (Fig 4.7; n=10, $p<0.001$). Interaction between NK cells and IL-15 DC resulted in a 3.6-fold increase of the cytotoxic properties of NK cells against Daudi cells with a mean % killing of 73.6 ± 6.2 % versus 20.6 ± 2.6 % killing by resting NK cells (Fig 4.7; n=10). Importantly, autologous CD56-depleted PBL were not killed by IL-15 DC-activated NK cells (Fig 4.7; n=6). In summary, IL-15 DC-stimulated NK cells exert a significantly increased killing activity against NK-sensitive target cells and acquire the ability to significantly kill NK-resistant cells, while sparing autologous PBL.

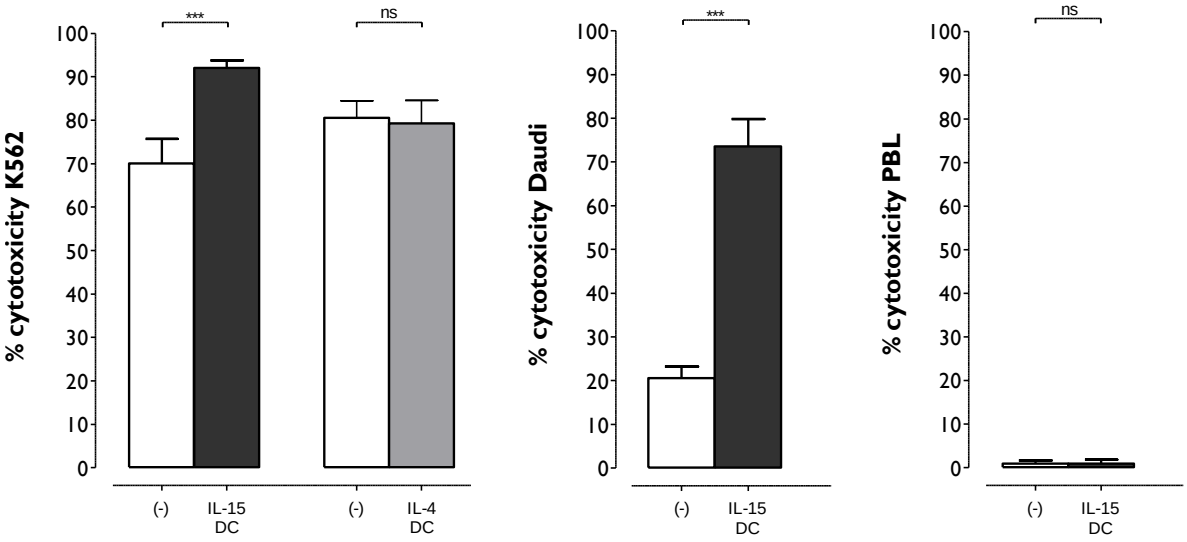

Figure 4.7. NK cell-mediated killing of NK-sensitive K562, NK-resistant Daudi and autologous cells. The % killing (±SEM) of K562 (n = 10), Daudi (n = 10) and autologous CD56-depleted PBL (n = 6) after 4 hours co-culture with NK cells alone (-) or co-cultures of NK cells with IL-15 DC or IL-4 DC are shown. (ns, not significant; ***, $P < 0.001$).

4.5. ACTIVATION OF NK CELL-MEDIATED TUMOR CELL KILLING BY IL-15 DC IS PREDOMINANTLY CONTACT-DEPENDENT

In order to verify whether the observed effects rely on cell-to-cell contact, Transwell conditions were performed. The enhanced killing of K562 after IL-15 DC-mediated NK cell stimulation is virtually completely abrogated when NK cells and IL-15 DC were not in close

contact (Fig 4.8). For the induction of NK cell-mediated killing of Daudi cells, a significant decrease of target cell killing was observed when NK cells and IL-15 DC were separated, but the killing was not completely blocked (Fig 4.8; n=10).

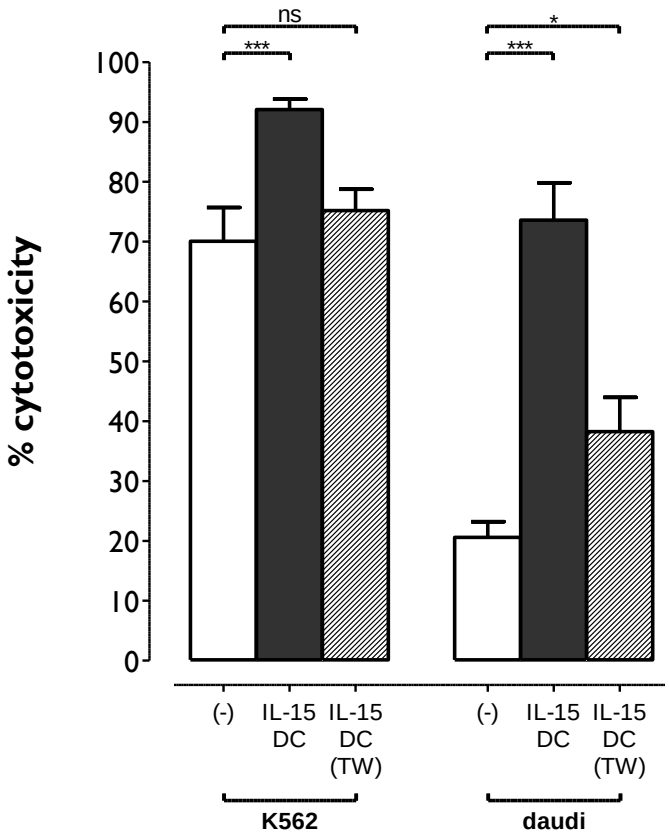

Figure 4.8. Enhancement of NK cell cytotoxicity after stimulation by IL-15 DC is predominantly contact-dependent. The % of killing (± SEM) of K562 and Daudi cells by NK cells (-), co-cultures of NK cells with IL-15 DC whether (IL-15 DC) or not in transwell (IL-15 DC (TW)) is shown. (n = 10; ns, not significant; *, $P < 0.05$; ***, $P < 0.001$).

4.6. MATURED IL-15 DC DO NOT SECRETE IFN-α

After 24 hours incubation free from differentiation factors and maturation cocktails, cell-free supernatant of mature IL-15 DC and IL-4 DC was analyzed for IFN-α (n=7). Concentrations IFN-α secreted by IL-15 DC and IL-4 DC were all below the detection limit (3.2 pg/mL; data not shown). A defined concentration of recombinant human IFN-α served as an internal control.

4.7. IL-15 DC EXPRESS MEMBRANE-BOUND IL-15 AND IL-15Rα

Here, we evaluated the surface expression of IL-15 and IL-15Rα on mature IL-15 DC and IL-4 DC. Flow cytometric analysis showed that IL-15 DC and not IL-4 DC express high amounts of membrane-bound IL-15 (81.67 ± 5.32 %; Fig 4.9). IL-15Rα is highly expressed

on IL-15 DC (36.80 ± 4.86 %), whereas IL-4 DC only have a low surface expression (7.46 ± 6.52 %). IL-15 and IL-15Rα expression is comparable for both $CD56^+$ and $CD56^-$ IL-15 DC.

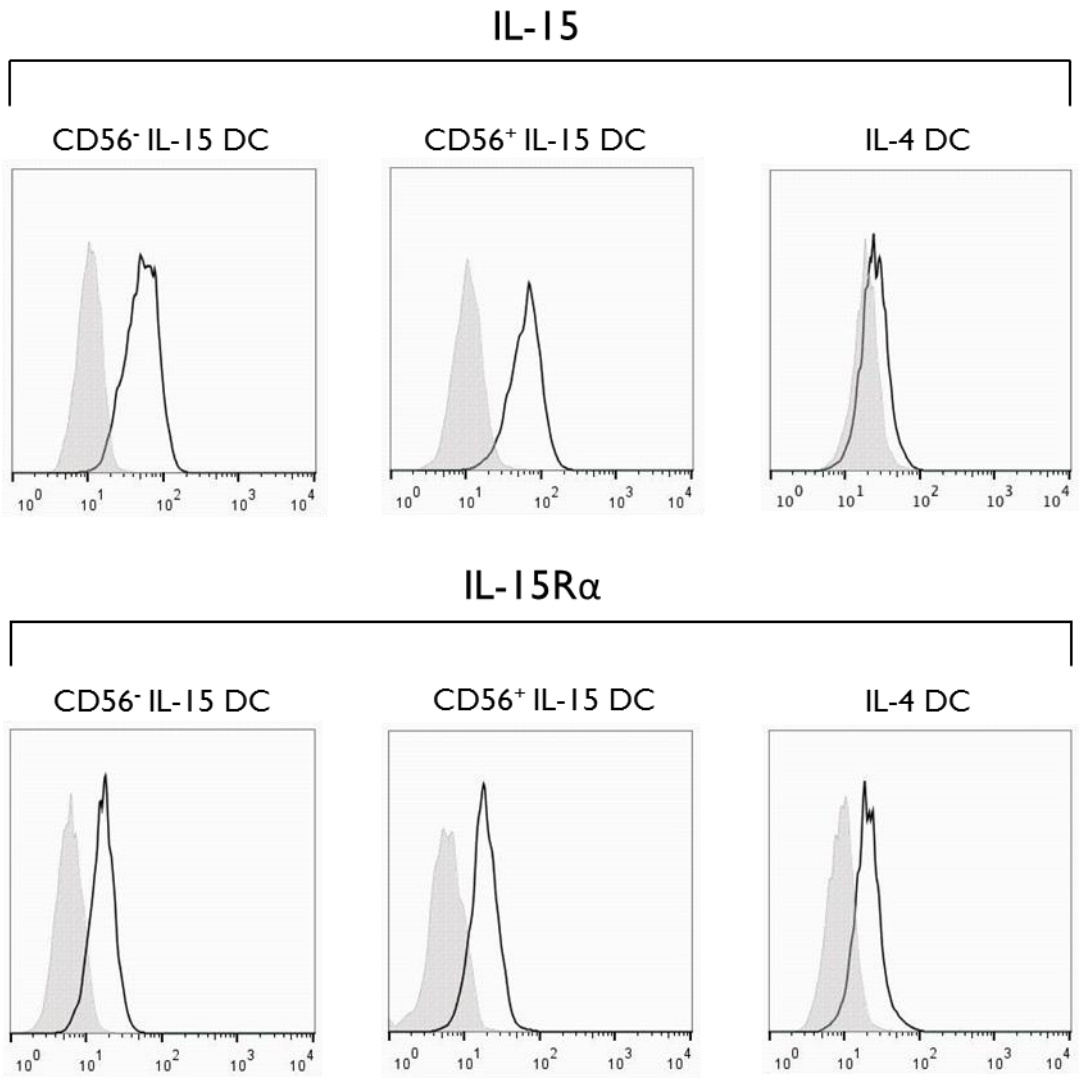

Figure 4.9. IL-15 and IL-15Rα expression on $CD56^-$ IL-15 DC, $CD56^+$ IL-15 DC and IL-4 DC. Histogram overlays illustrate a representative example (n=3) of the surface expression of IL-15 and IL-15Rα of the different mature DC types (solid black line) and their respective isotype controls (filled grey histograms).

5. DISCUSSION

In this thesis, we demonstrate that our previously established IL-15 DC vaccine manufacturing protocol (40) rapidly generates DC endowed with the capacity to activate NK cells and to harness NK cell cytotoxic effector function towards tumor cells. This adds to previous studies by our group and other groups which showed that IL-15 DC possess potent immunostimulatory functions and confirms that IL-15 DC can augment anti-tumor immunity not only through activation of the adaptive immune system (62, 89) and through a direct tumoricidal activity (62) but also through activation of the innate immune system. This is especially relevant in view of the consideration that innate immune cells, most notably NK cells, are important mediators of immune defense against tumors (63). For example, for AML, a growing body of evidence highlights the importance of NK cells in the generation of effective anti-leukemia immunity (19, 85). Our study clearly establishes that IL-4 DC, up until now the most commonly used DC formulation in clinical DC vaccine trials (17) are unable to mount an effective NK cell response against leukemia cells, which corroborates the current view that IL-4 DC are far from ideal DC for immunotherapeutic use (47, 49, 99). IL-15 DC, by contrast, display a potent ability to attract NK cells and induce both phenotypic and functional activation of NK cells.

The ability of DC to recruit NK cells is an important prerequisite to effective NK-DC crosstalk (100, 101). Here we show that the culture supernatant of IL-15 DC attracts NK cells, whereas IL-4 DC fail to induce NK cell migration. This confirms that IL-15 DC provide a favorable chemokine milieu for recruiting NK cells. However, whether or not this is related to the IL-15 used for DC differentiation or to the TLR7/8 agonist R848 used for inducing IL-15 DC maturation remains to be determined. On the one hand, IL-15 itself has indeed been shown to induce the secretion of NK cell-attracting chemokines (reviewed in (68)) by DC, such as CCL2 (102), CCL3 [MIP-1α] (103) and CCL5 [RANTES] (102, 103). TLR stimuli, on the other hand, can also enhance the production of chemokines by DC, resulting in NK cell migration (91, 100, 104, 105). For example, DC matured with bacterial fragments derived from Klebsiella pneumonia, which act through TLR2 and TLR4, were shown to recruit NK cells via production of CCL5 (91). DC stimulated with the TLR3 agonist poly(I:C) (104) or the TLR9 agonist CpG (106) also demonstrated a potent ability to attract NK cells; in both studies, CXCR3-binding chemokines were found to play a role in the induction of NK cell migration. Although one study found no significant increase in NK cell migration in response to stimulation with TLR7/8-activated DC (106), a possible involvement of R848 in the IL-15 DC-induced NK cell migration that was observed in this

thesis cannot be entirely excluded since R848-treated DC have been previously shown to secrete chemokines involved in NK cell migration, such as CCL2, CCL3, CCL5 and the CXCR3-binding chemokine CXCL10 (107, 108).

In addition to their ability to promote NK cell recruitment, IL-15 DC were found to induce a series phenotypic changes in NK cells. One interesting phenotypic finding was the marked increase of the $CD56^{bright}$ NK cell proportion relative to the $CD56^{dim}$ population after stimulation with IL-15 DC. This is in line with several other studies demonstrating preferential proliferation of the NK cell $CD56^{bright}$ population upon interaction with mature DC (92, 109, 110). Ferlazzo *et al.* previously showed that transpresentation of IL-15 by the IL-15Rα on the DC surface is involved in the selective proliferation and sustained survival of the $CD56^{bright}$ subset following DC/NK cell interaction (83). Given our finding that IL-15 DC express high-levels of membrane-bound IL-15 as well as IL-15Rα, it is tempting to speculate that IL-15 transpresentation also accounts for the observed relative increase in the $CD56^{bright}$ NK cell population after exposure to IL-15 DC. Strikingly, despite the relative decrease in the proportion of $CD56^{dim}$ NK cells, we found that both $CD56^{bright}$ and $CD56^{dim}$ NK cells subsets rapidly upregulated CD56 upon stimulation with IL-15 DC. Although the precise role of CD56, a 140-kDa isoform of the neural cell adhesion molecule (NCAM) (97), on NK cells is still largely unknown, our study corroborates emerging evidence that CD56 is a marker of NK cell activation and that its upregulation is paralleled by an enhanced NK cell effector function (111-113). This is congruent with our other phenotypic data which collectively indicate that IL-15 DC-exposed NK cells are in an enhanced activation state, as evidenced by the increased expression of the activation markers CD69 and HLA-DR on the total NK cell population and of CD25 on the $CD56^{bright}$ NK cell subset.

Apart from inducing an activated phenotype in NK cells, IL-15 DC also strongly upregulated the expression of NKG2D on both $CD56^{bright}$ and $CD56^{dim}$ subsets, whereas IL-4 DC-stimulated NK cells did not alter the expression of this activating receptor. The clinical significance of this finding and its potential implication for DC-based immunotherapy of cancer is emphasized by data showing that the level of NKG2D expression on NK cells correlates with clinical outcome following DC vaccination (110). Furthermore, NKG2D appears to be critically involved in AML cell recognition by NK cells and plays a key role in NK cell-mediated immunosurveillance of AML (114), providing a further rationale for exploring the potential utility of IL-15 DC for immunotherapy of AML. In line with the increased expression of NKG2D, we found that IL-15 DC could also

upregulate expression of the NCR NKp30 and NKp46 on $CD56^{bright}$ and $CD56^{dim}$ NK cells, respectively. Again, IL-4 DC did not lead to upregulation of these markers and, for the $CD56^{dim}$ NK cell subset, IL-4 DC were even found to have a detrimental effect on the expression of both NCR. This further underscores the need for caution with the use of IL-4 DC for cancer vaccination purposes. As described for NKG2D, NKp30 and NKp46 are also important in the NK cell-mediated immunosurveillance of AML; low expression of these NCR is correlated with poor NK cell cytotoxic effector function towards AML cells and is associated with an inferior prognosis in AML patients (115, 116). Whether or not IL-15 expression by IL-15 DC is implicated in the observed upregulation of NKG2D, NKp30 and NKp46 remains to be formally investigated, although it is tempting to draw a parallel between our study and two recent studies which showed that IL-15 can directly upregulate NKG2D and NCR expression on NK cells (117, 118).

With regard to the effect of IL-15 DC on NK cell function, we were unable to show that IL-15 DC enhance the capacity of NK cells to secrete IFN-γ. This was rather unexpected given the prominent phenotypic activation changes observed in the $CD56^{bright}$ NK cell population following IL-15 DC stimulation. $CD56^{bright}$ NK cells are indeed described to represent the main source of NK cell-derived IFN-γ. The production of IFN-γ by NK cells is, however, tightly regulated; under basal circumstances, NK cells will not produce IFN-γ, nor can the release of IFN-γ be triggered by exposing NK cells only to a tumor target cell (119-121). This was confirmed by our results, which showed no appreciable presence of IFN-γ in the supernatants of NK cell monocultures nor in the co-culture supernatants of NK cells with K562 leukemia cells. Resting NK cells generally require at least two "activating" signals to produce IFN-γ (e.g. K562 contact [signal 1] + IFN-α [signal 2]) (119). Our results indicate that IL-15 DC cannot provide the necessary second signal to trigger IFN-γ release from NK cells. This was confirmed by the observation that IFN-γ was not increased in the supernatants of IL-15 DC/NK cell/K562 co-cultures. In agreement with this, we found that IL-15 DC do not produce IFN-α, which is described to be an important "signal 2" cytokine (119-121). Another possible explanation for the observed lack of induction of IFN-γ secretion may be related to the fact that IL-15 DC are weak producers of IL-12 (40). Several studies have indeed indicated that IL-12 is an important cytokine for enabling DC to trigger NK cell IFN-γ secretion (42, 83, 91, 111, 122) . Perhaps the main cause for the depressed capacity of IL-15 DC to produce IL-12 relates to the use of PGE_2 during DC maturation. PGE_2 is usually included in DC maturation cocktails to promote DC migration (123), despite its known suppressive effect on the IL-12 production capacity of DC (124). Van

Elssen *et al.* confirmed that DC matured in the presence of PGE_2 have an impaired capacity to stimulate NK cell IFN-γ production, which could be ascribed to PGE_2-induced inhibition of IL-12 secretion by the DC (125). The low IL-12 production capacity of our DC and their inability to enhance NK cell IFN-γ production could likewise be related to the TLR7/8 agonist used in this study to induce DC maturation. This is supported by a recent study that compared the effect of 3 different non-PGE_2-containing, TLR-based DC maturation cocktails on DC-induced NK cell activation and found that only DC activated through TLR3 or TLR4, but not TLR7/8, were capable of producing significant amounts of IL-12 and of triggering NK cells to secrete IFN-γ (42).

The same study also found that IL-12 is not required for DC mediated-enhancement of NK cell cytotoxicity (42), providing a plausible explanation why IL-15 DC in our study were capable of harnessing the cytotoxic effector function of NK cells without being able to augment the secretion of IFN-γ by NK cells. The observation that IL-15 DC, but not IL-4 DC, can enhance the cytotoxicity of NK cells is consistent with our phenotypic data showing increased expression of CD56 (a marker of cytotoxic function (126)) and of NKG2D and NKp46 (involved in target cell recognition and cytotoxicity) on the cell surface of IL-15 DC-stimulated NK cells. Given the fact that IL-15 DC themselves have been recently described to possess direct lytic activity towards K562 cells, the question arises whether the enhanced K562 killing observed in IL-15 DC/NK cell co-cultures could be credited to the IL-15 DC (62). However, it is important to note that the lytic activity of IL-15 DC against K562 only occurs at a high effector-to-target cell (E:T) ratio of 50:1 (62), while in this study a 10-fold lower E:T ratio was applied. Furthermore, "killer" DC are rather weak and slow inducers of tumor cell death (126-128), making it almost impossible that IL-15 DC had developed any significant cytotoxicity during the 4 hour time frame of our cytotoxicity assay. In view of the above, it seems highly unlikely that IL-15 DC, even partly, contributed to the cytotoxic effects observed. In addition to the prototypic NK cell target K562, IL-15 DC-stimulated NK cells also displayed potent cytotoxicity towards the Daudi tumor cell line, which is a rather intriguing observation given the fact that Daudi cells are normally resistant to NK cell-mediated lysis (63). This corroborates emerging evidence that DC, if appropriately stimulated, can expand the target cell spectrum of NK cells to include tumor cells that are otherwise resistant to NK cell killing (111, 129). Despite broadening the target cell spectrum of NK cells, IL-15 DC stimulation of NK cells does not appear to result in unwanted lysis of normal healthy cells. T cells were indeed found to be

spared from killing by IL-15 DC-activated NK cells, which is important when considering the exploitation of IL-15 DC for DC-based immunotherapy.

The precise mechanisms by which IL-15 DC induce phenotypic and functional activation of NK cells remain to be elucidated. Nevertheless, Transwell^{TM} experiments already indicated that effective NK cell activation by IL-15 DC requires direct cell-to-cell contact, whereas soluble factors only play a minor role. Several cell-to-cell contact mechanisms can be implicated in NK-DC crosstalk, including: MICA, MICB, NKp30L, NKp46L, DNAM-1L and/or CX3CL1 (65, 74, 130-132). Perhaps the most relevant mechanism for our system may be transpresentation of IL-15 via IL-15Rα. The preassembling of the IL-15 and IL-15Rα complex takes places in the endoplasmic reticulum or Golgi apparatus of the DC. Subsequently, the complexes are conveyed to the cell surface, where they can interact with the IL-15Rβ/γ on the NK cells (54, 55, 83, 133). In line with previous studies (89, 134), we found that IL-15 DC express both IL-15 and IL-15Rα on their surface, underlining their potential to transpresent IL-15.

6. CONCLUSION

During this master thesis, we demonstrated that monocyte-derived IL-15 DC differentiated with IL-15 and matured with a TLR7/8 agonist-based maturation cocktail are able to stimulate autologous resting peripheral blood NK cells. Importantly, IL-15 DC activate NK cells in a superior way as compared to the conventional monocyte-derived IL-4 DC used in clinical vaccination trials today. With the challenge to improve the clinical efficacy of DC vaccine protocols for AML, and cancer in general, we demonstrate that inclusion of IL-15 and a TLR7/8 agonist during the generation of monocyte-derived DC vaccines, renders highly potent NK cell-activating DC.

The first objective was to evaluate the NK cell-attracting capacity of IL-15 DC. Our results show that IL-15 DC release factors that recruit NK cells, providing an environment in which close encounter between NK cells and DC can take place and could promote NK-DC crosstalk. The second objective was to determine if IL-15 DC could phenotypically activate NK cells. Based on an NK cell panel of activation markers, we demonstrate that NK cells are highly activated following IL-15 DC stimulation, whereas IL-4 DC stimulation exert only moderate phenotypic activation. Moreover, activation is predominantly expressed in the $CD56^{bright}$ NK cell fraction. This phenotypic activation of NK cells after IL-15 DC stimulation is an important argument for the pre-eminence of IL-15 DC in comparison to the IL-4 DC. As third objective, we examined the functional NK cell-activating abilities of IL-15 DC. Here, we show that IL-15 DC but not IL-4 DC promote NK cell-mediated killing of NK-sensitive K562 target cells and induce killing of NK-resistant Daudi target cells, while sparing autologous peripheral blood lymphocytes. In this way, IL-15 DC fulfill an important quality recommended in NK cell-directed immunotherapeutic approaches. On the other hand, the helper function of NK cells (i.e. IFN-γ secretion) is not addressed by conventional IL-4 DC nor by IL-15 DC; additional signals are required to harness DC for activating this NK cell function. The fourth and last objective of this master thesis was to elucidate underlying mechanisms of the observed IL-15 DC-mediated NK cell activation. We demonstrate that the enhanced NK cell lytic activity following IL-15 DC stimulation is predominantly contact-dependent. Transpresentation of IL-15 by IL-15Rα is an acknowledged mechanism in NK-DC crosstalk. In this context, we also show that IL-15 DC have a high surface expression of both IL-15 and IL-15Rα. Neutralization experiments will confirm if transpresentation of IL-15 is the mechanism involved in the phenotypic and functional activation of NK cells by IL-15 DC.

In conclusion, we demonstrate that IL-15 DC are good candidates for NK cell-directed immunotherapeutic approaches, in favor of clearance of (resistant) target cells and of promoting NK-DC crosstalk.

7. SUMMARY

Acute myeloid leukemia (AML) is an aggressive blood cancer that carries a dreadful prognosis. More effective and less destructive strategies are warranted for the treatment and prevention of AML relapse. Given their prime role in the generation of antitumor immunity, dendritic cells (DC) have attracted great interest for anticancer vaccination. Since DC are known to be capable of inducing natural killer (NK) cell activation and the fact that AML is characterized by NK cell dysregulation, regaining full NK cell activity with DC vaccination is an attractive approach. In this study, we examined the NK cell-activating capacity of monocyte-derived DC alternatively induced by interleukin-15 (IL-15 DC) and compared it with that of conventionally generated IL-4 DC used in clinical vaccination trials today.

Our results show that IL-15 DC are able to stimulate autologous resting peripheral blood NK cells in a superior way as compared to IL-4 DC, evidenced by the recruitment, phenotypic and functional activation of NK cells. Based on a panel of NK cell activation markers, we demonstrate that NK cells are highly activated following IL-15 DC stimulation, whereas IL-4 DC exert only moderate phenotypic activation. Importantly, IL-15 DC but not IL-4 DC promote NK cell-mediated killing of NK-sensitive target cells and induce killing of NK-resistant target cells, while sparing autologous peripheral blood lymphocytes. On the other hand, the helper function of NK cells (i.e. interferon-γ secretion) is not addressed by IL-4 DC nor by IL-15 DC. Finally, we demonstrate that the enhanced NK cell lytic activity following IL-15 DC stimulation is predominantly contact-dependent.

In conclusion, these data show that the IL-15 DC generation protocol renders highly potent NK cell-activating DC and endorse IL-15 DC as good candidates for NK cell-directed immunotherapeutic approaches for AML, in favor of clearance of (resistant) target cells.

8. PERSPECTIVES

With the first objective of this thesis, we demonstrated that IL-15 DC are capable of recruiting autologous resting peripheral blood NK cells. In light of the different characteristics of the $CD56^{dim}$ and $CD56^{bright}$ NK cell populations, their differential surface expression of chemotactic receptors (68), as well as the fact that the phenotypic activation is most prominent in the $CD56^{bright}$ NK cell fraction after IL-15 DC stimulation, it would be interesting to examine the migratory capacity of each NK cell subset and compare if they are attracted to the same extent. In parallel, investigation of the expression of chemotactic receptors on $CD56^{bright/dim}$ NK cells and the chemokine secretion profile of IL-15 DC could provide important information on the mechanisms involved in IL-15 DC-mediated NK cell recruitment.

methodology – Migrated cells are stained with specific antibodies for CD56, CD16 and chemokine receptors for flow cytometric phenotype analysis. Supernatant of wash-out IL-15 DC is subjected to multiplex chemokine analysis.

The importance of cell-to-cell contact between NK cells and IL-15 DC was established in the context of DC-induced NK cell-mediated killing of NK-sensitive and NK-resistant target cells (fourth objective). The precise mechanisms of this interaction, however, remain to be elucidated. Several receptor-ligand interactions that have been described to be involved in NK-DC crosstalk, qualify to be involved in IL-15 DC-mediated NK cell activation. In this context, we could already demonstrate that IL-15 DC highly express both IL-15 and IL-15Rα on their surface. Transpresentation of IL-15 is an acknowledged mechanism in the promotion of DC-mediated NK cell activation (54, 55). Further in-depth analysis of this pathway is therefore warranted. Another possibly involved ligand-receptor interaction in our experimental design is the MIC A/B-NKG2D interaction. It was demonstrated that IL-15 induces DC expression of MIC A/B (81). Since our DC generation protocol includes IL-15, evaluation of MIC A/B surface expression is argumentative.

methodology – To determine the involvement of the ligand-receptor interactions, neutralizing antibodies are added to mature DC cultures prior to co-culture with NK cells, followed by downstream analysis of NK cell-mediated killing. Additionally, the kinetics of IL-15/IL-15Rα and MIC A/B on IL-15 DC are surveyed during the generation of monocyte-derived DC (following the protocol under 3.7.1.2. on different time points; max 1 week), to evaluate the time frame in which NK-DC interaction through these mechanisms could take place.

Given the fact that IL-15 is a known inducer of NK cell proliferation (135, 136) and that induction of NK cell proliferation by human monocyte-derived DC has been demonstrated (83, 92, 130), the next step in the assessment of the NK cell-activating capacity of our IL-15/IL-15Rα-expressing IL-15 DC is to determine their NK cell proliferation-promoting ability.

methodology - To determine the capacity of IL-15 DC to induce NK cell proliferation, a 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution protocol is performed. NK cells are labeled with CFSE cell tracker and co-cultured with autologous mature DC. After 8 days, the CFSE fluorescence intensity of divided cells is analyzed on a flow cytometer.

Finally, translation of the *in vitro* findings with immune cells of healthy controls to a human AML model are indispensable. Given the NK cell dysregulation in AML patients (7, 19), the obtained results need to be validated with patient material to evaluate to what extent IL-15 DC derived from AML (remission) patients can reinforce or boost NK cell function. Employing immune cells of patients with *de novo* AML will prove the NK cell-activating potency of IL-15 DC. Considering the fact that IL-15 DC vaccination will likely be administered in the consolidation phase (to prevent relapse), usage of immune cells of AML patients in remission will reflect the clinical setting.

methodology - The functional and mechanistic findings obtained with healthy subjects will be validated with immune cells of patients with *de novo* AML and of AML patients in remission. The anti-leukemic activity will be set out against primary AML blasts and AML cell lines.

BIBLIOGRAPHY

1. Smyth MJ, Sullivan LC, Brooks AG, Andrews DM. Non-classical MHC Class I molecules regulating natural killer cell function. Oncoimmunology. 2013;2(3):e23336.
2. World Health Organisation [internet]. Geneva2013 [updated January 2013; cited 2013 March 2]. Available from: http://www.who.int/mediacentre/factsheets/fs297/en/.
3. Tallman MS, Gilliland DG, Rowe JM. Drug therapy for acute myeloid leukemia. Blood. 2005;106(4):1154-63.
4. Smits EL, Berneman ZN, Van Tendeloo VF. Immunotherapy of acute myeloid leukemia: current approaches. The oncologist. 2009;14(3):240-52.
5. Deschler B, Lubbert M. Acute myeloid leukemia: epidemiology and etiology. Cancer. 2006;107(9):2099-107.
6. Thein MS, Ershler WB, Jemal A, Yates JW, Baer MR. Outcome of older patients with acute myeloid leukemia: An Analysis of SEER Data Over 3 Decades. Cancer. 2013.
7. Anguille S, Lion E, Smits E, Berneman ZN, van Tendeloo VF. Dendritic cell vaccine therapy for acute myeloid leukemia: questions and answers. Human vaccines. 2011;7(5):579-84.
8. Estey E, Dohner H. Acute myeloid leukaemia. Lancet. 2006;368(9550):1894-907.
9. Roboz GJ. Current treatment of acute myeloid leukemia. Current opinion in oncology. 2012;24(6):711-9.
10. Zhu X, Ma Y, Liu D. Novel agents and regimens for acute myeloid leukemia: 2009 ASH annual meeting highlights. Journal of hematology & oncology. 2010;3:17.
11. Al-Mawali A, Gillis D, Lewis I. The role of multiparameter flow cytometry for detection of minimal residual disease in acute myeloid leukemia. American journal of clinical pathology. 2009;131(1):16-26.
12. van der Pol MA, Pater JM, Feller N, Westra AH, van Stijn A, Ossenkoppele GJ, et al. Functional characterization of minimal residual disease for P-glycoprotein and multidrug resistance protein activity in acute myeloid leukemia. Leukemia. 2001;15(10):1554-63.
13. Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115(3):453-74.
14. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. Blood. 2008;112(12):4371-83.
15. Van Tendeloo VF, Van de Velde A, Van Driessche A, Cools N, Anguille S, Ladell K, et al. Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(31):13824-9.
16. Barrett AJ. Understanding and harnessing the graft-versus-leukaemia effect. British journal of haematology. 2008;142(6):877-88.
17. Anguille S, Willemen Y, Lion E, Smits EL, Berneman ZN. Dendritic cell vaccination in acute myeloid leukemia. Cytotherapy. 2012;14(6):647-56.
18. Chan L, Hardwick NR, Guinn BA, Darling D, Gaken J, Galea-Lauri J, et al. An immune edited tumour versus a tumour edited immune system: Prospects for immune therapy of acute myeloid leukaemia. Cancer immunology, immunotherapy : CII. 2006;55(8):1017-24.
19. Lion E, Willemen Y, Berneman ZN, Van Tendeloo VF, Smits EL. Natural killer cell immune escape in acute myeloid leukemia. Leukemia. 2012;26(9):2019-26.

20. Morris JC, Waldmann TA. Antibody-based therapy of leukaemia. Expert reviews in molecular medicine. 2009;11:e29.
21. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nature reviews Cancer. 2008;8(4):299-308.
22. Blaser BW, Caligiuri MA. Autologous immune strategies to reduce the risk of leukemic relapse: consideration for IL-15. Best practice & research Clinical haematology. 2006;19(2):281-92.
23. Anguille S, Lion E, Willemen Y, Van Tendeloo VF, Berneman ZN, Smits EL. Interferon-alpha in acute myeloid leukemia: an old drug revisited. Leukemia. 2011;25(5):739-48.
24. Anguille S, Van Tendeloo VF, Berneman ZN. Leukemia-associated antigens and their relevance to the immunotherapy of acute myeloid leukemia. Leukemia. 2012;26(10):2186-96.
25. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nature reviews Cancer. 2012;12(4):265-77.
26. Abbas AK, Lichtman AH, Pillai S. Cellular and Molecular Immunology. sixth ed. Philadelphia: Saunders; 2010.
27. Van Brussel I, Berneman ZN, Cools N. Optimizing dendritic cell-based immunotherapy: tackling the complexity of different arms of the immune system. Mediators of inflammation. 2012;2012:690643.
28. Wu L, Liu YJ. Development of dendritic-cell lineages. Immunity. 2007;26(6):741-50.
29. Hubo M, Trinschek B, Kryczanowsky F, Tuettenberg A, Steinbrink K, Jonuleit H. Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells. Frontiers in immunology. 2013;4:82.
30. Sabado RL, Bhardwaj N. Dendritic cell immunotherapy. Annals of the New York Academy of Sciences. 2013;1284(1):31-45.
31. Gerrits JH, Athanassopoulos P, Vaessen LM, Klepper M, Weimar W, van Besouw NM. Peripheral blood manipulation significantly affects the result of dendritic cell monitoring. Transplant immunology. 2007;17(3):169-77.
32. Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nature medicine. 2004;10(5):475-80.
33. Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature. 2007;449(7161):419-26.
34. Moretta L, Ferlazzo G, Bottino C, Vitale M, Pende D, Mingari MC, et al. Effector and regulatory events during natural killer-dendritic cell interactions. Immunological reviews. 2006;214:219-28.
35. Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunology today. 1999;20(12):561-7.
36. Tuyaerts S, Aerts JL, Corthals J, Neyns B, Heirman C, Breckpot K, et al. Current approaches in dendritic cell generation and future implications for cancer immunotherapy. Cancer immunology, immunotherapy : CII. 2007;56(10):1513-37.
37. Collins M, Ling V, Carreno BM. The B7 family of immune-regulatory ligands. Genome biology. 2005;6(6):223.
38. Croft M. The role of TNF superfamily members in T-cell function and diseases. Nature reviews Immunology. 2009;9(4):271-85.
39. Wing K, Yamaguchi T, Sakaguchi S. Cell-autonomous and -non-autonomous roles of CTLA-4 in immune regulation. Trends in immunology. 2011;32(9):428-33.

40. Anguille S, Smits EL, Cools N, Goossens H, Berneman ZN, Van Tendeloo VF. Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties. Journal of translational medicine. 2009;7:109.
41. Gilboa E. DC-based cancer vaccines. The Journal of clinical investigation. 2007;117(5):1195-203.
42. Massa C, Seliger B. Fast Dendritic Cells Stimulated with Alternative Maturation Mixtures Induce Polyfunctional and Long-Lasting Activation of Innate and Adaptive Effector Cells with Tumor-Killing Capabilities. J Immunol. 2013.
43. Jonuleit H, Kuhn U, Muller G, Steinbrink K, Paragnik L, Schmitt E, et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. European journal of immunology. 1997;27(12):3135-42.
44. Cilloni D, Messa F, Arruga F, Defilippi I, Gottardi E, Fava M, et al. Early prediction of treatment outcome in acute myeloid leukemia by measurement of WT1 transcript levels in peripheral blood samples collected after chemotherapy. Haematologica. 2008;93(6):921-4.
45. Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, Van Broeckhoven C, et al. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood. 2001;98(1):49-56.
46. National Library of Medicine [internet]. Rockville Pike, Bethesda, USA [updated June 3 2013; cited 2013 June 5]. Available from: http://www.clinicaltrials.gov/ct2/show/NCT01686334?term=acute+myeloid+leukemia+dendritic+cell+vaccine&rank=8.
47. Lee JJ, Kook H, Park MS, Nam JH, Choi BH, Song WH, et al. Immunotherapy using autologous monocyte-derived dendritic cells pulsed with leukemic cell lysates for acute myeloid leukemia relapse after autologous peripheral blood stem cell transplantation. Journal of clinical apheresis. 2004;19(2):66-70.
48. Li L, Giannopoulos K, Reinhardt P, Tabarkiewicz J, Schmitt A, Greiner J, et al. Immunotherapy for patients with acute myeloid leukemia using autologous dendritic cells generated from leukemic blasts. International journal of oncology. 2006;28(4):855-61.
49. Kitawaki T, Kadowaki N, Fukunaga K, Kasai Y, Maekawa T, Ohmori K, et al. A phase I/IIa clinical trial of immunotherapy for elderly patients with acute myeloid leukaemia using dendritic cells co-pulsed with WT1 peptide and zoledronate. British journal of haematology. 2011;153(6):796-9.
50. Roddie H, Klammer M, Thomas C, Thomson R, Atkinson A, Sproul A, et al. Phase I/II study of vaccination with dendritic-like leukaemia cells for the immunotherapy of acute myeloid leukaemia. British journal of haematology. 2006;133(2):152-7.
51. Van Driessche A, Van de Velde AL, Nijs G, Braeckman T, Stein B, De Vries JM, et al. Clinical-grade manufacturing of autologous mature mRNA-electroporated dendritic cells and safety testing in acute myeloid leukemia patients in a phase I dose-escalation clinical trial. Cytotherapy. 2009;11(5):653-68.
52. Jakobisiak M, Golab J, Lasek W. Interleukin 15 as a promising candidate for tumor immunotherapy. Cytokine & growth factor reviews. 2011;22(2):99-108.
53. Ben Ahmed M, Belhadj Hmida N, Moes N, Buyse S, Abdeladhim M, Louzir H, et al. IL-15 renders conventional lymphocytes resistant to suppressive functions of regulatory T cells through activation of the phosphatidylinositol 3-kinase pathway. J Immunol. 2009;182(11):6763-70.
54. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity. 2007;26(4):503-17.

55. Mortier E, Woo T, Advincula R, Gozalo S, Ma A. IL-15Ralpha chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. The Journal of experimental medicine. 2008;205(5):1213-25.
56. Harris KM. Monocytes differentiated with GM-CSF and IL-15 initiate Th17 and Th1 responses that are contact-dependent and mediated by IL-15. Journal of leukocyte biology. 2011;90(4):727-34.
57. Smits EL, Ponsaerts P, Berneman ZN, Van Tendeloo VF. The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. The oncologist. 2008;13(8):859-75.
58. Lion E, Anguille S, Berneman ZN, Smits EL, Van Tendeloo VF. Poly(I:C) enhances the susceptibility of leukemic cells to NK cell cytotoxicity and phagocytosis by DC. PloS one. 2011;6(6):e20952.
59. Schreibelt G, Tel J, Sliepen KH, Benitez-Ribas D, Figdor CG, Adema GJ, et al. Toll-like receptor expression and function in human dendritic cell subsets: implications for dendritic cell-based anti-cancer immunotherapy. Cancer immunology, immunotherapy : CII. 2010;59(10):1573-82.
60. Smits EL, Cools N, Lion E, Van Camp K, Ponsaerts P, Berneman ZN, et al. The Toll-like receptor 7/8 agonist resiquimod greatly increases the immunostimulatory capacity of human acute myeloid leukemia cells. Cancer immunology, immunotherapy : CII. 2010;59(1):35-46.
61. Burdek M, Spranger S, Wilde S, Frankenberger B, Schendel DJ, Geiger C. Three-day dendritic cells for vaccine development: antigen uptake, processing and presentation. Journal of translational medicine. 2010;8:90.
62. Anguille S, Lion E, Tel J, de Vries IJ, Coudere K, Fromm PD, et al. Interleukin-15-induced CD56(+) myeloid dendritic cells combine potent tumor antigen presentation with direct tumoricidal potential. PloS one. 2012;7(12):e51851.
63. Lion E, Smits EL, Berneman ZN, Van Tendeloo VF. NK cells: key to success of DC-based cancer vaccines? The oncologist. 2012;17(10):1256-70.
64. De Maria A, Bozzano F, Cantoni C, Moretta L. Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(2):728-32.
65. Wehner R, Dietze K, Bachmann M, Schmitz M. The bidirectional crosstalk between human dendritic cells and natural killer cells. Journal of innate immunity. 2011;3(3):258-63.
66. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends in immunology. 2001;22(11):633-40.
67. Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology. 2009;126(4):458-65.
68. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, et al. The trafficking of natural killer cells. Immunological reviews. 2007;220:169-82.
69. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood. 2010;115(11):2167-76.
70. Spits H, Lanier LL. Natural killer or dendritic: what's in a name? Immunity. 2007;26(1):11-6.
71. Zanoni I, Granucci F, Foti M, Ricciardi-Castagnoli P. Self-tolerance, dendritic cell (DC)-mediated activation and tissue distribution of natural killer (NK) cells. Immunology letters. 2007;110(1):6-17.
72. Benjamin JE, Gill S, Negrin RS. Biology and clinical effects of natural killer cells in allogeneic transplantation. Current opinion in oncology. 2010;22(2):130-7.

73. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nature immunology. 2008;9(5):503-10.
74. Raulet DH. Interplay of natural killer cells and their receptors with the adaptive immune response. Nature immunology. 2004;5(10):996-1002.
75. Yokoyama WM, Kim S. Licensing of natural killer cells by self-major histocompatibility complex class I. Immunological reviews. 2006;214:143-54.
76. Varchetta S, Oliviero B, Mavilio D, Mondelli MU. Different combinations of cytokines and activating receptor stimuli are required for human natural killer cell functional diversity. Cytokine. 2013.
77. Byrd A, Hoffmann SC, Jarahian M, Momburg F, Watzl C. Expression analysis of the ligands for the Natural Killer cell receptors NKp30 and NKp44. PloS one. 2007;2(12):e1339.
78. Girart MV, Fuertes MB, Domaica CI, Rossi LE, Zwirner NW. Engagement of TLR3, TLR7, and NKG2D regulate IFN-gamma secretion but not NKG2D-mediated cytotoxicity by human NK cells stimulated with suboptimal doses of IL-12. J Immunol. 2007;179(6):3472-9.
79. Kato N, Tanaka J, Sugita J, Toubai T, Miura Y, Ibata M, et al. Regulation of the expression of MHC class I-related chain A, B (MICA, MICB) via chromatin remodeling and its impact on the susceptibility of leukemic cells to the cytotoxicity of NKG2D-expressing cells. Leukemia. 2007;21(10):2103-8.
80. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: "l'union fait la force". Blood. 2005;106(7):2252-8.
81. Jinushi M, Takehara T, Kanto T, Tatsumi T, Groh V, Spies T, et al. Critical role of MHC class I-related chain A and B expression on IFN-alpha-stimulated dendritic cells in NK cell activation: impairment in chronic hepatitis C virus infection. J Immunol. 2003;170(3):1249-56.
82. Wai LE, Garcia JA, Martinez OM, Krams SM. Distinct roles for the NK cell-activating receptors in mediating interactions with dendritic cells and tumor cells. J Immunol. 2011;186(1):222-9.
83. Ferlazzo G, Pack M, Thomas D, Paludan C, Schmid D, Strowig T, et al. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(47):16606-11.
84. Ferlazzo G, Munz C. Dendritic cell interactions with NK cells from different tissues. Journal of clinical immunology. 2009;29(3):265-73.
85. Smits EL, Lee C, Hardwick N, Brooks S, Van Tendeloo VF, Orchard K, et al. Clinical evaluation of cellular immunotherapy in acute myeloid leukaemia. Cancer immunology, immunotherapy : CII. 2011;60(6):757-69.
86. Ruggeri L, Mancusi A, Burchielli E, Aversa F, Martelli MF, Velardi A. Natural killer cell alloreactivity in allogeneic hematopoietic transplantation. Current opinion in oncology. 2007;19(2):142-7.
87. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene. 2008;27(45):5932-43.
88. Costello RT, Fauriat C, Sivori S, Marcenaro E, Olive D. NK cells: innate immunity against hematological malignancies? Trends in immunology. 2004;25(6):328-33.
89. Dubsky P, Saito H, Leogier M, Dantin C, Connolly JE, Banchereau J, et al. IL-15-induced human DC efficiently prime melanoma-specific naive CD8+ T cells to differentiate into CTL. European journal of immunology. 2007;37(6):1678-90.
90. Hardy MY, Kassianos AJ, Vulink A, Wilkinson R, Jongbloed SL, Hart DN, et al. NK cells enhance the induction of CTL responses by IL-15 monocyte-derived dendritic cells. Immunology and cell biology. 2009;87(8):606-14.

91. Van Elssen CH, Vanderlocht J, Frings PW, Senden-Gijsbers BL, Schnijderberg MC, van Gelder M, et al. Klebsiella pneumoniae-triggered DC recruit human NK cells in a CCR5-dependent manner leading to increased CCL19-responsiveness and activation of NK cells. European journal of immunology. 2010;40(11):3138-49.
92. Vujanovic L, Szymkowski DE, Alber S, Watkins SC, Vujanovic NL, Butterfield LH. Virally infected and matured human dendritic cells activate natural killer cells via cooperative activity of plasma membrane-bound TNF and IL-15. Blood. 2010;116(4):575-83.
93. Rahman M. Introduction to Flow Cytometry. United Kingdom: MorphoSys UK Ltd; 2009.
94. Shapiro HM. Practical Flow Cytometry. fourth ed. New Jersey: John Wiley & Sons. Inc. ; 2003.
95. Vermes I, Haanen C, Reutelingsperger C. Flow cytometry of apoptotic cell death. Journal of immunological methods. 2000;243(1-2):167-90.
96. Overton WR. Modified histogram subtraction technique for analysis of flow cytometry data. Cytometry. 1988;9(6):619-26.
97. Caligiuri MA. Human natural killer cells. Blood. 2008;112(3):461-9.
98. Seidel UJ, Schlegel P, Lang P. Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies. Frontiers in immunology. 2013;4:76.
99. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nature medicine. 2004;10(9):909-15.
100. Vujanovic L, Ballard W, Thorne SH, Vujanovic NL, Butterfield LH. Adenovirus-engineered human dendritic cells induce natural killer cell chemotaxis via CXCL8/IL-8 and CXCL10/IP-10. Oncoimmunology. 2012;1(4):448-57.
101. Liu C, Lou Y, Lizee G, Qin H, Liu S, Rabinovich B, et al. Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice. The Journal of clinical investigation. 2008;118(3):1165-75.
102. Gil M, Park SJ, Chung YS, Park CS. Interleukin-15 enhances proliferation and chemokine secretion of human follicular dendritic cells. Immunology. 2010;130(4):536-44.
103. Saikh KU, Khan AS, Kissner T, Ulrich RG. IL-15-induced conversion of monocytes to mature dendritic cells. Clinical and experimental immunology. 2001;126(3):447-55.
104. Gustafsson K, Ingelsten M, Bergqvist L, Nystrom J, Andersson B, Karlsson-Parra A. Recruitment and activation of natural killer cells in vitro by a human dendritic cell vaccine. Cancer research. 2008;68(14):5965-71.
105. Siddiqui N, Hope J. Differential recruitment and activation of natural killer cell sub-populations by Mycobacterium bovis-infected dendritic cells. European journal of immunology. 2013;43(1):159-69.
106. Persson CM, Chambers BJ. Plasmacytoid dendritic cell-induced migration and activation of NK cells in vivo. European journal of immunology. 2010;40(8):2155-64.
107. Ahonen CL, Gibson SJ, Smith RM, Pederson LK, Lindh JM, Tomai MA, et al. Dendritic cell maturation and subsequent enhanced T-cell stimulation induced with the novel synthetic immune response modifier R-848. Cellular immunology. 1999;197(1):62-72.
108. Jensen SS, Gad M. Differential induction of inflammatory cytokines by dendritic cells treated with novel TLR-agonist and cytokine based cocktails: targeting dendritic cells in autoimmunity. J Inflamm (Lond). 2010;7:37.

109. Vitale M, Della Chiesa M, Carlomagno S, Romagnani C, Thiel A, Moretta L, et al. The small subset of CD56brightCD16- natural killer cells is selectively responsible for both cell proliferation and interferon-gamma production upon interaction with dendritic cells. European journal of immunology. 2004;34(6):1715-22.
110. Osada T, Clay T, Hobeika A, Lyerly HK, Morse MA. NK cell activation by dendritic cell vaccine: a mechanism of action for clinical activity. Cancer immunology, immunotherapy : CII. 2006;55(9):1122-31.
111. Bontkes HJ, Kramer D, Ruizendaal JJ, Meijer CJ, Hooijberg E. Tumor associated antigen and interleukin-12 mRNA transfected dendritic cells enhance effector function of natural killer cells and antigen specific T-cells. Clin Immunol. 2008;127(3):375-84.
112. Loza MJ, Perussia B. The IL-12 signature: NK cell terminal CD56+high stage and effector functions. J Immunol. 2004;172(1):88-96.
113. Marquez ME, Millet C, Stekman H, Conesa A, Deglesne PA, Toro F, et al. CD16 cross-linking induces increased expression of CD56 and production of IL-12 in peripheral NK cells. Cellular immunology. 2010;264(1):86-92.
114. Pende D, Spaggiari GM, Marcenaro S, Martini S, Rivera P, Capobianco A, et al. Analysis of the receptor-ligand interactions in the natural killer-mediated lysis of freshly isolated myeloid or lymphoblastic leukemias: evidence for the involvement of the Poliovirus receptor (CD155) and Nectin-2 (CD112). Blood. 2005;105(5):2066-73.
115. Fauriat C, Just-Landi S, Mallet F, Arnoulet C, Sainty D, Olive D, et al. Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. Blood. 2007;109(1):323-30.
116. Costello RT, Sivori S, Marcenaro E, Lafage-Pochitaloff M, Mozziconacci MJ, Reviron D, et al. Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. Blood. 2002;99(10):3661-7.
117. Boyiadzis M, Memon S, Carson J, Allen K, Szczepanski MJ, Vance BA, et al. Up-regulation of NK cell activating receptors following allogeneic hematopoietic stem cell transplantation under a lymphodepleting reduced intensity regimen is associated with elevated IL-15 levels. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2008;14(3):290-300.
118. Szczepanski MJ, Szajnik M, Welsh A, Whiteside TL, Boyiadzis M. Blast-derived microvesicles in sera from patients with acute myeloid leukemia suppress natural killer cell function via membrane-associated transforming growth factor-beta1. Haematologica. 2011;96(9):1302-9.
119. Mailliard RB, Son YI, Redlinger R, Coates PT, Giermasz A, Morel PA, et al. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. J Immunol. 2003;171(5):2366-73.
120. Lion E, Smits EL, Berneman ZN, Van Tendeloo VF. Quantification of IFN-gamma produced by human purified NK cells following tumor cell stimulation: comparison of three IFN-gamma assays. Journal of immunological methods. 2009;350(1-2):89-96.
121. Lion E, Smits EL, Berneman ZN, Van Tendeloo VF. Acute myeloid leukemic cell lines loaded with synthetic dsRNA trigger IFN-gamma secretion by human NK cells. Leukemia research. 2009;33(4):539-46.
122. Gerosa F, Gobbi A, Zorzi P, Burg S, Briere F, Carra G, et al. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. J Immunol. 2005;174(2):727-34.

123. Boullart AC, Aarntzen EH, Verdijk P, Jacobs JF, Schuurhuis DH, Benitez-Ribas D, et al. Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E2 results in high interleukin-12 production and cell migration. Cancer immunology, immunotherapy : CII. 2008;57(11):1589-97.
124. Muthuswamy R, Mueller-Berghaus J, Haberkorn U, Reinhart TA, Schadendorf D, Kalinski P. PGE(2) transiently enhances DC expression of CCR7 but inhibits the ability of DCs to produce CCL19 and attract naive T cells. Blood. 2010;116(9):1454-9.
125. Van Elssen CH, Vanderlocht J, Oth T, Senden-Gijsbers BL, Germeraad WT, Bos GM. Inflammation-restraining effects of prostaglandin E2 on natural killer-dendritic cell (NK-DC) interaction are imprinted during DC maturation. Blood. 2011;118(9):2473-82.
126. Roothans D, Smits E, Lion E, Tel J, Anguille S. CD56 marks human dendritic cell subsets with cytotoxic potential. Oncoimmunology. 2013;2(2):e23037.
127. Shi J, Ikeda K, Fujii N, Kondo E, Shinagawa K, Ishimaru F, et al. Activated human umbilical cord blood dendritic cells kill tumor cells without damaging normal hematological progenitor cells. Cancer science. 2005;96(2):127-33.
128. Manna PP, Mohanakumar T. Human dendritic cell mediated cytotoxicity against breast carcinoma cells in vitro. Journal of leukocyte biology. 2002;72(2):312-20.
129. Fernandez NC FC, Crépineau F, Angevin E, Vivier E, Zitvogel L. Dendritic cells (DC) promote natural killer (NK) cell functions: dynamics of the human DUNK cell cross talk. Eur Cytokine Netw. 2002;13(1):17-27.
130. Amakata Y, Fujiyama Y, Andoh A, Hodohara K, Bamba T. Mechanism of NK cell activation induced by coculture with dendritic cells derived from peripheral blood monocytes. Clinical and experimental immunology. 2001;124(2):214-22.
131. Pallandre JR, Krzewski K, Bedel R, Ryffel B, Caignard A, Rohrlich PS, et al. Dendritic cell and natural killer cell cross-talk: a pivotal role of CX3CL1 in NK cytoskeleton organization and activation. Blood. 2008;112(12):4420-4.
132. Pende D, Castriconi R, Romagnani P, Spaggiari GM, Marcenaro S, Dondero A, et al. Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: relevance for natural killer-dendritic cell interaction. Blood. 2006;107(5):2030-6.
133. Mortier E, Quemener A, Vusio P, Lorenzen I, Boublik Y, Grotzinger J, et al. Soluble interleukin-15 receptor alpha (IL-15R alpha)-sushi as a selective and potent agonist of IL-15 action through IL-15R beta/gamma. Hyperagonist IL-15 x IL-15R alpha fusion proteins. The Journal of biological chemistry. 2006;281(3):1612-9.
134. Harris KM. Monocytes differentiated with GM-CSF and IL-15 initiate Th17 and Th1 responses that are contact-dependent and mediated by IL-15. Journal of leukocyte biology. 2011;90(4):727-34.
135. Dubois S, Patel HJ, Zhang M, Waldmann TA, Muller JR. Preassociation of IL-15 with IL-15R alpha-IgG1-Fc enhances its activity on proliferation of NK and CD8+/CD44high T cells and its antitumor action. J Immunol. 2008;180(4):2099-106.
136. Waldmann TA, Dubois S, Tagaya Y. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. Immunity. 2001;14(2):105-10.