Design of tumour-specific immunotherapies using dendritic cells – analyses of IL15-DC

Rania Al-Mahdi

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University of Bergen
Institute for Biomedicin
Broegelmann Research Laboratory,
The Gade Institute

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To my husband, Mohammed, and sons, Nasr and Sam. Thank you for your unconditional love and support.

You made this possible.

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Summary

Immunotherapy of malignancies aims at activating the patient's own immune system to fight the tumour affecting the patient. Even though the use of dendritic cells (DC) has shown promising results, the DC vaccination strategy needs improvement, as only few relevant clinical responses could be documented so far. **Aim:** In this study, the standard protocol to generate monocyte derived DC using GM-CSF and IL-4 was compared to the use of GM-CSF and IL-15. **Methods:** Monocytes were isolated by plastic adherence from peripheral blood mononuclear cells and cultured for 6 days with GM-CSF and either IL-4 (IL4-DC) or IL-15 (IL15-DC). A fraction of the IL4-DC was stimulated with a cytokine cocktail, while a fraction of the IL15-DC was stimulated by adding TNF-α 24 hours before harvesting. The phenotypes of the four DC populations were determined using flow cytometry. Intracellular signalling pathways were investigated using phospho-specific antibodies in a Western blot. IL-12 production was analysed in an ELISA. Migratory capacity was determined in a chemotaxis assay. **Results:** Monocytes cultured with GM-CSF and IL-15 developed a DC-like morphology. Phenotypic analyses revealed that both IL4-DC and IL15-DC had down-regulated CD14 expression and up-regulated CD1a expression, although IL15-DC to a lesser extent. IL15-DC showed an enhanced surface expression of co-stimulatory molecules CD80 and CD86 whereas no difference was observed in surface expression of MHC class II and CD40. Upon stimulation, an up-regulation of the maturation marker CD83 and CD86 were observed on IL4-DC only. The IL15-DC had more phosphorylated JNK and ERK than IL4-DC whereas the phosphorylation level of p38 in both IL4-DC and IL15-DC were approximately the same. None of the cell populations produced IL-12 or showed chemotaxis towards CCL19. **Conclusions:** The generation of IL15-DC turned out to be more problematic compared to the generation of IL4-DC. The stimulatory activity of IL-15 on T-cell proliferation resulted in a high degree of contamination with T -cells in the IL15-DC cultures as I did not have a pure monocyte population to start with. This problem might be overcome by using either alternative monocyte isolation protocols or by reducing the culture period from 5-6 to 3-4 days. Because of the variation in the results of the experiments the data needs to be confirmed with this approach.

Abbreviations

Abbreviation	*Name*	*Abbreviation*	*Name*
Ag	Antigen	LC	Langerhans cells
APC	Antigen presenting cells	LPS	Lipopolysaccharide
APS	Ammonium persulfate	mAb	Monoclonal antibody
BSA	Bovine serum albumin	MHC	Major histocompatibility complex
CFDA SE	Carboxyfluorescein diacetate succinimidyl ester	MLR	Mixed leukocyte reaction
CFSE	Carboxyfluorescein succinimidyl ester	NAC	Non adherent cells
CTL	Cytotoxic T-lymphocytes	NK	Natural killer
DC	Dendritic cells	NKT	Natural killer T-cells
dH_2O	Distilled water	PAMP	Pathogen associated molecular patterns
DMSO	Dimethyl sulfoxide	RT	Room temperature
EDTA	Ethylenediaminetetraacetic acid	SDS	Sodium dodecyl Sulfate
FBS	Fetal bovine serum	SSC	Side scatter channel
FSC	Forward scatter channel	TCR	T-cell receptors
GM-CSF	Granulocyte macrophage colony stimulating factor	T_H cells	T helper cells
IFN-γ	Interferon gamma	TLR	Toll-like receptors
IL	Interleukin	TNF	Tumour necrosis factor
IL-1β	Interleukin 1 beta	TSLP	Thymic stromal lymphopoietin

1. Introduction

1.1 Immune system

The human body is always exposed to pathogenic infectious agents and, yet, the body usually can resist the invasion of these agents. It is our immune system that protects us against these infectious agents. The immune system has two main branches, innate or non-specific immune system and adaptive or specific immune system. Vertebrates are the only organism having an adaptive immune system. The main function of the immune system is to distinguish between self and non-self antigen, which is a vital step in protecting our body from invading microbes or/and elimination of altered cells, e.g. tumour cells.

1.1.1 Innate immunity

Innate immunity is the first line of the host immune defence. In fact, innate immunity can eliminate and prevent infections of the host as well as initiate adaptive immune responses. The innate immunity mechanism acts immediately and mounts a response against the antigen. However, the innate immune system does not generate lasting protective immunity (1).

The principle components of the innate immunity are (i) the anatomic barrier, e.g. epithelia of the internal and external surfaces of the body, (ii) blood proteins, e.g. the complement system, (iii) cellular barrier, e.g. the phagocytes that can engulf and digest invading microorganisms, and (iv) cytokines which are proteins, peptides or glycoproteins. If the innate immunity for one reason or another fails to get rid of the infection, the response of the adaptive immune system takes place.

The innate immunity lacks specificity, but recognises foreign pathogens as it identifies structures that are common to groups of related microbes. Therefore, cells of the innate immune system use a variety of pathogen-associated molecular patterns (PAMP) recognition receptors to recognise the patterns shared between pathogens. One of the major classes of the pattern recognition receptors are the Toll-like receptors (TLR).

The innate immunity also does not induce immunological memory and therefore each time the innate immune system acts against microbes as it would the first time.
The principle effector cells of the innate immunity are neutrophils, macrophages, endothelial cells, dendritic cells and natural killer cells.

1.1.2 Adaptive immunity

The adaptive immunity is characterised by having the so-called immunological memory. This is a distinctive feature of the specific immune system and is defined as the ability to remember an encounter with a foreign antigen for a period of time or whole lifetime. This can help fight re-infection with the same microorganism. Another important feature of the adaptive immune system is its specificity as it has a diverse repertoire of rearranged receptors (2). The adaptive immune system depends on innate immune cells that are able to present antigens and this function is mainly controlled by antigen-presenting cells (APC). There are three different main types of professional APC, namely macrophages, B-cells and dendritic cells. The main cells of the adaptive immune system are T- and B-lymphocytes. T-lymphocytes arise from bone marrow and migrate to and mature in thymus, and they are the mediator of cellular immunity. On the other hand, B-lymphocytes arise and have their early stage of maturation in bone marrow and are the mediator of humoral immunity. B-lymphocytes are the immune cells that produce antibodies.
After naïve T- and B-lymphocytes emerge from the thymus and bone marrow, respectively, they migrate into the secondary lymphoid organs where they can be activated by different antigens presented to them and thereafter proliferate and differentiate into effector and memory cells. The effector cells include $CD4^+$ helper T-cells, $CD8^+$ cytotoxic T-cells and antibody-secreting B-cells.

1.1.3 Dendritic cells

Dendritic cells (DC) are the most potent antigen presenting cells that possess the ability to stimulate naïve T-cells. The DC capture antigens and process the antigens into peptides as they mature during their journey from the peripheral tissue to the lymphatic organs where the T-lymphocytes can recognise these peptides on the major histocompatibility complex (MHC) molecules which is important for the initiation of the immune response (3, 4). DC are critical for the induction of T-cell responses resulting in cell-mediated immunity. The term DC comes from the shape and motility that DC exhibit. These cells are mobile, stellate in shape (Figure. 1.1) and these features facilitate the cells' function (5).

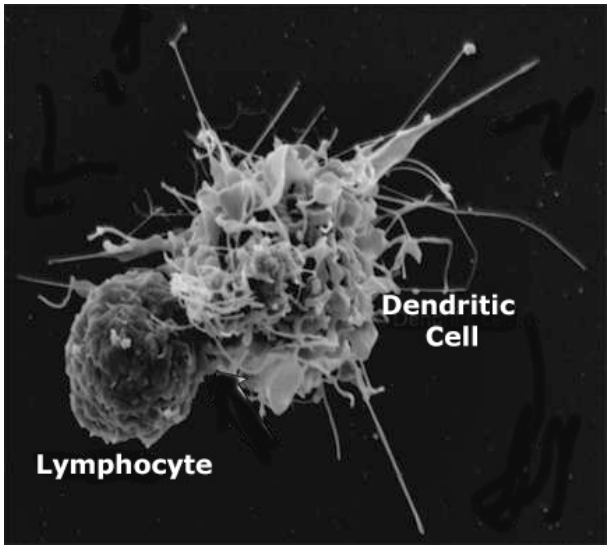

Figure 1.1. Dendritic cell presenting antigen to a lymphocyte. The process involves intimate contact between the two cells in which DC projections might facilitate this function. Lymphocyte is stimulated by DC in order to proliferate and differentiate into effector cell. From**:** www.bcrfund.org/ images/dendriticcellb.jpg (accessed 5th April 2009)

Few DC are required to stimulate the response of T-lymphocytes; it is estimated that one DC is able to stimulate 100-3000 T-lymphocytes (5).

1.1.3.1 DC subsets

The different DC subsets are presented herein for just simplification of the issue, although, in real situation the picture is very complicated and a clear distinction is not always the case. DC precursors develop in the bone marrow and then home to a large variety of tissues. DC are present almost everywhere in the body, but mainly they reside in the peripheral tissue, the secondary lymphoid organs, and circulating in blood (6). All DC subsets originate from haematopoietic stem cells (HSC). DC are divided into subsets according to certain surface markers, these subsets have common features i.e. the DC morphology, expression of high amount of MHC class II and co-stimulatory molecules as well as T-cell stimulation (7). Recent studies have characterised the DC subsets as belonging to the myeloid or lymphoid lineage (8). The myeloid DC contain subtypes including Langerhans cells (LC), which are located in the epidermis, interstitial DC, monocyte-derived DC and blood DC. The blood DC contain two subsets, mDC1 that is characterised by BDCA1 (CD1c) expression and mDC2 which are positive for BDCA3 (CD141) (9). The myeloid precursors also give rise to monocytes which in turn can differentiate into DC (10). Monocytes are not a major source of DC *in vivo* in the steady state. However, during infection the monocytes can differentiate into DC and they are called monocyte-derived DC. Finally, plasmacytoid DC are derived from the lymphoid lineage (8). These cells are remarkable in their ability to secrete large amounts of type I interferon (11).

1.1.3.2 Antigen capturing, processing and presentation by DC

DC are the sentinels of immune system and they are the link of the innate immunity to the adaptive immunity. The immature DC are present in the peripheral tissues where they can capture antigens, e.g. bacteria and virus. Immature DC use several pathways in order to capture antigens. These pathways are (i) macropinocytosis in which the cell membrane forms a pocket and pinches off into the cell to form a vesicle that travels into the cytosol and fuses with either endosomes or lysosomes and releases its content, (ii) receptor-mediated endocytosis through C-type lectin receptors or Fcγ receptor type I and II (12, 13), and (iii) phagocytosis in which the cell changes its

shape by sending out projections which are called pseudopodia. These pseudopodia will surround the antigen and form an intracellular vesicle.

When DC undergo the maturation process, the DC morphology will start to change. For example, the expression of receptors that are responsible for the phagocytic and endocytic process will be down-regulated (13). In addition, the co-stimulatory molecules CD40, CD80 and CD86 will be up-regulated, the MHC II compartment will start to change and the MHC class II-peptide complex will be translocated to the cell surface (14). Moreover, chemokine receptors, e.g. CCR7 that regulate DC trafficking will be up-regulated. Furthermore, the change of DC morphology will be associated with the secretion of chemokines that are responsible for the attraction of different effectors of the immune system as well as the secretion of cytokines such as IL-12 (15, 16). These maturation markers and the secretion of chemokines and cytokines will allow the optimal interaction between the DC and the T-cells.

There are several factors that play a role in the induction and maturation of DC. These include (i) inflammatory cytokines e.g. tumour necrosis factor (TNF-α), interleukin (IL)-1, IL-6 and prostaglandin E_2 (PGE_2), (ii) pathogen-related molecules e.g. bacterial DNA, lipopolysaccharide (LPS) and double stranded RNA, (iii) antigen-antibody complexes and (iv) the ligation of CD40 on DC by its ligand CD40L (13, 17).

The antigen that is captured by the immature DC will be processed and presented on the MHC molecules. Intracellular antigen that is in the cytosol of DC will bind to the MHC class I molecules and be presented to the $CD8^+$ cytotoxic T-lymphocytes (CTL). However, antigens that have been captured and internalized into the endocytic pathway will bind to MHC class II molecules and subsequently be presented to the $CD4^+$T helper cells (T_H). This in turn give help to other cells such as B-lymphocytes to start responding against these antigens (5, 18).

When the DC present a peptide to a T-cell, the latter will induce a response called cellular immune response and that include both $CD4^+T_H$ cells and $CD8^+$ CTL (19). The T-cells need to recognise three signals in order to proliferate and differentiate into T_H cells and CTL. The first signal in this process is the binding of MHC- peptide complexes to the T-cell receptors (TCR) and the second signal is the binding of the appropriate co-stimulatory receptor CD28 on the T-cells to the co-stimulatory ligands

CD80 or CD86 on the APC. A third signal delivered from the APC will determine the differentiation of T-cells into effector cells, e.g. the secretion of IL-12 from APC will differentiate the $CD4^+$ T-cells into T_H1 effector cells (Figure 1.2). The DC is not restricted to T-lymphocytes but can also activate B-lymphocytes (20, 21), natural killer (NK) cells (22), and natural killer T (NKT) cells (23).

The immune system should recognise dangerous foreign antigens but should also be able to discriminate between self and non-self antigens to avoid autoimmunity. The DC are of critical importance in inducing T-cell tolerance. As it was mentioned above the T-cells need three signals in order to be activated, and if only signal 1 (binding of MHC- peptide complexes to the TCR) is delivered, this will cause T-cell inactivation leading to tolerance. Thus, DC have a great role in the contrast states of immunity and tolerance (5, 13).

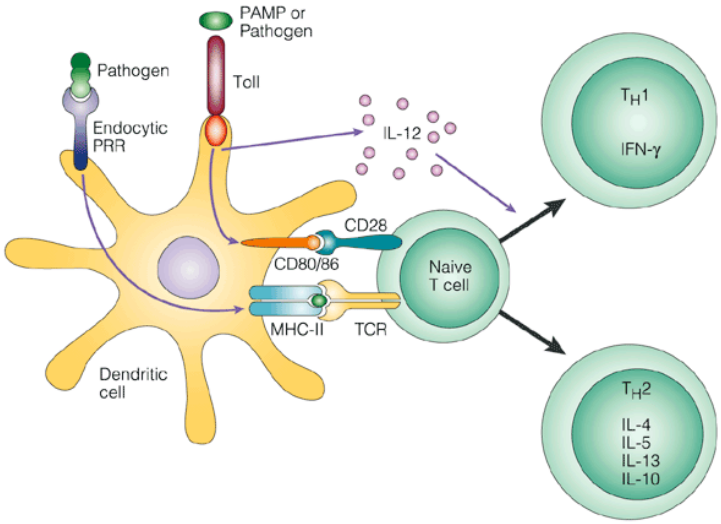

Figure 1.2. Three signals should be delivered from the DC in order to differentiate the T-cells into effector cells. Toll-like receptors (TLR) sense the presence of infection through recognition of PAMP (pathogen-associated molecular patterns). Recognition of PAMP by TLR expressed on DC will lead to the up-regulation of the MHC II molecules that present the antigenic peptide to the T-cells (signal 1) and the up-regulation of the co-stimulatory molecules CD80 and CD86 (signal 2). TLR also induce expression of cytokines, such as IL-12 (signal 3), and trigger many other events associated with DC maturation. All of the three signals lead to the activation of T-cells and the secretion of IL-12 contributes to the differentiation of activated T-cells into T helper (T_H)1 effector cells. From: Ruslan Medzhitov, 2001 (24).

1.1.3.3 Ex-vivo generation of DC

There are several sources from which we can generate DC *ex vivo*, e.g. from $CD34^+$ haematopoietic progenitors or from monocytes isolated from blood. It is known that the combination of the cytokines granulocyte macrophage colony-stimulating factor (GM-CSF) with IL-4 will allow the differentiation of monocytes into DC (IL4-DC) and it is a standard protocol of *ex-vivo* generation of DC. In order to induce the IL4-DC maturation, a cytokine cocktail composed of IL-6, TNF-α, IL-1β, and PGE_2 is one of the acknowledged protocols and is regarded as a gold standard. (25). Finding the DC subset(s) that can make the optimal vaccines is a very critical step in order to improve DC-based vaccines. Much work has been done trying to test different cytokines or stimuli to the monocyte cultures in order to influence their differentiation *ex vivo* into DC with different phenotypes. For example, adding GM-CSF with IL-15 to the monocytes will skew the differentiation of the monocytes into IL15-DC (Figure 1.3) (26). Moreover, in the course of monocyte activation with, for example, GM-CSF, the monocytes can differentiate into different types of DC according to the different cytokines that will be added such as TNF (TNF-DC) (27), IFN-α (IFN-α-DC) (28) and thymic stromal lymphopoietin (TSLP-DC) (29) (Figure 1.3).

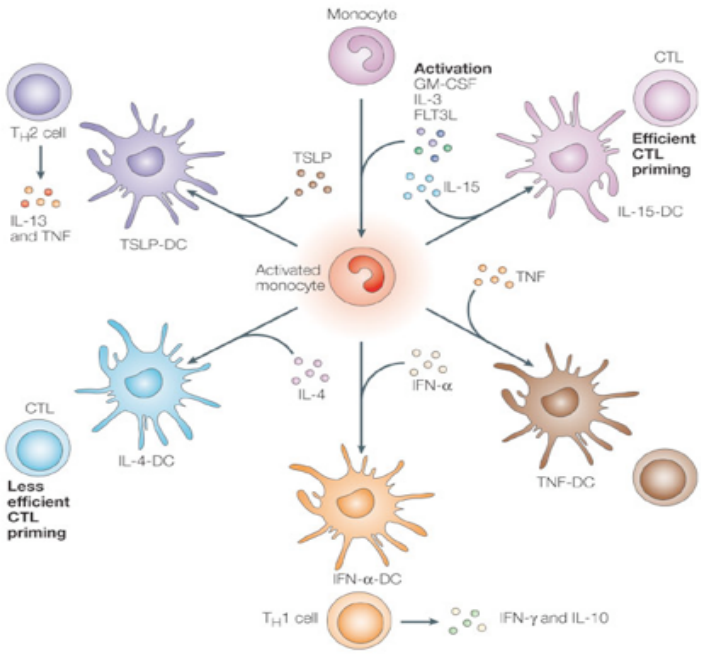

Figure 1.3. Subtypes of monocyte-derived DC. Activated monocytes with e.g. either GM-CSF, IL-3 or FLT3L can differentiate into different DC subtypes after encounter with different cytokines such as IL-4 (IL4-DC), IL-15 (IL15-DC), TNF (TNF-DC), TSLP (TSLP-DC) and IFN-α (IFN-α-DC). These DC subtypes will differentiate the lymphocytes into immune effectors with different functions, leading to varied immune responses. From: Banchereau and Palucka, 2005 (30).

1.1.4 Tumour immunology

Cancer is a group of diseases that are characterised by the unregulated and uncontrolled cell proliferation. Altered cells arise on a regular basis but a vigilant immune system can eliminate them before they become harmful. Cells that evade immunosurveillance will develop into tumours, benign or malignant. Tumours vary in their immunogenicity, and even tumours with antigens that can be recognised by the host immune system can evade elimination. Cancer cells can use many mechanisms in order to evade T-cell responses, either to avoid being recognised by T-cells or trying to disable the effector T-cells (31). Moreover, the tumour may not display any danger signals that can activate the DC or other immune system components that be capable of acting against the tumour.

An example of how the tumour cells avoid being recognised by T-cells, is the absence of MHC class I expression due to the mutations of the β2-microglobulin gene, or the down-regulation of MHC class I expression (32, 33). It was shown that many tumours progressed due to their suppression of the DC functions by IL-10, transforming growth factor-β (TGF-β) or vascular endothelial growth factor (VEGF) (34, 35). Another example is the signal transducer and activator of transcription (STAT) 3 that is activated in several tumour cases leading to the inhibition of DC maturation (36). Furthermore, the tumour microenvironment can induce immune tolerance as IL-10 is able to convert the DC function toward the induction of T-cell anergy (37).

1.1.5 Immunotherapy

Immunotherapy seeks to harness and enhance the patients' own immune system against diseases including cancer. It is known that both chemotherapy and

radiotherapy usually lead to undesirable side effects and most of these side effects are the consequence of the cell damage brought by the chemotherapy agents or irradiation. These side effects constitute serious drawbacks of chemotherapy and radiotherapy. Initial studies using DC-based vaccinations have proven its safety and shown that it is well tolerated by patients. Certain cytokines and antibodies can be used as immunotherapy as well as vaccines. Transferring the immune effectors cells (T-cells) or proteins (antibodies) is called passive immunization, whereas active immunization is the ability to stimulate the patient's own immune effector cells, e.g. DC-based vaccination (17). Vaccines intend to induce specific, non-toxic and long-lasting immune responses to prevent the infections and/or ameliorate the symptoms of the diseases vaccinated against (7). The same principle can be applied to vaccines against cancer. In the following section only immunotherapy concerning cancer will be discussed.

Many studies were conducted and others are still going on after it had been shown in murine studies that the immune system can recognise and elicit potent anti-tumour immunity (38, 39). It is documented that *ex-vivo* generated DC that had been loaded with tumour antigens in mouse have the ability to induce protective (prophylactic vaccines) and therapeutic (therapeutic vaccines) anti-tumour immunity (40). It is shown also that the *ex-vivo* generated DC have the ability to induce therapeutic anti-tumour immunity in humans (17). For the purpose of vaccination, the *ex-vivo*-generated DC can be loaded or pulsed with specific tumour antigen(s) and then reintroduced back into the patient in order to stimulate the patient's own immune system (Figure 1.4).

To improve DC-based immunotherapy there are questions that need to be addressed. These are; the type of DC, the type of antigen(s) and their formulation, and the type of DC-activation signal as well as the route of injection (17). It was observed that distinct DC subsets would induce distinct types of immune response.

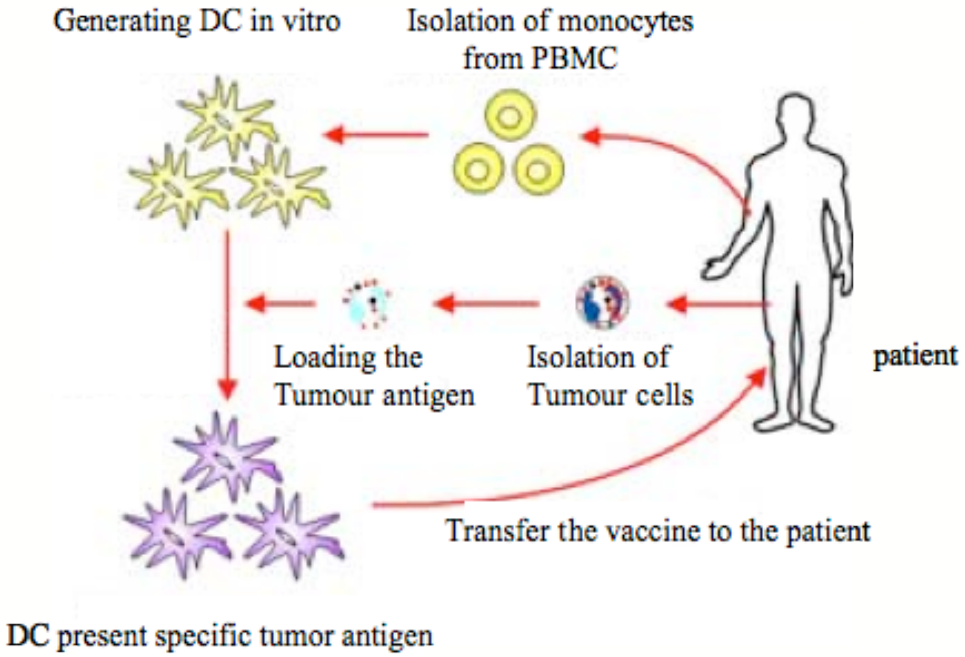

Figure 1.4. The principles of immunotherapy using DC-based vaccination. DC are generated from monocytes that were isolated from peripheral blood mononuclear cells. DC will then be loaded with specific tumour antigen followed by their injection to the patient in order to activate effector T-cells that can eliminate tumour cells. Modified from**:** www.ehealthandhealing.com/html/cancervaccines/cancervaccines.html. (accessed 20th April 2009)

The route of injection is another important parameter that is unsolved and it is still unclear which route is optimal. Many injection routes have been tested, e.g. skin injections such as subcutaneous and intradermal injections, intravenous injections, injections into lymph nodes and intratumoural injections (41, 42). However, it has been shown that the subcutaneous injection is much less effective than intradermal and intranodal injections (43).

It is known that a competent immune response is one that fulfils the following criteria (i) the ability to produce effector cells that are multifunctional, (ii) induce a broad range of effector cells that are specific for several types of tumour antigens, (iii) inhibit the tumour antigen-specific regulatory T-cell function and (iv) generate a tumour antigen-specific immune memory (17).

DC-based vaccine taking into clinical trials and early studies showed that vaccines

using DC that were generated *ex vivo* from blood precursors result in a specific immune response in treating patients having tumour (44, 45). Recently, a phase III clinical trial also demonstrated a survival benefit in patients with metastatic hormone-refractory prostate cancer treated with DC-based immunotherapy (46). In another randomized phase III trail, autologous peptide-loaded DC vaccination had to be prematurely closed. It was concluded that the DC-based vaccination was not more effective than DTIC chemotherapy in stage IV melanoma patients. However, they came out with future suggestions that might help for more effective vaccination (47).
It has been observed that the outcomes of DC-based vaccination are variable. In many cases, a tumour-specific immune response was detected although the clinical benefit was limited (48, 49).
Furthermore, an important factor that should be considered during the design of the DC-based vaccination is the ability of DC for migration. It was shown that less than 1% of intradermally injected DC migrated rapidly to the regional lymphatics (50). In order to overcome this problem, previous studies tried to use different activation signals, e.g. PGE_2 that can induce CCR7 expression; therefore increase the ability of DC to respond to CCL19 and CCL21 (51, 52). It is still a challenge to design vaccines that induce the optimal effective immune system by using DC-based vaccination, as the optimal type of DC that can be used for vaccination is not determined yet.

1.2 Aim of the study

Immunotherapy of malignancies aims at activating the patient's own immune system to fight the tumour affecting the patient. Even though the use of DC has shown promising results, the DC vaccination strategy needs improvement, as only few relevant clinical responses could be documented so far. In this study, the standard protocol to generate monocyte-derived DC using GM-CSF and IL-4 was compared to the use of GM-CSF and IL-15, which is thought to be better at inducing antigen-specific cytotoxic T-lymphocyte differentiation *in vivo*.

2. Material

2.1 Media and buffers

Name	*Composition*
10x TBS	0.1 M Tris pH 8.0
	1.4 M NaCl
1x Blotting buffer pH 8.3	Running buffer without SDS
	20% Methanol
1x Running buffer	0.1% SDS
	25 mM Tris
	192 mM Glycine
1x TBST (0.5%)	1 x TBS
	0.5% Tween20
6x Lämmli buffer	375mM Tris HCl pH 6,8
	9 % SDS
	50% Glycerol
	9% β- Mercaptoethanol
	0,03% Bromphenolblue
Blocking buffer	5% skimmed milk
	1x TBST (0.5%)
FACS buffer	0.5% BSA in PBS
PBS pH7.4	137mM NaCl
	2.7mM KCl
	8.1mM Na_2HPO_4
	1.5mM KH_2PO_4
RIPA buffer	50 mM Tris pH7.4
	1% NP40
	0.25 % Sodium deoxycholate
	150 mM NaCl
	1mM EDTA
	freshly added before use:

	1X proteinase inhibitor complete ®
	1mM PMSF
	1mM Na-orthovanadate
	1mM NaF
RP10	RPMI 1640 with ultraglutamine 1
	10% FBS
	50 units/ml Penicillin G sodium
	50 μg/ml streptomycin sulfate
Stripping buffer	2% SDS
	62.5 mM Tris/HCL pH 6.7
	100 mM β- Mercaptoethanol
Washing buffer	0.05% Tween-20 in PBS

2.2 Cell culture plastic

Name	*Company*
6-well plate	Nunc, Denmark
75cm^2 flask	Nunc, Denmark
96-well plate	Nunc, Denmark

2.3 Softwares

Name	*Company*
Flowjo	Tree Star, Inc.
Quantity one	Bio-Rad, Hercules, California

2.4 Antibodies

2.4.1 FACS antibodies

Name	*Clone*	*Company*
CD14 FITC	UCHM1	AbD Serotec, Oxford, UK
CD1a PE	NA1/34-HLK	AbD Serotec, Oxford, UK
CD38 APC	AT13/5	AbD Serotec, Oxford, UK
CD4 APC	MEM-241	Immunotools, Germany
CD40 FITC	LOB7/6	AbD Serotec, Oxford, UK
CD8 PE	LT8	AbD Serotec, Oxford, UK
CD80 APC	MEM-233	Immunotools, Germany
CD83 PE	HB15e	AbD Serotec, Oxford, UK
CD86 FITC	BU63	AbD Serotec, Oxford, UK
CCR7 PE	150503	R&D system, USA
HLA-DR APC	HL-39	AbD Serotec, Oxford, UK

2.4.2 Western blot antibodies

Name	*Company*
AKT (Pan)(C67E7), Rabbit mAb	Cell signalling, USA
P38 MAP Kinase antibody	
P44/42 MAP Kinase, (137F5), Rabbit mAb	
phosho-AKT, (Ser473)(D9E), Rabbit mAb	
phosho-p38 MAPK, (Thr180/Tyr182), (3D7) Rabbit mAb	
Phospho-p44/42 MAP Kinase, (Thr202/tyr204), Rabbit mAb	
phospho- SAPK/JNK, (Thr183/Tyr185) (81E11), Rabbit mAb	
SAPK/JNK, (56G8), Rabbit mAb	
Goat anti-rabbit (HRP)	Bio-Rad, Hercules, California

2.5 Reagents

Name	*Company*
0.5M Tris-HCL buffer pH6.8	Bio-Rad, Hercules, California
1.5M Tris-HCL buffer pH8.8	Bio-Rad, Hercules, California
30%Acrylamide/Bis solution	Bio-Rad, Hercules, California
25x Proteinase inhibitor,complete EDTA free	Roche, Germany
Albumin, bovine serum (BSA**)**	Sigma, USA
Ammonium persulfate (APS)	Bio-Rad, Hercules, California
β-Mercaptoethanol	Sigma, USA
CCL19	Immunotools, Germany
Dimethyl sulfoxide (DMSO)	Sigma, UK
Ethylenediaminetetraacetic acid (EDTA) 0.5M	Sigma, USA
Glycine (Electrophoresis Purity Reagent)	Bio-Rad, Hercules, California
GM-CSF	Immunotools, Germany
IL-1β	Strathmann Biotec, Germany
IL-15	Immunotools, Germany
IL-4	Immunotools, Germany
IL-6	Immunotools, Germany
Isopropanol prima	Arcus, Oslo, Norway
Ionomycin	Sigma Aldrich, USA
Lymphoprep	Axis- shield poc AS, Norway
Pencillin/ strepotomycin	Invitrogen, USA
PGE_2	Sigma Aldrich, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich, USA
Ponceau S	Sigma Aldrich, USA
Precision plus protein kaleidoscope standards	Bio-Rad, Hercules, California
RPMI with Ultraglutamine 1	BioWhttaker, Lonza, Belgium
Sodium dodecyl Sulfate (SDS)	Bio-Rad, Hercules, California
TNF-α	Immunotools, Germany
Tris (Electrophoresis Purity Reagent)	Bio-Rad, Hercules, California
Tween20	Merck, Germany

2.6 Equipment

Name	*Company*
Cabinet	Nuair, biological safety cabinets
Cell counter CASY®	Schärfe System GmbH, Germany
Centrifuges	- KUBOTA 8700, Tokyo, Japan - Thermo, Heraeus multifuge 1S-R, USA - Thermo, Heraeus Fresco 17, USA
ChemiDoc	Bio-Rad, Hercules, California
Flowcytometer, BDFACS Canto I	BD Biosciences, USA
Incubator	Forma Scientific, USA
Microscope	Leica, Germany
Mini-PROTEAN 3 cell	Bio-Rad, Hercules, California
Mini-Trans-Blot Electrophoric Transfer Cell	Bio-Rad, Hercules, California
Nitrocellulose trans-blot membrane (0.2μm 7x10 cm)	Bio-Rad, Hercules, California
Transwell® permeable supports 8 μm pore size	Corning, NY, USA
Water bath	GFL, Germany

2. 7 Kits

Name	*Company*
BCA protein Assay Kit	PIERCE, USA
BD Compbeads	BD Biosciences, USA
CFSE (Vybrant™ CFDA SE, cell Tracer kit)	Invitrogen, USA
ELISA IL-12p70	BioLegend, USA
Immuno-Star WesternC Chemiluminescent kit	Bio-Rad, Hercules, California

Materials that are not listed were either purchased from Bio-Rad, Sigma or Merck.

3. Methods

3.1 Isolation of peripheral blood mononuclear cells (PBMC) from buffy coat

Buffy coat was diluted ~1:4 with phosphate buffered saline pH7.4 (PBS) at room temperature (RT). Approximately 33ml of the diluted buffy coat was carefully layered on top of 12ml lymphoprep. They were then centrifuged at 800g with no brake for 30 minutes at 22°C. The PBMC were recovered from the plasma/lymphoprep interface and transferred into 50ml tubes. The PBMC were washed three times with cold PBS at 400g for 5 minutes at 4°C, suspended in RP10 medium and counted.

3.2 Ex-vivo generation of different dendritic cell (DC) populations

Dendritic cell generation started with the plating of $1x10^8$ PBMC in a $75cm^2$ cell culture flask. Monocytes were isolated from PBMC by plastic adherence during the one-hour incubation at 37°C, 5% CO_2 in humidified atmosphere while the lymphocytes remained floating. The non-adherent cells (NAC) were then transferred into a 50ml tube. The attached monocytes were washed with PBS (RT) 2-3 times until all floating cells were removed. To differentiate the monocytes into different DC populations, monocytes were cultured in new RP10 medium, 100 ng/ml of GM-CSF and either 20 ng/ml of IL-4 or 200 ng/ml of IL-15 to differentiate into IL4-DC or IL15-DC, respectively. The cells were then incubated for 5-6 days in a humidified atmosphere, 5% CO_2, at 37°C. The cytokines were replenished every 2-3 days. 24 hours before harvesting the cells a fraction of IL4-DC was matured by adding a cytokine cocktail consisting of IL-6 (1×10^3 IU/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml) and PGE_2 (1μg/ml) (CYTO-DC), whereas a fraction of IL15-DC was stimulated by 10 ng/ml of TNF-α (IL15+ TNFα-DC).

3.3 Freezing the cells

To freeze the non-adherent cells, 90% of FBS and 10% of dimethyl sulfoxide (DMSO) were added to the cells. The solution should be aliquoted within 2-3 minutes, once the cells had come in contact with the DMSO. Approximately $5x10^7$ cells/ml were frozen. The cell suspension was then transferred into cryo tubes which were placed into an isopropanol containing freezing container. The isopropanol containing freezing container was then stored at -80°C. This provides a relatively constant rate of freezing (-1°C \ min), thus improving viability.

3.4 Cell counting

Cell counting was done by using an automated cell counter (CASY), in which the cell suspension was diluted in casytone buffer. In addition, cell counting was done manually under the microscope using a Neubauer chamber, and the cell suspension was diluted in PBS and trypan blue.

3.5 Harvesting cells

After the incubation of monocytes for 6 days with the appropriate cytokines, the cells had developed into IL4-DC, IL15-DC, mature IL4-DC (CYTO-DC) and IL15+ TNF-α-DC. To harvest each population, the cell suspension was transferred into 50ml tubes and centrifuged at 400g for 5 minutes at 4°C. Immediately PBS with 2mM EDTA was added to the flasks to harvest the remaining adherent cells. After centrifugation, the supernatant was transferred into cryo tubes to be stored at -20°C and the remaining cells in the PBS with 2mM EDTA were transferred to the obtained pellet. The tube was then centrifuged at 400g for 5 minutes at 4°C. The supernatant was discarded; PBS was added twice and centrifuged at 400g for 5 minutes at 4°C for the washing steps. DC were then counted.

3.6 Immunostaining for Flow cytometry

To analyse the phenotype of the dendritic cell subsets, all DC populations were immunostained and analysed by flow cytometry.
For each staining, $1x10^5$ cells were resuspended in 50µl FACS buffer and 2µl of FcR block per $1x10^5$ cells was added to block the Fc receptors. The surface markers that were detected by specific antibodies were: CD14, CD1a, HLA-DR, CD80, CD86, CD83, CD40, CCR7 and CD38. The IgG isotype controls included were labelled with FITC, PE and APC, and were used as a negative control. Compensation beads were used for the compensation control. The cells were then incubated in the dark for 10 minutes at RT. After incubation the cells were washed twice with FACS buffer at 400g for 5 min at 4°C. Finally, the cells were resuspended in 175µl of FACS buffer and transferred into FACS tubes and analysed on a FACS Canto flow cytometer within one hour.

3.7 Chemotaxis

During maturation of DC, some surface markers are up-regulated. CCR7 is a chemokine receptor up-regulated upon DC maturation that is a prerequisite for DC migration towards the CC-chemokine ligand CCL19. We tested the ability of the different DC subsets to migrate towards CCL19 by adding $5x10^4$ DC to a 8-µm pore-size transwell plate in 80µl of RP10 medium. Each DC subset was tested against CCL19, as well as against the RP10 medium alone. The lower compartment therefore contained 235µl of RP10 either with or without 100 ng/ml CCL19, respectively. The cells were incubated for 18 hours in a humidified atmosphere, 5% CO_2 at 37°C. The migrated cells were then harvested from the lower chamber and counted using a CASY cell counter.

3.8 Mixed leukocyte reaction (MLR)

3.8.1 Thawing and harvesting non-adherent cells (NAC)

The NAC vial was thawed in 37°C water bath until a small lump of ice remained. NAC were added to 9ml warm RP10 and centrifuged at 400g, 5 minutes at room temperature before being washed again in RP10. The pellet was then dissolved in 5ml warm RP10 and transferred into a 25cm^2 cell culture flask. The NAC were incubated at 37°C, 5% CO_2 humidify atmosphere over night. NAC were harvested into a 50ml tube and centrifuged at 400g for 5 minutes at room temperature. The cells were resuspended in 10ml RP10 medium and counted.

3.8.2 Labelling the NAC with Carboxyfluorescein succinimidyl ester (CFSE)

The NAC were resuspend in warm PBS/0.1% BSA at a final concentration of 1x10^6 NAC / ml and 1µl of a 10µM stock carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) solution was added. The cells were then incubated for 10 minutes at 37°C. The CFDA SE will be converted by the cell into CFSE. The cells retain the CFSE in the cytoplasm and with each cell division the fluorescence intensity of the CFSE will decrease by half.
To quench the reaction, 5 times of ice-cold RP10 medium was added before the cells were incubated on ice for 5 minutes. The labelled NAC were washed 3 times at 400 g for 5 minutes at 4°C with 20ml RP10. The labelled NAC were then counted. They were resuspended at a concentration of 2x10^6/ml RP10 medium.

3.8.3 Co-culture of DC and allogenic NAC

After harvesting, each DC population was added into a 96-well cell-culture plate in duplicate i.e. 8 wells in total. Each well contained 5x10^4 DC in 100µl RP10 medium. 2x10^5 of allogenic CFSE labelled NAC were added only to one well of each DC subset. DC without NAC were used as a negative control to detect proliferation of

lymphocytes that originated from the DC culture. Moreover, three wells of labelled NAC were prepared as a negative control to detect lymphocytes proliferation that was independent from allogenic DC co-culture. In addition three wells with unlabelled NAC were prepared to be used later for compensation in the FACS analysis. Furthermore, one well of labelled NAC was stimulated with Phorbol 12-myristate 13-acetate (PMA; 25ng) and Ionomycin (1μg) as a positive control. RP10 medium was added to a final volume of 200μl in each well. The NAC and DC were co-cultured for 6 days. The cells were then harvested, washed and stained with CD4 APC and CD8 PE antibodies. Thereafter, the cells were analysed by flow cytometry as described in 3.6.

3.9 Protein lysis

After harvesting, the DC were washed once with PBS. The supernatants then were discarded; RIPA (Radio-Immunoprecipitation Assay) buffer was added to the cells (approximately 100μl of RIPA buffer per million cells). The protein lysates were then incubated on ice for 5-10 minutes. After the incubation the protein lysates were centrifuged at 17000g for 5 minutes at 4°C. Each supernatant was transferred into a new tube and a 5μl aliquot was taken for the BCA protein assay to determine the protein concentration. Both tubes were stored at -80°C until used in further analysis.

3.10 BCA Protein Assay

To determine the protein concentration of the protein lysates, we used the bicinchoninic acid (BCA) protein assay. The standard was prepared by making a two-fold serial dilution of 1.5 mg/ml bovine serum albumin (BSA) into seven 1.5-ml tubes, and using the RIPA buffer as a blank. 10μl of each standard were transferred into a 96-well plate in duplicates, and 10μl of RIPA buffer were transferred in duplicate as a blank.

The protein lysate of each DC population was diluted 1:5 with PBS. Thereafter, 10μl from each sample were transferred into the 96-well plate in duplicate.

50 parts of BCA reagent A was mixed with 1 part of BCA reagent B to prepare the working reagent. After that, 200µl of the working reagent was added to each well of the standards and samples. It was then incubated at 37°C for 30 minutes. Finally, the absorbance was measured at 595nm on a plate reader and the sample's protein concentration was calculated based on the standard.

3.11 SDS-PAGE

The main idea of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is to separate the proteins according to their size. Using SDS will denature the proteins as well as it will apply a negative charge in proportion to their mass. The proteins will move then through the gel according to their sizes toward the positive pole after an electrical field is applied.

3.11.1 Gel preparation

The gel was consisting of two types of gels, the resolving gel and the stacking gel.
To make 2 minigels of resolving gel, 10ml of 12% resolving gel was prepared as follows:

dH_2O	3.4ml
30% Acrylamide/Bis	4.0ml
1.5M Tris-HCL pH 8.8	2.5ml
10% SDS	100µl
10% APS	50µl
TEMED	5µl

The solution was mixed well and was transferred to each glass cassette. The resolving gel was then layered with isopropanol to even the surface. The gel surface was rinsed after its polymerisation. The polymerisation took approximately 15-30 minutes.
For the two minigels of stacking gel, 5ml of 5% stacking gel was prepared as follows:

dH_2O	2.85ml
30% Acrylamide/Bis	0.85ml
0.5M Tris-HCL pH 6.8	1.25ml
10% SDS	50µl
10% APS	25µl
TEMED	5µl

The stacking gel solution was poured on top of the resolving gel and the comb was inserted carefully. The gel was left to polymerise between 15-30 minutes.

3.11.2 Sample loading

The samples were prepared by adding 6x Lämmli buffer to 20µg of protein lysate of each DC population and denaturing them at 100°C for 5 minutes. The marker and the samples were then loaded into the wells of the gel. The electrophoresis was run at 150V for approximately 1:15 hours or until the blue running front had left the gel.

3.12 Western blot

After the proteins were separated by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane. The proteins were transferred from the gel to the nitrocellulose membrane with a current of 250mA for one hour. The nitrocellulose membrane was then washed with 1xTBST (0.5%) and the proteins were visualised by incubating the membrane in a Ponceau S solution. The membrane was washed with 1xTBST (0.5%) until we got rid of the Ponceau S solution colour. The nitrocellulose membrane was then soaked in the blocking buffer for one hour to block the non-specific binding of proteins. The proteins were then detected one by one using specific primary antibody and secondary enzyme labelled antibody. The proteins that were detected are: AKT, ERK, JNK, P38 and their phosphorylated forms. All the primary antibodies were diluted 1:1000 in 5% BSA in 1xTBST (0.5%). The incubation time was for the phospho-proteins over night at 4°C. For the non-phospho

proteins, it was one hour at room temperature. After incubation, the nitrocellulose membrane was washed 5 minutes with 1xTBST (0.5%), 4 times. The secondary antibody goat anti-rabbit HRP was diluted 1:1000, added and incubated for 1 hour. The nitrocellulose membrane was then washed 5 minutes with 1xTBST (0.5%) and it was repeated for 4 times. The fifth time of washing was with 1xTBS. For the detection of proteins, Immuno-Star WesternC Chemiluminescent kit was used and the proteins were then analysed by ChemiDoc. The band intensity was measured for both the phospho and non-phospho forms of each protein that I detected in the four DC populations. CYTO-DC bands for both phospho and non-phospho forms of the proteins were set to 100%. The ratio between the phospho proteins and the non-phospho proteins was then calculated. The ratio of the phospho to the non-phospho protein of CYTO-DC was therefore 1. Other DC populations were then compared to CYTO-DC.

3.12.1 Stripping of the membrane

The antibodies were removed from the membrane by stripping in order to allow detection of other proteins of interest with similar size. The membrane was incubated 30 minutes in stripping buffer at 60°C with shaking followed by 5 times of 5 minutes each time of washing with 1xTBST (0.5%). The membrane was then blocked for 1 hour before proceeding with the detection of another protein on the membrane.

3.13 Enzyme-linked immunosorbent assay (ELISA)

The amount of the cytokine IL-12p70 production for each DC population was analysed using a commercially available ELISA. A monoclonal anti-human IL-12p70 antibody was first coated on a 96-well plate and incubated overnight at 2-8°C and the plate was then washed with the washing buffer 0.05% Tween-20 in PBS for 4 times. For blocking, Assay Diluent was added, and incubated at room temperature for 1 hour, after that the plate was washed with washing buffer for 4 times. The standard was prepared by making six two-fold serial dilutions of a 1000 pg/ml human recombinant

IL-12p70 solution. The conditioned medium from CYTO-DC, IL15-DC and IL15+TNF-α-DC were diluted 1:100. The standard, the diluted and undiluted samples were then added to the appropriate wells in duplicates and incubated at room temperature for 2 hours, followed by 4 times washing with washing buffer. The detection antibody biotinylated anti-human IL-12 p40/p70 was added to the wells and incubated at room temperature for 1 hour, followed by 4 times washing with washing buffer. Avidin-horseradish peroxidase (HRP) conjugate was added to the wells and incubated for 30 minutes. The plate was then washed 5 times, 1 minute per wash, with washing buffer. After that 3,3',5,5' - tetramethylbenzidine (TMB) substrate solution was added to each well, and incubated in the dark for 15 minutes to produce a blue colour in proportion to the amount of IL-12 p70 that is present in the samples. Finally the stop solution 1M H_2SO_4 was added to each well to change the reaction colour from blue to yellow and the absorbance was read at 450nm.

4. Results

4.1 IL15-DC have the morphology of DC

In order to see the influence of IL-15 on the differentiation of monocytes into DC and compare such effect to the standard protocol using IL-4, the monocytes were cultured in RP10 medium supplemented with GM-CSF together with either IL-4 (IL4-DC) or IL-15 (IL15-DC). A fraction of the IL4-DC was incubated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 for 24 hours (CYTO-DC). A fraction of IL15-DC was stimulated with TNF-α for 24 hours (IL15+TNFα-DC). All four cell populations had the morphology of DC after 6 days of culturing. However, the size of IL15-DC was smaller than IL4-DC and that was detected using the automated cell counter CASY and flow cytometry (Figure 4.1).

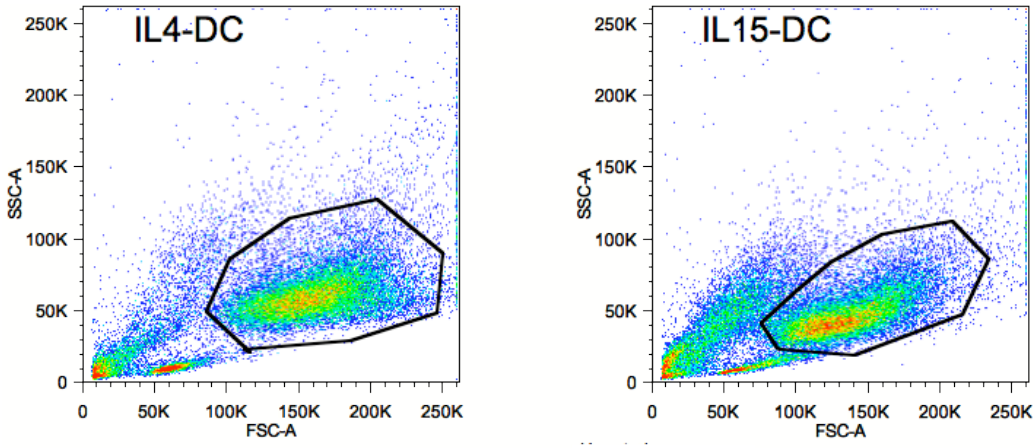

Figure 4.1. IL15-DC have a smaller size than IL4-DC. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) or IL-15 (IL15-DC) for 6 days. The figure shows the forward scatter channel (FSC) and the side scatter channel (SSC) of the IL4-DC (left panel) and the IL15-DC (right panel). The FSC of the IL15-DC showed that the IL15-DC cells are smaller than the IL4-DC cells. Both IL15-DC and IL4-DC had the same FSC and SSC setting. The data of one representative experiment is shown. DC were gated using FSC/SSC.

4.2 IL15-DC express less DC marker CD1a than IL4-DC

IL15-DC expressed approximately the same level of the monocyte/macrophage surface marker CD14 as IL4-DC. However, the DC surface maker CD1a was expressed more in the IL4-DC. CYTO-DC expressed less CD14 but more CD1a comparing to IL15-DC. The stimulated IL15-DC with TNF-α expressed approximately the same levels of both CD14 and CD1a as IL15-DC (Figure 4.2).

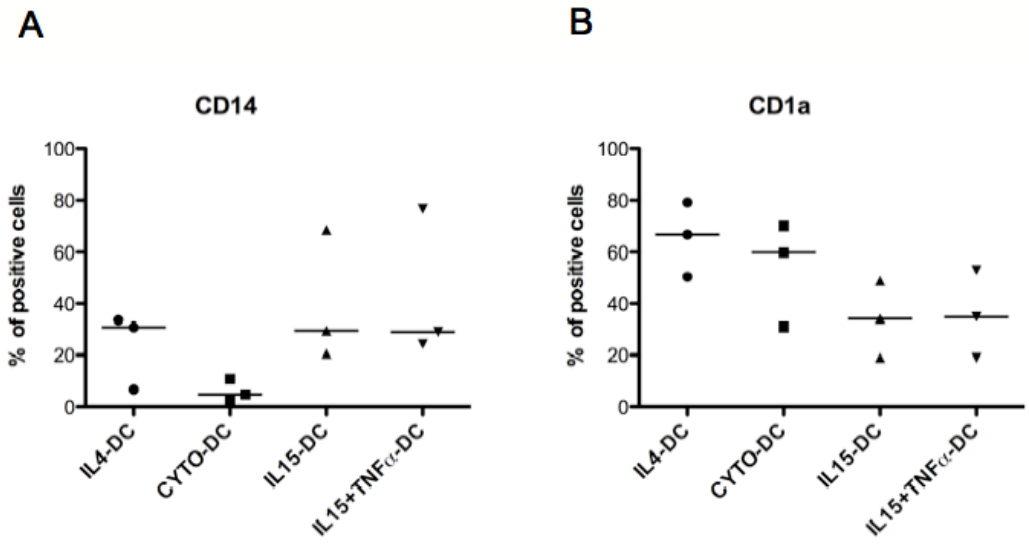

Figure 4.2. IL15-DC express less DC marker CD1a than IL4-DC. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days, and 24 hours before harvesting, a fraction of IL4-DC was stimulated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 (CYTO-DC). Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days and a fraction of IL15-DC was stimulated for 24 hours with TNF-α. The figure shows 3 independent experiments and the median is indicated by a bar. Flow cytometry was used to determine the percentage of cells that expressed CD14 (A) and CD1a (B). IL15-DC expressed similar level of monocyte/macrophage marker CD14 and less DC marker CD1a compared to IL4-DC. CYTO-DC expressed less CD14 but more CD1a compared to IL15-DC. The stimulated IL15-DC with TNF-α expressed approximately the same levels of both CD14 and CD1a as IL15-DC.

4.3 IL15-DC express more maturation markers than IL4-DC

The DC maturation markers CD83 and HLA-DR were expressed more on IL15-DC than IL4-DC. The CYTO-DC expressed higher levels of CD83 and HLA-DR than the other populations. The stimulated IL15-DC with TNF-α expressed similar levels of CD83 and HLA-DR as IL15-DC (Figure 4.3). However, the median fluorescence intensity (MFI) of HLA-DR was higher on IL15-DC than the CYTO-DC and IL4-DC (Figure 4.4).

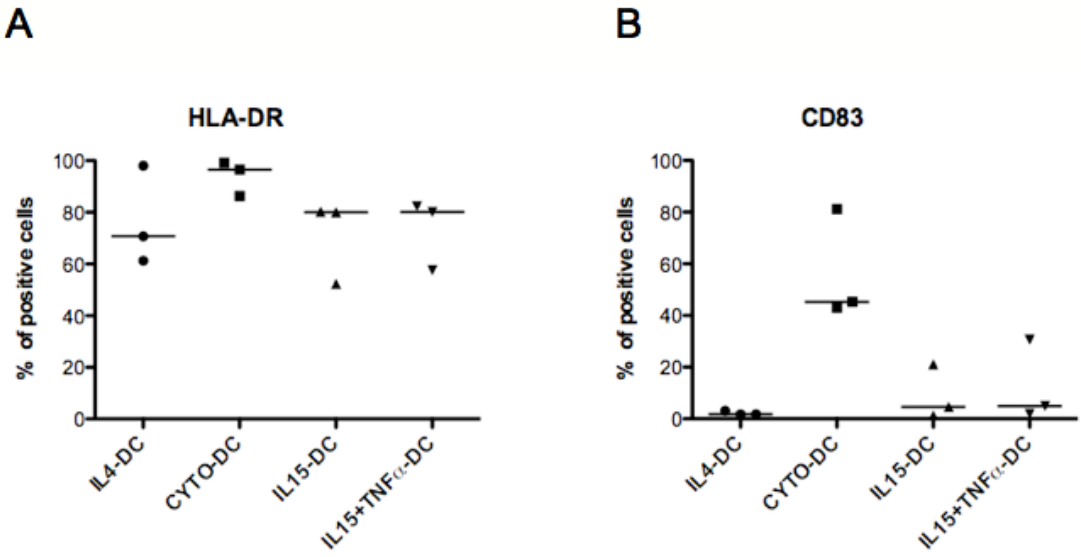

Figure 4.3. IL15-DC express more maturation markers than IL4-DC. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days, and 24 hours before harvesting, a fraction of IL4-DC was stimulated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 (CYTO-DC). Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days and a fraction of IL15-DC was stimulated for 24 hours with TNF-α. The figure shows 3 independent experiments and the median is indicated by a bar. Flow cytometry was used to determine the percentage of cells that expressed HLA-DR (A) and CD83 (B). IL15-DC expressed more maturation surface markers HLA-DR and CD83 than IL4-DC. CYTO-DC showed more expression of the maturation surface markers HLA-DR and CD83 than the other populations. The stimulated IL15-DC with TNF-α expressed similar levels of HLA-DR and CD83 as IL15-DC.

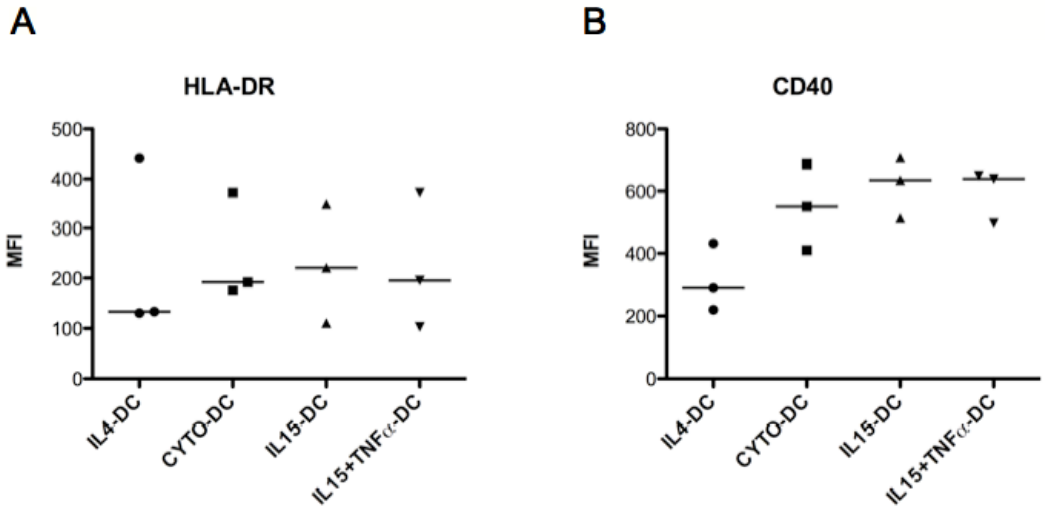

Figure 4.4. The median fluorescence intensities of CD40 and HLA-DR are higher for IL15-DC than IL4-DC and CYTO-DC. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days, and 24 hours before harvesting, a fraction of IL4-DC was stimulated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 (CYTO-DC). Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days and a fraction of IL15-DC was stimulated for 24 hours with TNF-α. The figure shows 3 independent experiments and the median is indicated by a bar. Flow cytometry was used to determine the median fluorescence intensity (MFI) of HLA-DR (A) and CD40 (B). The MFI of HLA-DR and CD40 were higher on the IL15-DC than both the IL4-DC and the CYTO-DC. The MFI of CD40 and HLA-DR on the stimulated IL15-DC with TNF-α was approximately the same as IL15-DC.

The co-stimulatory molecules CD86 and CD80 were expressed more on IL15-DC than IL4-DC. The CYTO-DC expressed higher level of CD86, whereas CD80 was expressed more on IL15-DC. Adding TNF-α to the IL15-DC resulted in the up-regulation of CD86. The IL15-DC expressed more CD80 than the stimulated IL15-DC with TNF-α (Figure 4.5). All DC populations expressed approximately the same level of the co-stimulatory molecule CD40 (Figure 4.5), but the MFI of CD40 was higher on IL15-DC than both IL4-DC and CYTO-DC (Figure 4.4). The MFI of CD40 on the stimulated IL15-DC with TNF-α was approximately the same as on IL15-DC (Figure 4.4).

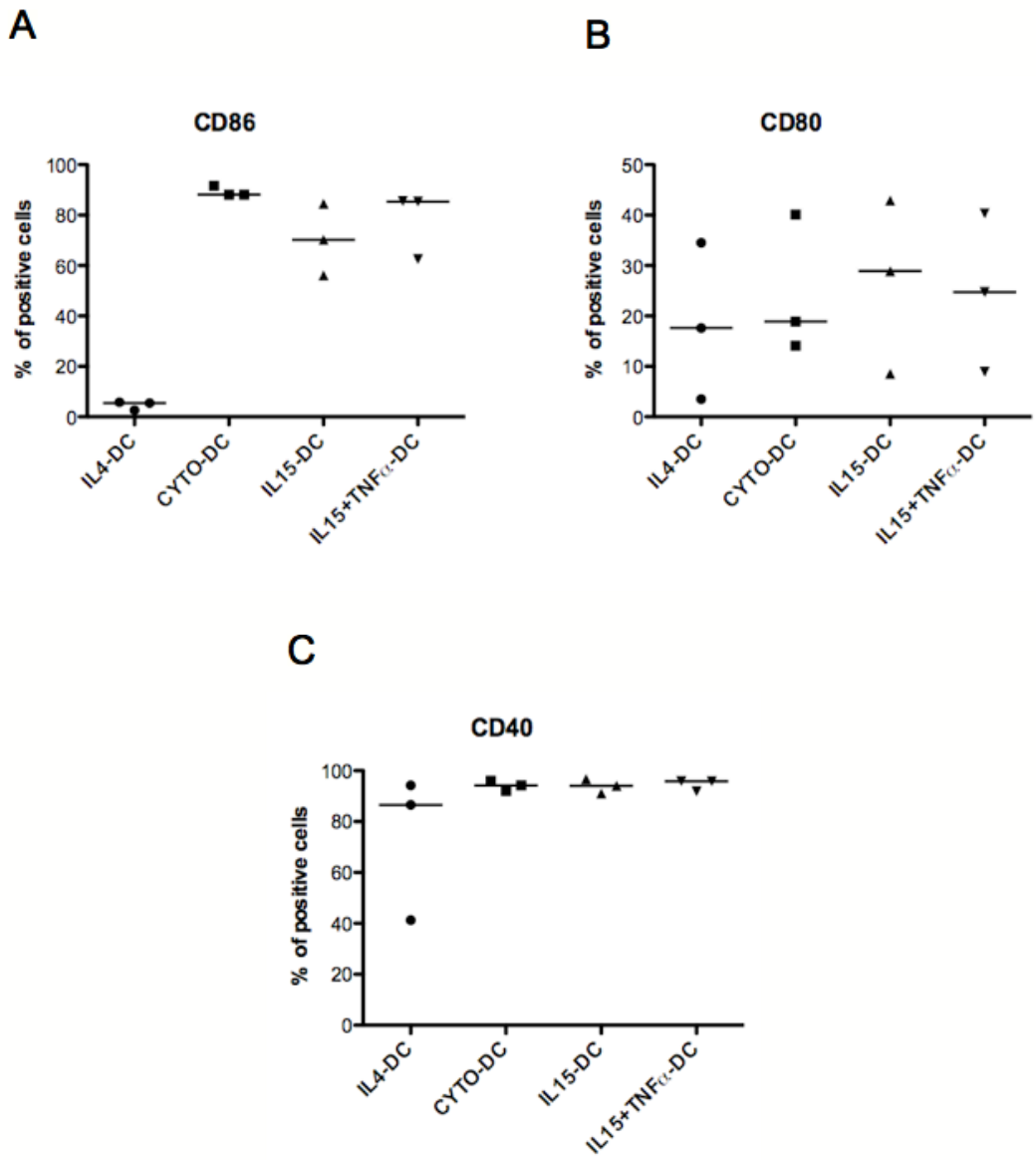

Figure 4.5. IL15-DC express more CD80 whereas CYTO-DC express more CD86. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days, and 24 hours before harvesting, a fraction of IL4-DC was stimulated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 (CYTO-DC). Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days and a fraction of IL15-DC was stimulated for 24 hours with TNF-α. The figure shows 3 independent experiments and the median is indicated by a bar. Flow cytometry was used to determine the percentage of cells that expressed CD86 (A), CD80 (B) and CD40 (C). CYTO-DC expressed the highest level of the co-stimulatory molecule CD86. IL15-DC expressed more CD80 than IL4-DC and CYTO-DC. The stimulated IL15-DC with TNF-α showed more CD86 and less CD80 expressions than IL15-DC. All DC populations showed approximately the same expression of the co-stimulatory molecule CD40.

4.4 CYTO-DC express more CCR7 whereas IL15-DC express more CD38

The migration marker CCR7 was expressed more on the CYTO-DC than the other DC populations. IL15-DC expressed approximately the same level of CCR7 as the un-stimulated IL4-DC (Figure 4.6). IL15-DC showed the highest expression of the migration marker CD38. The addition of cytokine cocktail to the IL4-DC resulted in the down-regulation of CD38. The stimulated IL15-DC with TNF-α expressed approximately similar levels of CCR7 and CD38 as IL15-DC (Figure 4.6).

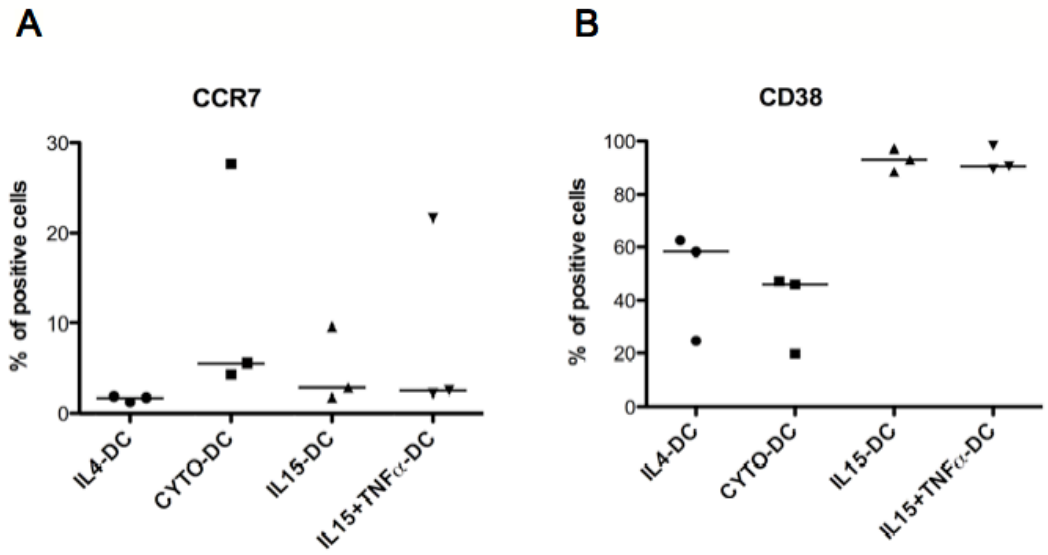

Figure 4.6. CYTO-DC express more CCR7 whereas IL15-DC express more CD38. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days, and 24 hours before harvesting, a fraction of IL4-DC was stimulated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 (CYTO-DC). Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days and a fraction of IL15-DC was stimulated for 24 hours with TNF-α. The figure shows 3 independent experiments and the median is indicated by a bar. Flow cytometry was used to determine the percentage of cells that expressed CCR7 (A) and CD38 (B). CYTO-DC showed the highest expression of CCR7, and both IL15-DC and IL4-DC had approximately the same level of CCR7 expression. IL15-DC showed the highest expression of CD38. Stimulated IL4-DC with cytokine cocktail showed down-regulation of CD38. The stimulated IL15-DC with TNF-α expressed approximately similar levels of CCR7 and the CD38 as IL15-DC.

4.5 Analyses of MAPK and AKT signalling pathways

The intracellular MAPK signalling pathways involved in DC maturation were investigated using phospho-specific antibodies in a Western blot. The stimulated IL15-DC with TNF-α had more phosphorylated ERK 42/44 (P-ERK) than the other DC populations. However, IL15-DC had similar levels of P-ERK as CYTO-DC. IL4-DC showed less P-ERK than all DC populations (Figure 4.7). All DC populations had approximately the same ratio of phosphorylated p-38 (P-p38) to p38 (Figure 4.8).

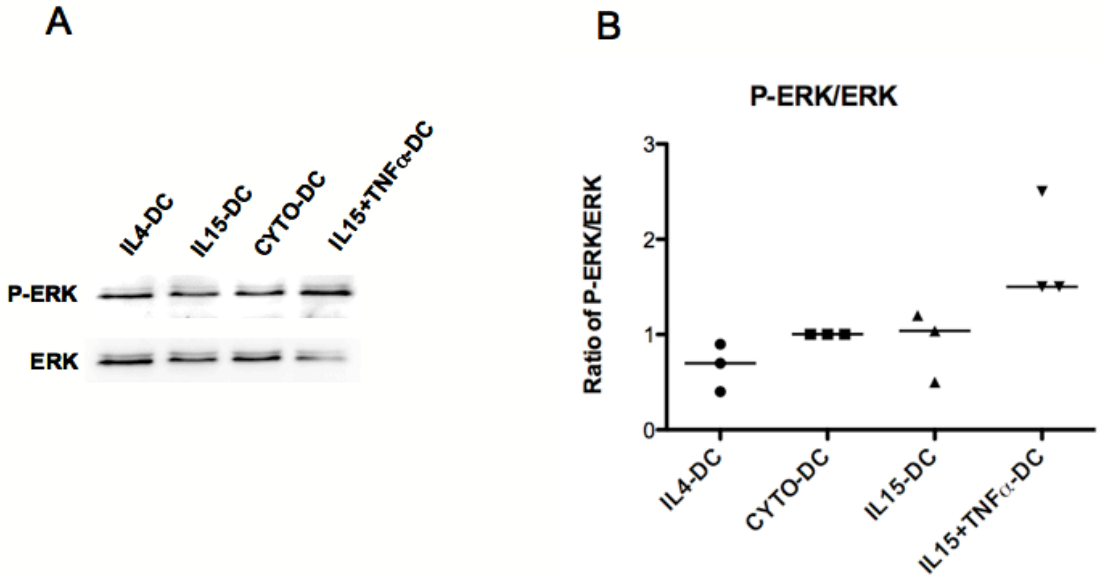

Figure 4.7. IL15-DC shows a similar level of P-ERK like CYTO-DC. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days, and 24 hours before harvesting, a fraction of IL4-DC was stimulated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 (CYTO-DC). Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days and a fraction of IL15-DC was stimulated for 24 hours with TNF-α. The phosphorylation of the protein kinase ERK42/44 was analysed using Western blot. (A) shows representative bands of ERK and P-ERK for each DC population. (B) shows the results of 3 independent experiments and the median is indicated by a bar. The intensity of the bands was measured and CYTO-DC was set to 100%. The ratio of the phospho to the non-phospho protein was then calculated. The ratio of the phospho to the non-phospho ERK of CYTO-DC is therefore 1. IL4-DC, IL15-DC and stimulated IL15-DC with TNF-α were compared to CYTO-DC. IL15-DC showed similar levels of P-ERK as CYTO-DC. IL4-DC had less P-ERK than all DC populations. The stimulated IL15-DC with TNF-α had the highest level of P-ERK.

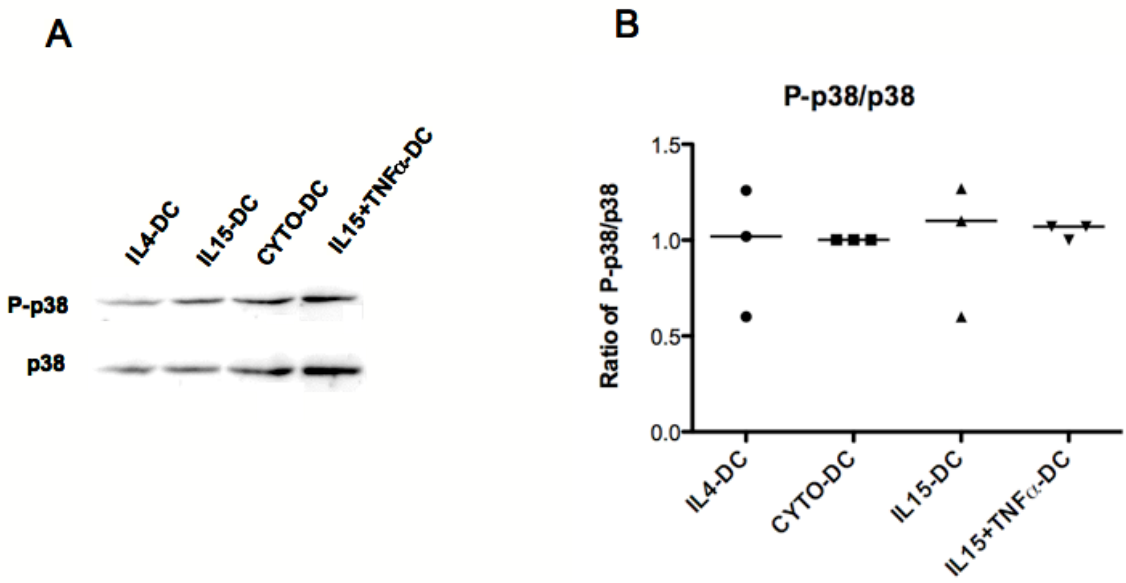

Figure 4.8. All DC populations have approximately the same ratio of phosphorylated p-38 to p38. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days, and 24 hours before harvesting, a fraction of IL4-DC was stimulated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 (CYTO-DC). Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days and a fraction of IL15-DC was stimulated for 24 hours with TNF-α. The phosphorylation of the protein kinase p38 was analysed using Western blot. (A) shows representative bands of p38 and P-p38 for each DC population. (B) shows the results of 3 independent experiments and the median is indicated by a bar. The intensity of the bands was measured and CYTO-DC was set to 100%. The ratio of the phospho to the non-phospho protein was then calculated. The ratio of the phospho to the non-phospho p38 of CYTO-DC is therefore 1. IL4-DC, IL15-DC and stimulated IL15-DC with TNF-α were compared to CYTO-DC.

The CYTO-DC had more P-JNK 46/54 than the other DC populations. IL15-DC had more P-JNK 46/54 than the IL4-DC. However, the stimulated IL15-DC with TNF-α had less P-JNK 46/54 than IL15-DC (Figure 4.9). The AKT signalling pathway was also investigated using Western blot. However, phosphorylated AKT protein kinase was not detected in any of the DC populations (data not shown).

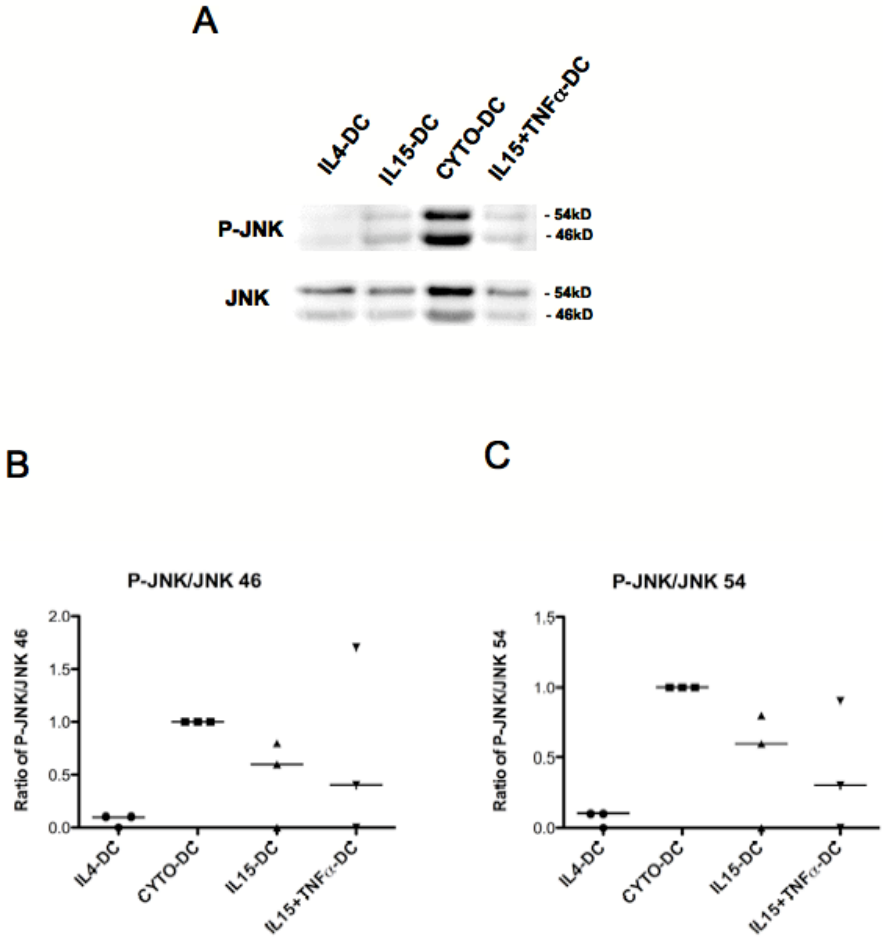

Figure 4.9. CYTO-DC have the highest level of phosphorylated JNK 46/54 (P-JNK). Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days, and 24 hours before harvesting, a fraction of IL4-DC was stimulated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 (CYTO-DC). Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days and a fraction of IL15-DC was stimulated for 24 hours with TNF-α. The phosphorylation of the protein kinase JNK 46/54 was analysed using Western blot. (A) shows representative bands of JNK 46/54 and P-JNK 46/54 for each DC population. (B and C) show the results of 3 independent experiments and the median is indicated by a bar. The intensity of the bands was measured and CYTO-DC was set to 100%. The ratio of the phospho to the non-phospho protein was then calculated. The ratio of the phospho to the non-phospho JNK 46/54 of CYTO-DC is therefore 1. IL4-DC, IL15-DC and stimulated IL15-DC with TNF-α were compared to CYTO-DC. The stimulated IL15-DC with TNF-α had a lower level of P-JNK 46/54 than IL15-DC. IL15-DC had more P-JNK 46/54 than IL4-DC.

4.6 IL-12p70 is not present in the cell culture supernatants of the four DC populations

The cell culture supernatants of the four DC populations were analysed by ELISA for the presence of IL-12p70. There was no IL-12 detected in the cell culture supernatants of IL4-DC, IL15-DC, CYTO-DC and IL15-DC with TNF-α.

4.7 No DC chemotaxis is detected in response to CCL19

The ability of the four DC populations to migrate towards CCL19 was tested in order to determine whether the CCR7 is functioning. However, the four DC populations did not show any migratory ability towards CCL19.

4.8 Lymphocytes co-cultured with CYTO-DC showed higher percentage of proliferation in a MLR

To test the ability of DC to stimulate lymphocyte proliferation, a mixed leukocyte reaction (MLR) was set up. CFSE labelled allogenic lymphocytes (2×10^5) were co-cultured with each DC population (5×10^4) for 6 days. CFSE is retained in the cytoplasm and with each round of cell division the fluorescence intensity of the CFSE will decrease by half. Lymphocytes that had been co-cultured with CYTO-DC showed the highest percentage of cell division as seen by reduction of CFSE positive cells. Lymphocytes that had been co-cultured with IL4-DC had a higher percentage of proliferation than both IL15-DC and the stimulated IL15-DC with TNF-α (Figure 4.10). The lowest percentage of lymphocyte proliferation showed lymphocytes that had been co-cultured with the stimulated IL15-DC with TNF-α. For further analyses the CFSE labelled allogenic lymphocytes were stained with CD4 and CD8 antibodies. However, the cell counts were not high enough to be further analysed.

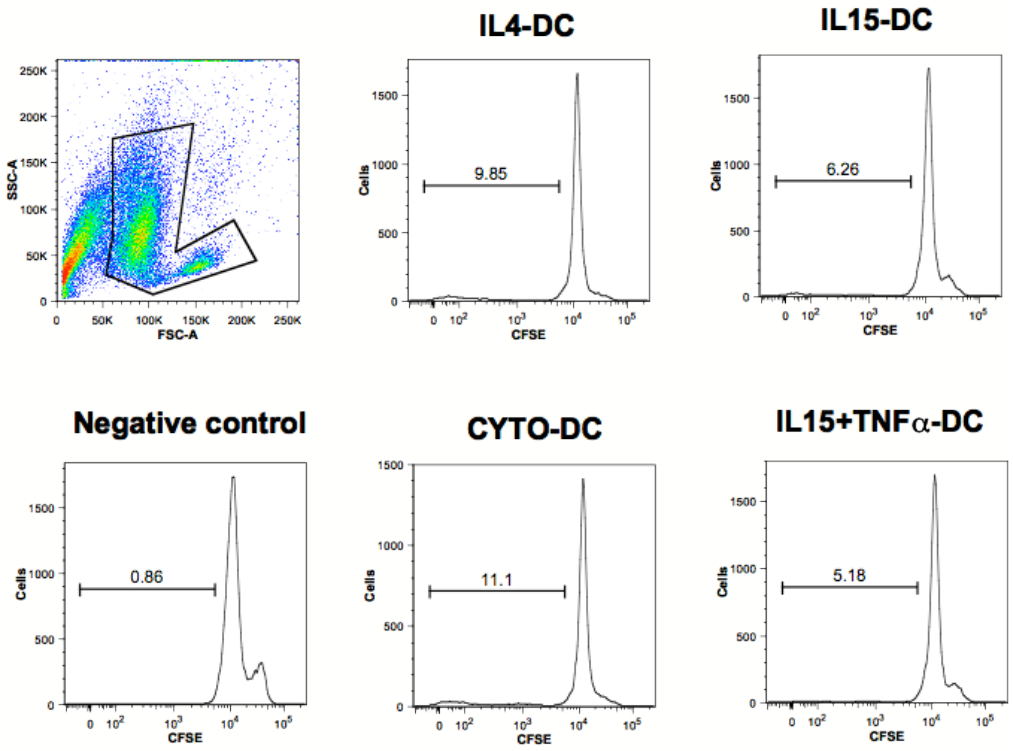

Figure 4.10. Lymphocytes co-cultured with CYTO-DC showed highest percentage of proliferation in a MLR. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days, and 24 hours before harvesting, a fraction of IL4-DC was stimulated with the cytokine cocktail IL-6, IL-1β, TNF-α and PGE_2 (CYTO-DC). Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days and a fraction of IL15-DC was stimulated for 24 hours with TNF-α. CFSE labelled allogenic lymphocytes (2×10^5) were co-cultured with each DC population (5×10^4) for 6 days. Flow cytometry was used to determine the percentage of lymphocyte proliferation. Lymphocytes were gated using FSC/SSC. The figure shows the percentage of lymphocyte proliferation after co-culturing allogenic lymphocytes with the indicated DC population. The negative control is CFSE labelled NAC without allogenic DC. Lymphocytes that had been co-cultured with IL4-DC had a higher percentage of proliferation than both IL15-DC and the stimulated IL15-DC with TNF-α. The lowest percentage of lymphocyte proliferation showed lymphocytes that had been co-cultured with the stimulated IL15-DC with TNF-α.

5. Discussion

DC are the most potent antigen presenting cells that possess the ability to stimulate naïve T-cells. The DC maturation process represents a crucial step in the initiation of adaptive immune responses. DC maturation is accompanied by changes of their morphological, phenotypic, and functional properties. In order to clinically apply DC vaccine in cancer immunotherapy, choice of precursor cells, type of maturation stimuli involved in the development from immature to mature DC, and which DC subsets to be used, are yet to be optimised.

In this study, I examined the phenotype, cytokine secretion, and T-cell stimulatory capacity of monocyte derived DC generated using GM-CSF and IL-15 (IL15-DC), and compared it to DC generated after the standard protocol using GM-CSF and IL-4 (IL4-DC). 24 hours before harvesting, a fraction of IL15-DC was stimulated with TNF-α, while a fraction of IL4-DC was stimulated with a cytokine cocktail of IL-6, TNF-α, IL-1β and PGE_2 (CYTO-DC). A previous study has shown that DC that were generated by using IL-15 were more efficient at T-cell induction as well as in priming cytotoxic T-lymphocytes, a process that may help to overcome the tumour escape mechanisms (53). However, in that study, LPS was used for stimulation. LPS is a good stimulant for the DC but it is questionable to put back DC that might have residual LPS to patients.

The results show that IL15-DC have a more mature phenotype than IL4-DC. On the other hand, IL15-DC have a less mature phenotype than CYTO-DC.

The results show that culturing monocytes for 5-6 days with GM-CSF and IL-15 led to their differentiation into cells that had the morphology and phenotypic properties of DC. After harvesting the IL15-DC, the cells were smaller in size than the IL4-DC.

Monocyte-derived DC that are generated by adding GM-CSF and IL-4 are characterised by being $CD14^-$ $CD1a^+$. CD14 is a monocyte/macrophage surface marker that is down-regulated upon differentiation of monocytes into DC. However, as shown in figure 4.2, all IL4-DC unexpectedly still expressed a relatively high amount of CD14. This could be attributed to non-specific binding of CD14 antibodies to Fc receptors. It could also be attributed to antibodies drying out, which could happen if a master mix of antibodies was not used. In my experiments, CD14

antibodies were the first antibodies to be pipetted to the wells. Other antibodies were then added one by one to the wells. So the probability of CD14 antibodies to be dry was high because of the time taken to add all other antibodies. The use of antibody master mix solved this particular problem, yet other problems still existed that were difficult to deal with because of time limitation. In figure 5.1 A, the $CD14^+$ population in the IL4-DC looks as if it is caused by unspecific staining as both CD1a positive and CD1a negative cell populations have shifted towards being $CD14^+$. Compared to that, a distinct CD14 positive CD1a negative population of the IL15-DC is shown in figure 5.1 B.

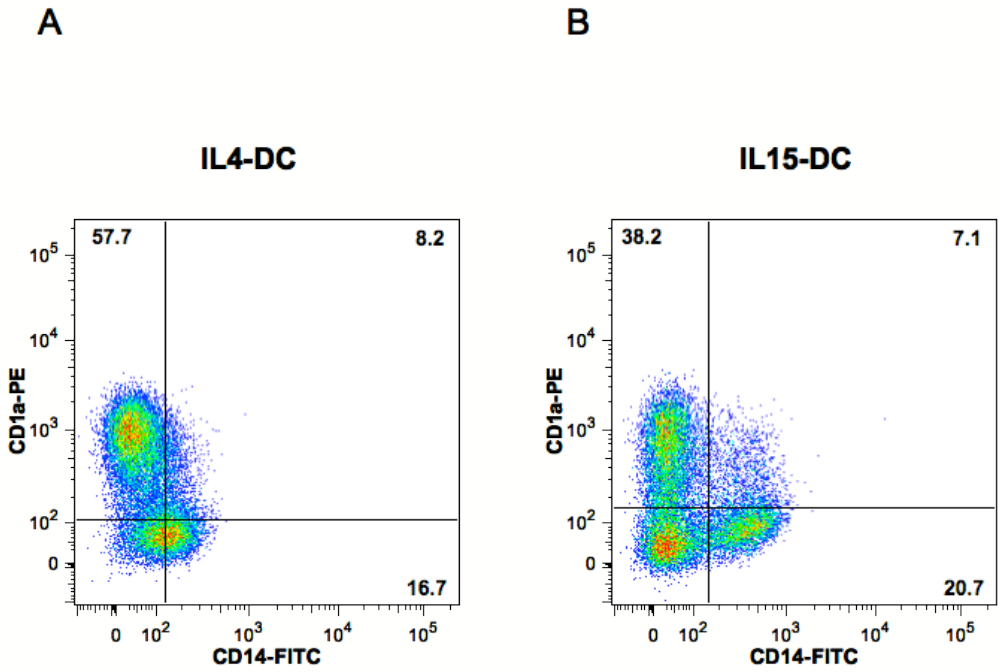

Figure 5.1 A distinct $CD14^+$ cell population present in IL15-DC but not in IL4-DC. Monocytes were cultured with GM-CSF and IL-4 (IL4-DC) for 6 days. Monocytes were cultured with GM-CSF and IL-15 (IL15-DC) for 6 days. The figure shows a representative experiment of three experiments. Flow cytometry was used to determine the percentage of cells that expressed CD14 (X axis) and CD1a (Y axis). (A) Both CD1a positive and CD1a negative cell populations have shifted towards being $CD14^+$ in the IL4-DC. (B) A distinct CD14 positive CD1a negative population is present in the IL15-DC.

Phenotypic analyses by flow cytometry showed that CYTO-DC expressed more HLA-DR, CD86, and CD83 than IL15-DC. However, the IL15-DC expressed more

maturation markers, i.e. HLA-DR, CD80, CD83, and CD86, than IL4-DC. The results show that the stimulated IL15-DC with TNF-α showed no difference from the non-stimulated IL-15-DC in the expression of most of the surface markers except for the surface marker CD86 which was up-regulated.

It is the mature DC and not the immature DC that can be utilized in vaccination against cancer. Taking this into consideration, it is very important that DC maturation markers are checked for their level of expression after DC are stimulated. Highly expressed maturation markers could be an indicator for a higher probability of good DC efficiency in T-cell activation. The co-stimulatory molecules CD80 and CD86 are responsible for delivering activation signals to the T-cells through the binding to CD28, the co-stimulatory receptor. This binding will deliver a potent co-stimulatory signal that leads to T-cell responses. These responses include the differentiation of naïve T-cells into effector and memory cells as well as production of cytokines such as IL-12. The CD83 molecule is another important DC maturation surface marker and it is thought that it plays an important role in the activation of certain immune responses (54).

The results show that CD80 was higher expressed in the IL15-DC population than in the IL4-DC population, which is in line with a previous study (53). However, the same study also showed that CD86 was expressed more on IL4-DC than IL15-DC which is in contrast to our findings. In another study, IL15-DC showed a lower expression of CD80 and CD83, but a considerably high expression of CD86 (26). An explanation for the different results could be related to the use of different stimuli. In this study, the cytokine cocktail IL-6, TNF-α, IL-1β and PGE_2 and TNF-α were used to stimulate IL4-DC and IL15-DC, respectively. In two previous studies, LPS was used to stimulate both IL4-DC and IL15-DC (26, 53). Other differences are that in the first study the monocytes were cultured for 3-4 days, i.e. less time than that used in this study, whereas in the second study, the lymphocytes and $CD1a^+$ DC were depleted in order to get a pure monocyte population, i.e. a different purification protocol than that used in this study (26, 53).

We speculated that the stimulation of IL15-DC with TNF-α might lead to the up-regulation of the surface maturation markers. My results show, however, that CD80 was down-regulated, CD83 was not changed, and CD86 was the only surface marker

that was up-regulated. Other studies showed that up-regulation of CD80, CD83 and CD86 occurred when IL15-DC were stimulated with LPS (26, 53).

The results show that the IL15-DC expressed more HLA-DR, i.e. MHC class II than IL4-DC, but yet less than that of CYTO-DC. When we examined the median fluorescence intensity (MFI) of the four populations, we found that the MFI of the IL15-DC was higher than both IL4-DC and CYTO-DC. MHC class II molecules are very important as they present antigens to $CD4^+$ T-cells. In the current study, all DC populations showed the same expression level of CD40 but when it comes to the MFI it was found to be higher in the IL15-DC than in both the IL4-DC and the CYTO-DC. CD40 molecules are expressed on DC and are up-regulated during the process of DC maturation. CD40 functions as a trigger for the expression of the co-stimulatory molecules which are required for efficient T-cell activation. In addition to, the CD40 ligation of DC has the capacity also to induce high level of IL-12 secretion (55).

The results show that CCR7 was expressed more on CYTO-DC and approximately equally on both IL4-DC and IL15-DC. CCR7 is one of the necessary chemokine receptors for DC migration. However, all the four DC populations were generally considered to have low expression of CCR7, which was confirmed by the chemotaxis assay result. The four DC populations did not show any migratory ability towards CCL19 (data not shown). It has been shown previously that the migration of skin DC toward the lymph nodes is impaired in CCR7-deficient mice under inflammatory conditions (56).

The surface marker CD38 was also analysed. The results show that CD38 was down-regulated on CYTO-DC while there were no detectable differences between the stimulated IL15-DC with TNF-α and the non-matured IL15-DC. A previous study showed that CD38 could regulate adaptive immunity by controlling the migration of DC and DC precursors (57). Moreover, another study showed that the CD38 could up-regulate CD83 expression and IL-12 secretion (58). However, this is in contrast to my result. In my study IL15-DC expressed a high level of CD38 but low level of CD83. Moreover, IL15-DC did not produce IL-12 (data not shown). The presence of IL-12 in the cell culture supernatants of all the DC populations was analysed. The results show that IL-12 was not detected in the supernatants of any of these populations. It has been shown before that DC produce various amount of IL-12

depending on the stimulus used (59). In this study, it was expected that CYTO-DC might not produce IL-12 as previously documented (59). The IL-12 production by IL15-DC was assessed for the first time in this study.

The phosphorylation of three distinct mitogen-activated protein kinase (MAPK) signalling cascades, including the extracellular signal-regulated kinase (ERK), p38, and the c-Jun N-terminal kinase (JNK) pathways were analysed in all DC populations by Western blotting. The results show that even after 24 hours of incubation of DC for stimulation, which is a long time for phosphorylation signalling analysis, the P-JNK in the CYTO-DC and the P-ERK in the stimulated IL15-DC with TNF-α were detected. The analysis of the signalling pathways stimulation after a shorter period of incubation was not feasible in this study because of time limitations. The cells were stimulated for 24 hours in order to be able to do phenotypic analyses (FACS), chemotaxis assays and MLR. Several reports have shown that the activation of the three MAPK pathways occurs during DC maturation (60-62). However, the number of pathways activated depends on the stimuli (60-62). This is also shown in our result in which the CYTO-DC had the highest levels of P-JNK46/54. However, the CYTO-DC had not the highest levels regarding P-ERK42/44 and P-p38 expression.

The JNK and p38 pathways are activated by stress-inducing agents whereas the ERK signalling cascade regulates cell proliferation and differentiation in response to mitogens and growth factors (63). The results show that IL15-DC had similar levels of P-ERK42/44 and P-p38 as that of CYTO-DC. However, the CYTO-DC had the highest level of P-JNK46/54. The MAPK signalling pathway has a role in the maturation of DC. A recent study demonstrated that the blockage of the p38 MAPK pathway could inhibit the up-regulation of CD40, CD80, CD86, CD83, and HLA-DR (64). The inhibition of JNK also resulted in decreased expression of CD80, CD86, CD83 and increased expression of MHC class II on LPS stimulated DC (65). In a previous study, ERK pathway inhibitors increased the expression of MHC class II complex and co-stimulatory molecules (66). It is difficult to draw a conclusion out of my result since the signalling pathway analysis was done after 24 hours of DC stimulation which is not ideal. Therefore, further analyses in the future should be performed.

In order to analyse the T-cell stimulatory capacity of the different DC populations, a MLR was performed. The result shows that the lymphocytes that had been co-

cultured with allogenic CYTO-DC showed the highest percentage of lymphocyte proliferation. On the other hand, lymphocytes that had been co-cultured with IL4-DC had a higher percentage of proliferation than cells co-cultured with both IL15-DC and the stimulated IL15-DC with TNF-α. As this data was based on only one experiment, it needs to be confirmed in the future.

It is known that the maturation of DC is crucial for the initiation of T-cells, and from our results we can conclude that the CYTO-DC are the more efficient cells in T-cell stimulation compared to IL15-DC.

The variation in the results of the three experiments presented in this thesis regarding the expression levels of DC surface markers and signalling pathway analysis might be explained simply by donor related variations. It could be also because uneven distribution of the cytokines between cells. This could be either during the replenishing of the cytokines during generation of DC or during addition of the stimulating cytokines. In other words it could be that not all cells had received enough cytokines. Another explanation could be that the monocyte population was originally contaminated with lymphocytes, which can affect the analyses outcome of the IL15-DC. Furthermore, the variation in the surface marker levels of expression could be because of adding improper amount of surface marker antibodies or long incubation of DC with the surface marker antibodies.

A total of 21 experiments were performed in this study. However, due to different reasons, only 3 experiments could be presented in the current thesis. For example, in 5 experiments, few cells were obtained of which 2 of them had a lot of thrombocytes. Another 3 experiments were omitted because CYTO-DC had very low CD83 expression and in 5 experiments, the IL15-DC were lost after harvesting. Two experiments were omitted because of low HLA-DR expression in CYTO-DC and very high CD14 expression in IL4-DC, respectively, indicating that the generation of this cell population had not been successful. Finally, 3 experiments were taken away for other reasons.

Although it is known that monocytes strongly stick to the plastic, the loss of monocytes during the washing steps after adherence was one of the major problems. Several experiments were stopped because of the low amount of cells still in the flasks after washing. It was also observed that cells like thrombocytes adhered to the

plastic instead of monocytes which resulted in low numbers of monocytes. Monocyte isolation from PBMC by adherence is a common procedure. About 10% of PBMC are plastic-adherent monocytes. However, monocyte isolation by adherence can be associated with a high degree of contamination with lymphocytes (67). In this study, it was observed that lymphocyte contamination was one of the main problems during sample preparation in three experiments. It was thought that this could be because of improper wash of the monocytes, a critical step in order get red of the lymphocytes to start with a pure monocyte population.

Loss of IL15-DC during cell harvesting was another obstacle. In three out of five experiments, IL15-DC were present in the flasks and looked as a nice DC population. The proper number of IL15-DC was obtained and their viability was checked. However, during the FSC/SSC analyses of the flow cytometry no IL15-DC population was present. One explanation could be that IL15-DC were sticking strongly to the 96-well plate during the staining procedures preceding flow cytometry. It appears that IL15-DC were very sensitive and might be subjected to death if a short delay occurred. Some experimental procedures took longer time than we anticipated. For example, IL15-DC were very sticky to the flasks which, therefore, required more time to harvest. Thus, the IL15-DC were subjected to longer time of EDTA treatment to detach sticky cells. The EDTA is a chelating agent that can reduce cell viability if it stayed for too long.

Adding IL-15 to monocytes that are contaminated with lymphocytes will lead to the stimulation and proliferation of T-cells. It was observed that there was a colour change of the cell culture medium of the IL15-DC population in some experiments which might be related to the stimulation and proliferation of T-cells.

The technical problems in the experiments can be overcome in the future by using alternative techniques of monocyte isolation. One of these techniques can be the elutriation technique. In this technique, the PBMC are isolated first from whole blood by Ficoll gradient then the PBMC are separated by an elutriation procedure. Monocytes can also be isolated from PBMC by using CD14 magnetic beads. This method is simple and reliable and purity of up to 98% is reported (68). However, the method is expensive. In addition, this technique leads to the loss of the $CD14^{-}$ monocyte population. Another way of monocyte isolation from PBMC is by depletion of lymphocytes, i.e. T-, B-, and NK-cells. This technique is easy to use and yields a

high purity of monocytes but is even more expensive than positive selection of $CD14^+$ cells. The elutriation technique is the most suitable technique to be used in immunotherapy.

5.1 Conclusions and future perspectives

The generation of IL15-DC turned out to be more problematic compared to the generation of IL4-DC. The stimulatory activity of IL-15 on T-cell proliferation resulted in a high degree of contamination with T-cells in the IL15-DC cultures as we did not have a pure monocyte population to start with. This problem can in future be overcome by using either alternative monocyte isolation protocols or by reducing the culture period from 5-6 to 3-4 days. It might also be worth trying different stimuli to induce the maturation of IL15-DC as the use of TNF-α did not result in a remarkable stimulation. Moreover, due to the variation in the results between the experiments, my data needs to be confirmed with this approach.

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