Design of tumor-specific immunotherapies using dendritic cells – effect of bromelain on dendritic cell maturation

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Marie Karlsen

Contents

Abbreviations

APC	antigen presenting cells
APS	ammonium persulfate
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
CFDA SE	carboxyfluorescein diacetate succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
CTL	cytotoxic T lymphocytes
DC	dendritic cells
dH_20	distilled water
DMSO	dimethyl sulfoxide
DTIC	dacarbazine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FSC	forward scatter channel
FITC	fluorescein isothiocyanate
FBS	fetal bovine serum
Flt3-L	FMS-like tyrosine kinase 3 ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase conjugate
IL	interleukin
LC	Langerhans cells
LPS	lipopolysaccharid
MAPK	mitogen-activated protein kinase
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MIIC	MHC class II-rich compartments
MLR	mixed lymphocyte reaction
mDC	myeloid dendritic cells
NAC	non-adherent cells
NF-κB	factor kappa-light-chain-enhancer of activated B
NK cells	natural killer cells nuclear
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cells
PGE_2	prostaglandin E2
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethanesulphonylfluoride
RIPA	radio-immuno-precipitation assay
SDS	sodium dodecyl sulfate
SSC	sideward scatter channel
TBP	TATA binding protein
TBS	tris buffered saline
TBST	tris buffered saline tween
TCR	T-cell antigen receptor
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
TLR	toll-like receptors
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor alfa
VEGF	vascular endothelial growth factor

Abstract

Immunotherapy using dendritic cells (DC) has shown promising results in clinical trials, but few relevant successes are recorded. Therefore, the choice of an appropriate DC population is critical for the outcome of this treatment. The DC used today in immunotherapy are often matured with a cytokine cocktail consisting of TNF-α, IL-1β, IL-6 and PGE_2. These cells have deficits in their cytokine production, and also their migratory capacity *in vivo* needs improvement. After being introduced to bromelain and the effect it has shown on glioma cells, a curiosity about how it would affect DC maturation awoke. Bromelain is a pineapple stem extract that has been clinically used in adjuvant cancer treatment for a long time. The aim of this study was to analyze the effect of bromelain on DC maturation.

Immature monocyte derived DC were stimulated for 24 h with different concentrations of bromelain and compared to cells stimulated with the cytokine cocktail. The phenotype of the generated cell populations was investigated by flow cytometry. Moreover, the phosphorylation patterns of MAP kinase proteins and AKT, in addition to nuclear RelB expression, were examined by Western blot. The migratory capacity of the generated DC populations was analyzed in a chemotaxis assay toward CCL19, and IL-12p70 production was determined by ELISA. The T cell stimulatory capacity of the generated DC populations was investigated by a mixed lymphocyte reaction (MLR).

Bromelain treated DC showed a concentration dependent upregulation of maturation markers and co-stimulatory molecules. However, they had a less mature phenotype compared to DC stimulated with the cytokine cocktail. Phosphorylation patterns of MAP kinase proteins and AKT obtained from Western blotting indicated that bromelain stimulated DC were more similar to immature cells. Nuclear RelB expression of bromelain stimulated DC was lower compared to cytokine cocktail treated DC but higher compared to immature DC. All bromelain concentrations used in this study resulted in increased IL-12p70 secretion by DC compared to cytokine cocktail treated cells and immature DC. The chemotaxis assay revealed that nearly the same number of DC migrated without a chemokine gradient and the MLR revealed that bromelain stimulated DC can induce proliferation of allogeneic T cells comparable to DC treated with the cytokine cocktail.

Although bromelain stimulated DC seem to be less mature than DC treated with the cytokine cocktail, they did produce more IL-12p70. Since lack of IL-12 secretion is one of the drawbacks of using the cytokine cocktail, bromelain might be used as an additional stimulus, in combination with other stimuli during generation of DC used in immunotherapy.

1 Introduction

1.1 Immune system

The human immune system is an amazing instrument, defending our bodies from attacks caused by bacteria, microbes, parasites, viruses, toxins as well as dangerous cancer cells. The importance of this incredible system becomes clear when the consequences of death are considered. When death occurs, the immune system, in addition to all other functions of our body shuts down. The outcome of a non-functioning immune system is invasion of all possible infectious agents mentioned above, leading to decomposition of the body within weeks. This indicates how remarkable and essential the immune system is for our existence (1). The immune system consists of an innate and an adaptive immunity, and these two systems collaborate to generate a successful protection against all possible threats our bodies are exposed to.

1.1.1 Innate immunity

Innate immunity is the body's first response to infectious agents or foreign antigens. This resistance is non-specific and does not create memory or lasting protection against pathogens. The innate immune system is the phylogentically oldest protection against infection and provides two main functions. First, the innate immunity is the body's initial response and therefore attempts to prevent, control and eliminate infection or other threats. Second, the role of the innate immunity is to stimulate the adaptive immune response. Components of the innate immune response are (2):

1) Epithelial barriers that function as a physical fence, preventing entry of pathogens. The skin, in addition to mucosal surfaces of gastrointestinal and respiratory tracts are the major epithelial barriers in the human body.
2) The complement system consists of plasma proteins that circulate in the blood. These complement proteins are activated when they encounter microbes, eventuating in inflammation and destruction of the microbes.
3) Phagocytes (neutrophils and macrophages) identify, ingest and break down microbes. Natural killer (NK) cells also contribute to elimination of microbes by similar mechanisms.

4) The last component is cytokines, which regulate and control the activity of immune-cells. Macrophages, neutrophils and NK cells produce and secrete cytokines that enable cell communication between inflammatory cells and responding tissue cells.

Cells belonging to the innate immune response discover pathogens by recognizing patterns shared among pathogens through various pattern recognition receptors. These patterns include bacterial lipopolysaccharide (LPS) or teichoic acid, in addition to carbohydrates and viral RNA (3). There exist different classes of pattern recognition receptors, and the most comprehensive one is the family of toll-like receptors (TLR) (2). This family consists of eleven different TLR, named TLR 1 to 11. Some of these are located in the plasma membrane of the cells, while others are expressed in the endosomal membrane inside the cells. This enable the cell to recognize pathogens having already entered the cells, as well as those attacking from the outside. Several signaling pathways link signals received by TLR to transcription factors in the nucleus, and these transcription factors regulate expression of genes necessary for innate immune responses.

1.1.2 Adaptive immunity

The adaptive immune system is complementary to the innate immune system. Signals from the innate system activate the adaptive immune system, and an immune response is built in response to the signal. Since the immune response is developed after pathogen or antigen encounter, the adaptive response building up is specific towards these foreign substances. In addition, this response generates a memory that enables a more rapid and vigorous response to any repeated exposure.

1.1.2.1 Humoral and cell mediated responses

Adaptive immunity can be further divided into two types of responses, humoral immunity and cell-mediated immunity. These two responses are executed by different components of the immune system, and they destroy different kinds of microbes. The humoral immunity consists of antibodies. Antibodies are produced by B lymphocytes and they remove microbes by recognizing microbial antigens and target these microbes for degradation. This is partly achieved by the help of the complement system, which is a part of the innate immune system. The humoral immune response is the mechanism against extracellular microbes and the toxins these secrete. The other immune response, the cell-mediated response, is mediated by T

lymphocytes. This immune response is directed toward intracellular microbes like virus and some bacteria since these are inaccessible for antibodies in the blood. There exist two types of T lymphocytes, helper T lymphocytes that activate and help macrophages and other immune cells to kill phagocytosed microbes, and cytotoxic T lymphocytes (CTL) which kill infected cells to eliminate reservoirs of infection.

1.1.3 Linking innate and adaptive immunity through antigen presenting cells

To eliminate foreign substances, the innate and adaptive immune systems need to collaborate. This is necessary since the innate immunity often can not combat an infection alone, and the adaptive immunity is dependent on signals from the innate system to be activated. The bridging between these two responses is chiefly executed by antigen presenting cells (APC). The function of APC is to capture antigens, which they then transport and display to cells of the adaptive immune system. Dendritic cells (DC), B lymphocytes, and macrophages are all noted as professional APC because they are capable of activating T cells. Although all these cells have this ability, DC are superior concerning T cell stimulation, because of their ability to stimulate naïve T cells This has been confirmed by mixed lymphocyte reactions (MLR) where the T cell stimulatory capacity of these different APC have been compared (4).

1.1.4 Dendritic cells

DC are of crucial importance in our immune responses. They are the sentinels of the immune system, and have the function of bridging the innate and the adaptive immunity.

DC have been named according to their physical appearance, which is characterized by large amounts of long, membranous extensions on the cell surface. They have the role of capturing antigens and display these to the lymphocytes of the adaptive immune response. This interaction between a DC and a lymphocyte is demonstrated in figure 1.1 (A), where the characteristic appearance of DC is visualized. Figure 1.1 (B) shows schematically how this interaction takes place. Several co-stimulatory molecules and adhesion proteins are required for activation of the T cell. In addition, peptides presented by APC to T cells need to be coupled to MHC molecules.

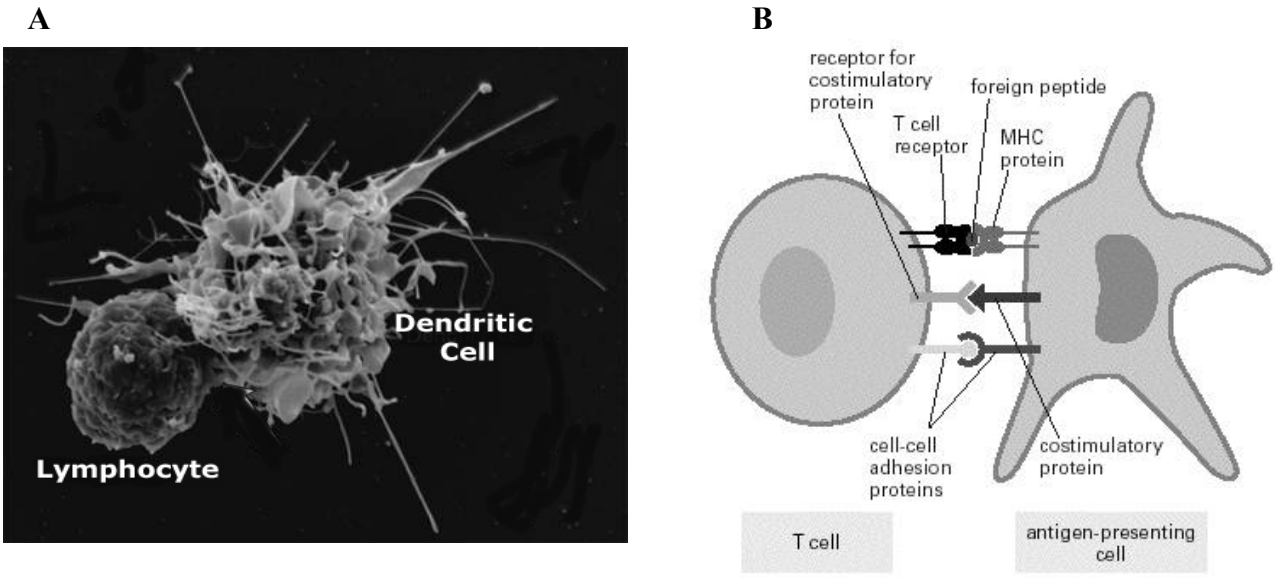

Figure 1.1: **Interaction between DC and lymphocytes.** The connection between a DC and a lymphocyte are shown in (A), the characteristic appearance of DC with its membranous projections are also visualized. (B) shows the different molecules involved in antigen presentation between a DC and a T cell. T cell ability to recognize peptides presented by APC requires the peptides to be displayed in connection with MHC proteins on the APC. In addition co-stimulatory and adhesion molecules are necessary for activation of the T cell. Figure A from: www.bcrfund.org/Story006.htm, and figure B from: www.homepages.ucl.ac.uk/~ucbpdsb/Essay2/TCR-MHC.jpg

DC are complex immune cells and they exist in immature and mature states. Immature DC are the sentinels that patrol the periphery, seeking antigens to capture. After immature DC have been exposed to an antigen and have captured this, they migrate to lymphoid organs where they present these antigens to naïve T lymphocytes. During this journey to the lymphoid organs, the DC mature and start presenting antigens instead of capturing them. Without the ability of DC to migrate, naïve T lymphocytes would not be able to achieve contact with peripheral antigens (5).

1.1.4.1 DC origin and subsets

Immature DC are continuously produced in the bone marrow from hematopoietic stem cells (6). DC can be generated *ex-vivo* from blood monocytes when cultured with appropriate cytokines (7). Since the DC population in the blood is a very small population, this method enables generation of higher numbers of DC.

There exist two main lineages of DC, myeloid DC (mDC) and plasmacytoid DC (pDC) (8). These two lineages have different morphology, function and expression of markers. Despite these differences, the two DC lineages express the same adhesion-, co-stimulatory-, and

inhibitory molecules, in addition to activation markers (9). The mDC can be found almost everywhere in the body. Some are located in peripheral tissues, others are found in secondary lymphoid organs, while a few mDC also circulate in the blood. These are the ones that handle pathogen entry in the skin and mucosal tissue. The pDC all circulate in the blood, and from here they can enter the T cell areas in the lymphoid organs through high endothelial venules. These DC may become specialized to capture blood borne pathogens in addition to recognize self-antigens (8, 9).

Langerhans cells (LC) are a subset of mDC. They reside in the epidermis and are involved in the immune response toward several different infections (skin transmitted viral, bacterial and parasitic), dermatoses and skin cancers. LC were discovered in 1868, but they were not connected to the DC system until Steinman and Cohn around 100 years later identified splenic DC (8, 10). DC consist of a number of different lineages and subsets and are therefore a heterogeneous cell population. Still there is a lot unknown about the DC subsets and their classification. However, it is clear that DC reside in many areas of the body where they carry out antigen capture and subsequent presentation to lymphocytes, and they are a necessity to achieve high-quality immune responses.

1.1.4.2 Antigen capture, processing and presentation

Immature DC can capture antigens using several pathways. First they can utilize phagocytosis to take up particles and microbes, second they can internalize extracellular fluid and solutes by micro- and macro-pinocytosis. (11, 12). The third mechanism of DC antigen uptake is receptor mediated endocytosis. DC express a number of different receptors that mediate this function of antigen capturing, including C-type lectin receptors and Fcγ and Fcε receptors (3, 12). The fourth manner DC can pick up antigens is through engulfment of apoptotic bodies (13, 14). Immediately after antigen uptake immature DC undergo a change that leads to maturation of the cells. This change involves reduction of the ability to capture antigens and upregulation of major histocompatibility complex (MHC) molecules and other surface molecules necessary for T-cell priming (5, 12). While B lymphocytes can recognize antigens directly through their B-cell receptors, T lymphocytes are dependent on antigen processing and presentation mediated by an APC. This is necessary since the T cell receptors (TCR) lack the ability to recognize antigens directly. Instead the TCR recognize antigen peptides bound to MHC molecules (12). DC are the most effective APC since they are able to produce larger amounts of MHC class II peptide complexes compared to other types of APC (15).

One mechanism of antigen processing by DC is done in endosomes. Generated peptides are transported to MHC class II-rich compartments (MIIC) residing inside DC. MIIC are late-endosomal structures where antigen peptides are connected to MHC clas II molecules (12). The peptide loaded MHC class II molecule is then transported to the plasma membrane (9).

There exist two types of MHC molecules. MHC class II molecules present peptides to $CD4^+$ helper T cells while peptides presented on MHC class I molecules stimulate $CD8^+$ CTL. Antigens to be presented on MHC class I molecules are ubiquitinated and then degraded by the proteasome before they are transported as peptides to the endoplasmic reticulum (ER). MHC class I molecules are loaded with peptides directly in the ER where they are synthesized (3, 12). Peptide loaded MHC class I molecules move through the golgi apparatus and are transported to the cell surface by exocytic vesicles. Both processes of peptide coupling to these MHC molecules are shown in figure 1.2.

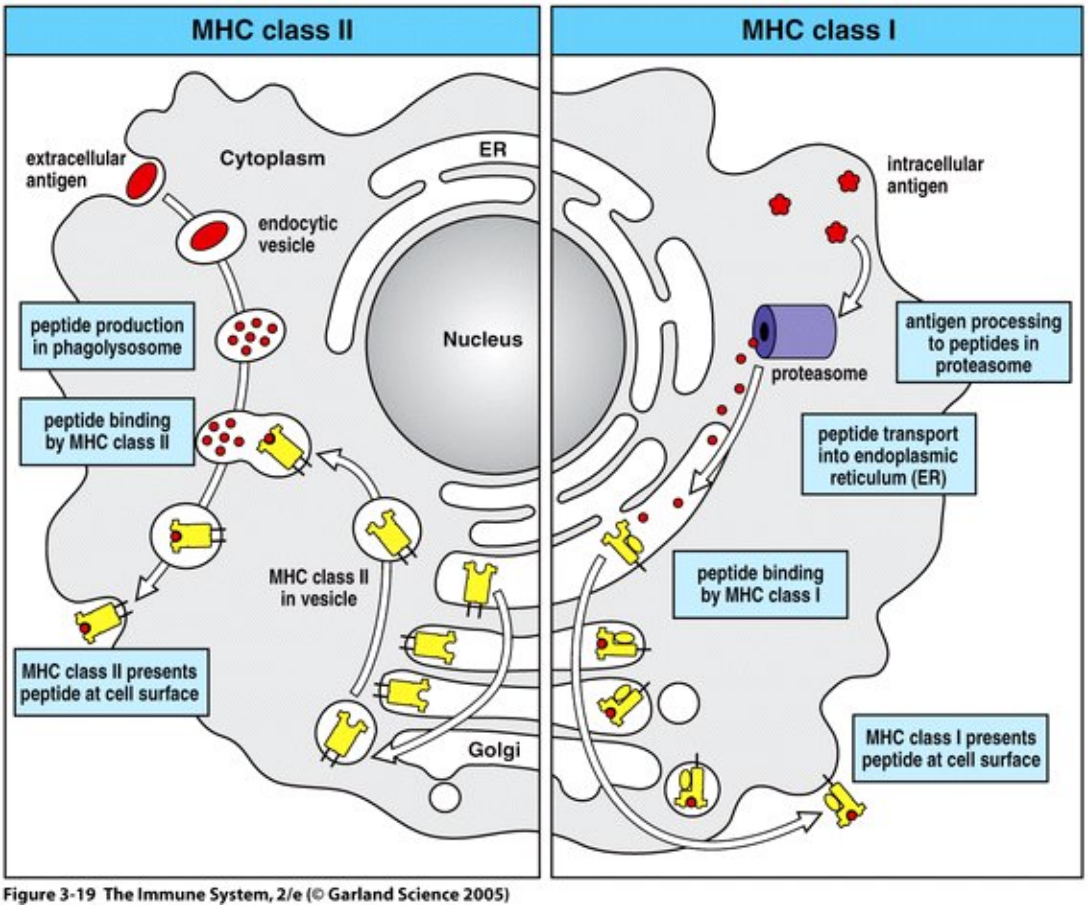

Figure 1.2: **Processing and presentation of MHC class I and MHC class II peptides.** Antigens captured are processed and then coupled to either MHC class I or MHC class II molecules. The right part of the figure shows how intracellular antigens are bound to MHC class I molecules. These antigens are ubiquitinated and degraded by the proteasome before linked to the MHC proteins in the ER. This complex is then transferred to the cell surface where the peptide is presented to $CD8^+$ T lymphocytes. The left side of the figure explains how extracellular antigens are processed into peptides in phagolysosomes, before transported to MIIC where they are bound to MHC class II molecules. These peptide-MHC complexes are transferred to the cells surface where they are recognized by $CD4^+$ T lymphocytes. Figure from Garland Science 2005.

1.1.4.3 Maturation of DC

At the same time as antigens are processed and coupled to MHC molecules, other changes also occur with the DC. Immediately after antigen capture, the maturation process begins. While immature DC have the ability to capture antigens, only mature DC have the ability to present antigens and prime immune responses (16).

During maturation DC start expressing new molecules on the surface while others are removed. Mannose receptor (CD206), Fcγ receptor (CD32) as well as the C-type lectin DC-SIGN (CD209), all involved in antigen uptake, have shown reduced expression on DC that have been stimulated and matured (17). MHC class II molecules and co-stimulatory molecules like CD40, CD80 and CD86 are upregulated on stimulated DC. The receptors of these co-stimulatory molecules are present on T cells, and the connections between these are required for T cell commitment (3, 5). In addition to these molecules, CCR7 is rapidly expressed on matured DC, and this chemokine receptor directs DC migration into the lymph nodes where DC release cytokines and chemokines that attract T and B cells (6, 9, 12).

Mature DC also enhance adaptive immune responses by synthesizing high levels of IL-12 (12), resulting in a stronger CTL response due to IL-12 activation of Th1 cells and the ability of these helper cells to induce proliferation of CTL. (18). Maturation signals also induce phosphorylation of mitogen-activated protein kinases (MAPK) in DC. There exist several MAPK signaling pathways, and they are activated through a cascade of phosphorylated kinases. When they are phosphorylated they gain the ability to act on downstream targets. As reviewed by Nakahara et al, studies have implied that MAPK signaling pathways regulate several parts of DC maturation, including phenotypic and functional maturation as well as cytokine production (19).

1.2 Tumor immunology

Tumor formation and cancer development is the result of a long chain of events. It is known that environmental factors such as chemicals and radiation can cause mutations resulting in cancer development. Other factors contributing to development of cancer and tumors include viral infection or genetic predisposition.

If a foreign antigen is discovered by cells of the immune system, it will initiate an immune response that leads to removal of affected cells and the pathogen. The immune system is capable of executing this because the antigen or pathogen is recognized as foreign. One of the problems concerning immune responses toward tumors is that some tumor antigens are recognized as self antigens by T cells. This will lead to destruction and removal of these T cells to avoid autoimmune reactions. These evasions from the immune response can occur since cancer cells are derived from normal cells and have been altered due to mutations. This mechanism known as tolerance is necessary to avoid the immune system attacking the body's own cells. Fortunately this is not true for all tumor antigens. Many tumor antigens have been shown to be recognized by the immune system as foreign or altered-self and thereby activate an immune response (20, 21).

This is not the only manner tumors use to evade the immune system. Many tumor cells lack or manage to loose tumor specific antigens. In addition to tumor specific antigens, also MHC class I has been shown to be deficient on cancer cells. Without MHC class I, cells of the immune system loose the ability to recognize tumor antigen presented on the surface of the cell and initiate immune responses (22).

Tumors also induce the generation of blocking factors and tumor specific regulatory T cells (Tregs). These inhibit interactions between effector cells and cancer cells (22). Tregs are a subpopulation of T lymphocytes, and these maintain system homeostasis in addition to tolerance by suppressing the activation of the immune system. The presence of Tregs in tumors is linked with reduced tumor specific immune responses, and poorer survival rates (23).

Defects in effector cells are also a problem concerning immune responses toward tumors. Tumor cells produce immunosuppressive molecules that affect the effector cells negatively. As a result, macrophages and natural killer (NK) cells loose their phagocytic ability and cytotoxity (22).

In addition to producing these immunosuppressive molecules, tumor cells also secrete soluble products that directly affect the function of immune cells. Vascular endothelial growth factor (VEGF) is one of the soluble factors secreted from tumor cells. In addition to its role in angiogenesis, VEGF has been shown to inhibit the functional maturation of DC. It was discovered that DC cultured in the presence of tumor cell supernatants had a significant reduction in their stimulatory capacity. This was confirmed when the cells were analyzed in an allogeneic MLR (24). Transforming growth factor beta (TGF-β) and interleukin (IL)-10

have also displayed a clear role in immune suppression. TGF-β is important in several cell functions; in addition it stimulates angiogenesis and suppresses the effect of the immune system. Cancerous cells often have increased TGF-β secretion. IL-10 is an immunoregulatory cytokine, and is known to target APC. It directly promotes tumorigenesis by affecting tumor cells, while it indirectly suppresses immune responses. The relative mRNA expression of VEGF, TGF-β and IL-10 in tumor tissue was compared with tumor free tissue, and it was shown to be frequently overexpressed in the tumor tissue (25).

1.3 Tumor immunotherapy

Tumor immunotherapy aims at activating the body's own immune system to fight the tumor. Therefore immunotherapy may not lead to as many negative side effects as conventional cancer treatments do by being more targeted to the cancer cells. Chemotherapy aims at eliminating cancer cells by killing rapidly dividing cells, while radiation therapy uses ionizing radiation to kill cancer cells and shrink tumors. Unfortunately it is not possible to direct this treatment specifically to cancer cells. The consequence is cell death also among healthy cells. This results in hair loss, nausea and leukopenia which affect the physical and mental wellbeing for the patient.

Immunotherapy can be either passive or active. Passive immunotherapy provides extra supplies to the immune response by use of antibodies or CTL. The disadvantage regarding passive immunotherapy is the short lived response. Since all components introduced have short half-lifes the treatment must be repeated to achieve desirable results. Active immunotherapy directly activates the immune system in the patient, by triggering proliferation and activation of residing T cells. Active immunotherapy therefore aims to achieve an endogenous immune response, where the immune system recognizes the tumor as foreign.

1.3.1 DC in tumor immunotherapy

Since DC are the most potent APC, many scientists designing or improving immunotherapeutic treatments are interested in DC. Increased understanding of the importance of DC in immune responses and their physiology has lead to optimism concerning their capacity in immunotherapy (26). In addition to generating a potent anti-tumor response

through activation of T cells, DC have also shown to be competent to directly eliminate cancer cells through expression of death receptor ligands (27).

This indicates that DC are interesting in multiple ways concerning cancer treatment and tumor elimination. DC in immunotherapy are utilized both by activation of DC *in vivo*, and by reinfusion of *ex vivo* generated DC. Methods stimulating DC inside the body are noted as *in vivo* techniques. One of the aims for *in vivo* approaches is to expand the DC pool in the patient since some patients have a lowered frequency of activated DC. Injections of DC specific growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), FMS-like tyrosine kinase 3 ligand (Flt3-L), granulocyte colony-stimulating factor G-CSF or CD40 ligand (CD40-L) can increase DC numbers and make the DC present tumor antigens in a more effective way (28-30). Another method tested in mouse models is engineering of tumor cells. Attempts where tumor cells have been transfected with co-stimulatory molecules from the B-7 and the TNF/TNF-R family, and MHC class II molecules have been performed, hoping to make the tumor cells express DC functions. By doing this, scientists desire to enhance the tumor antigen presentation by engineering the tumor cells to directly activate $CD4^+$ or $CD8^+$ T cells (28, 31).

1.3.1.1 Ex vivo approaches

In addition to the two main lineages of DC, there is one more population of DC, monocyte derived DC. After several years searching it was found that monocyte derived DC actually exist in the body. During an inflammation, monocytes in the blood are transformed into monocyte derived DC. Since DC are a rare cell population in the blood, this is today an established method exploited by scientists to generate large quantities of DC in cell culture. Monocytes are isolated from human blood, and by correct stimulation they will develop into DC over a period in cell culture. In theory tumor immunotherapy using *ex vivo* generation of DC is simple and executable, but unfortunately it is not that easy.

Figure 1.3 gives an overview of this method. Blood cells are harvested from the patient and peripheral blood mononuclear cells (PBMC) are isolated by density gradient centrifugation. Immature DC are generated from the monocytes in the PBMC. These cells are then pulsed with tumor material from the patient. This in addition to other stimulations results in matured DC that present tumor specific antigen. This other stimulation induces a functional maturation of DC, and this is important for the ability of the cells to generate strong immune responses when they are transferred back into the patient. In theory the cells will migrate from injection site to the lymph nodes, where they present the tumor antigen to lymphocytes and initiate an

immune response toward the tumor. Since the quality of the maturation stimulus is essential for proper immune responses, improvement of this is the aim of my project.

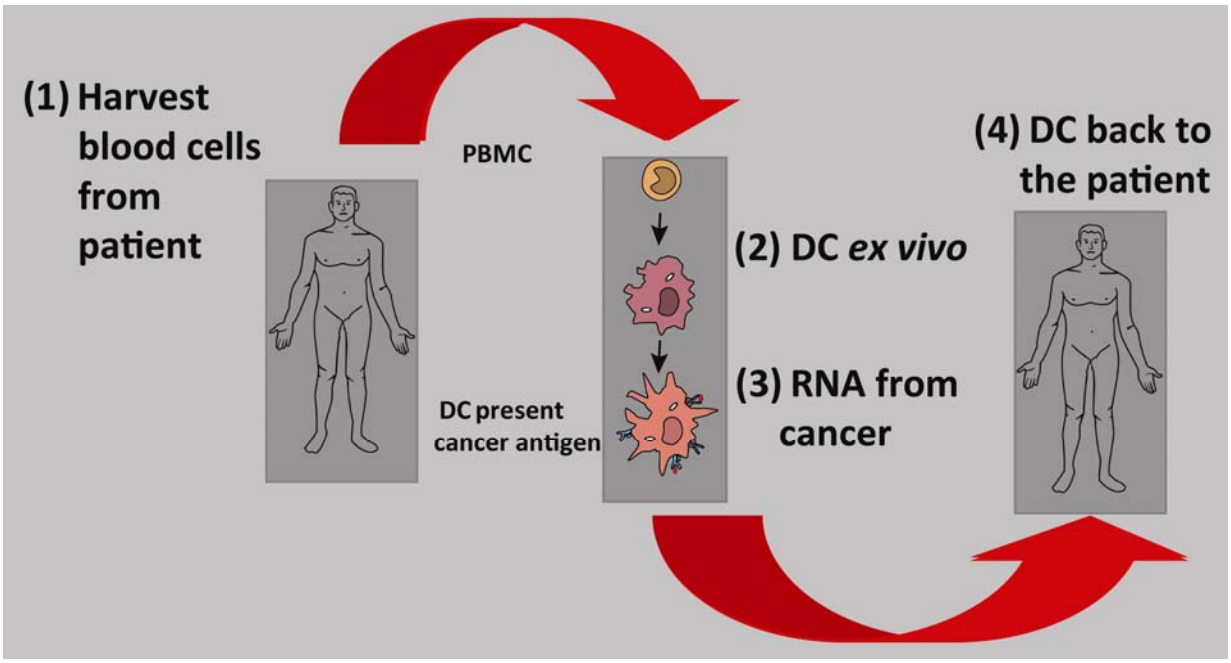

Figure 1.3: **DC vaccination strategy**. Blood cells are harvested and PBMC isolated. From the monocytes, immature DC are generated *ex-vivo*. These cells are stimulated to mature, and presented to tumor material from the patient before they are transferred back into the patient. DC will then migrate to lymph nodes where they interact with T lymphocytes and induce immune responses toward the tumor. Figure made by Arnt-Ove Hovden.

Several phase I studies using *ex vivo* generated DC have been carried out in humans, unfortunately, only few of these early trials have shown significant tumor regressions (32-34). Few phase III studies have been carried out using DC vaccines so far. One approach to investigate DC vaccination versus standard Dacarbazine (DTIC) treatment in stage IV melanoma was conducted between March 2000 and July 2003 (35). In this phase III trial, patients were divided into two groups. One of the groups received DTIC which is the standard first-line treatment given to metastatic melanoma patients. The other group received a DC vaccine. DC used in this vaccine was matured with a cytokine cocktail consisting of TNFα (10 ng/ml), IL-1β (2 ng/ml), IL-6 ($1x10^3$ IU/ml) and PGE_2 (1 µg/ ml), and then loaded with several MHC class I and II restricted peptides. No significant difference was shown between the two groups, and the study was ended after the first interim analysis due to low objective response in both groups (DTIC: 5,5%, DC: 3,8%) (35).

These results show that improvements of the DC vaccine still are necessary, although one of the main obstacles concerning these trials is that the patients used are all late-stage cancer patients. The result of this is that the patients already have an enormous tumor burden, and are

immunosuppressed by heavy use of conventional cancer therapies and therefore may not be able to develop a strong immune response.

Another issue hampering the success of DC vaccination is lack of knowledge concerning tumor antigens and antigen loading. Furthermore, to be able to initiate a response against the tumor, the DC injected back into a patient need to possess the ability to migrate from injection site to lymph nodes. Research has shown that mature DC have a superior migration capacity compared to immature DC (36). One possible explanation can be that mature DC upregulate the expression of chemokine (C-C motif) receptor 7 (CCR7). There is evidence confirming that this G-protein coupled receptor participates in the migration of DC from peripheral tissue to lymph nodes (37). The migratory pattern for mature DC still requires more investigation, in that way generated DC can be improved to fit the requirements necessary for successful migration. Obviously more research is necessary to develop a successful *ex vivo* generated DC vaccine.

Also the maturation step during generation of mature monocyte derived DC needs improvement. DC used today are stimulated with a "gold standard" cytokine cocktail consisting of TNFα, IL-1β, IL-6 and PGE_2, however this stimulation is not optimal. PGE_2 is supposed to induce and improve the migration of the cells, but has in addition the effect of inhibiting IL-12 secretion by the DC (38). Since IL-12 is an important stimulator for T cell activation, the stimulation used needs to be improved to be able to generate strong immune responses.

1.4 Aim of the study

The aim of this study was to analyze the effect of bromelain on DC maturation, concerning their phenotype, cytokine production and T cell stimulatory capacity. Bromelain is a pineapple stem extract, prepared from cooled pineapple juice by centrifugation, ultrafiltration and lyophilisation (39). Immunological and enzymological data indicate that crude pineapple stem extracts contain four different cysteine proteinases. These are stem bromelain, ananain, comosain and fruit bromelain. Of these, stem bromelain is the major component (40). Bromelain has been clinically used as additive to cancer treatment to reduce side effects of chemotherapy, and to reduce inflammation and edema as well as improve wound healing caused by radiotherapy and surgery (39, 41). Previous studies have shown that bromelain treated glioma cells have a significant reduction of adhesion, migration and invasion capacity without affecting the cells' viability (42). In addition, adhesion molecules on blood and

endothelial cells are known to change function after exposure to bromelain, and bromelain has shown to activate various immune cells and their cytokine production (39).

2 Materials

2.1 Equipment

Name	Company
Biological Safety Cabinet class II	Nuaire, USA
CASY cell counter	Schärfe System, Germany
Centrifuges	- KUBOTA 8700, Tokyo, Japan -Thermo Heraeus multifuge 1S-R - Mini-spin, Eppendorf, Germany - Thermo Heraeus Fresco 17, USA
ChemiDoc	BIO-RAD
Microplate reader, EMax	Molecular Devices
Flowcytometer: BDFACS Canto	BD Bioscience, USA
CO_2 Incubator	Forma Scientific, USA
Microscope: Leica DMIL	Leica, Germany
Mini-PROTEAN MP3 system	BIO-RAD, USA
Wather bath	GFL, Germany

2.2 Plastic ware

Name	Company
6 well plate	NUNC, Denmark
96 well cell culture plate	NUNC, Denmark
96 well V-shaped plate	NUNC, Denmark
Cryotubes 1ml/2ml	NUNC, Denmark
Microtubes 1.5ml clear	Axygen
HTS Transwell 96 system	Corning, USA
T25 Cell culture flask	NUNC, Denmark
T75 Cell culture flask	NUNC, Denmark

2.3 Software

Name	Company
FlowJo	Tree Star, Inc
Prism	GraphPad Software
Soft Max Pro	Molecular Devices
Quantity One	BIO-RAD, USA

2.4 Buffers and media

Name	Composition
10x TBS	0.1 M Tris pH 8.0
	1.4 M NaCl
1x Blotting buffer	1x Running buffer without SDS
	20% Methanol
1x Running buffer	0.1% SDS
	25 mM Tris
	192 mM Glycine
1x TBST (0.5%)	100 ml 10x TBS
	5ml Tween20
	900 ml H_2O
6x Lämmli buffer	375 mM Tris HCl pH 6.8
	9% SDS
	50% Glycerol
	9% β-Mercaptoethanol
	0.03% Bromphenolblue
Blocking buffer	5 % milk in 1xTBST (0.5%)
Buffer A (Nuclear extracts):	10 mM HEPES pH 7.9
	10 mM KCl
	0.1 mM EDTA
	0.1 mM EGTA
	+ freshly added before use:
	1 mM DTT
	0.5 mM PMSF

Buffer C (Nuclear extracts):	20 mM HEPES pH 7.9
	0.4 M NaCl
	1 mM EDTA
	1 mM EGTA
	+ freshly added before use:
	1 mM DTT
	1 mM PMSF
FACS buffer	PBS + 0.5% BSA
PBS pH 7.4	137 mM NaCl
	2.7 mM KCl
	8.1 mM Na_2HPO_4
	1.5 mM KH_2PO_4
RIPA buffer	50 mM Tris pH 7.4
	1% NP40
	0.25% Na-deoxycholat
	150 mM NaCl
	1 mM EDTA
	+ freshly added before use:
	1x proteinase inhibitor complete
	1 mM PMSF
	1 mM Na-orthovanadat
	1 mM NaF
RP10 medium	RPMI 1640 w/ ultraglutamine
	10 % FBS
	50 units/ml Penicillin sodium
	50µg/ml Streptomycin sulfate in 0.85% saline
Stripping buffer	2% SDS
	62.5 mM Tris/HCl pH 6.7
	100 mM β-Mercaptoethanol
Washing buffer	0.05% Tween20 in PBS

2.5 Reagents

Name	Company
0,5M Tris-HCl pH 6,8	BIO-RAD
1,5M Tris-HCl pH 8,8	BIO-RAD
25x Proteinase inhibitor, complete EDTA-free	Roche, Germany
Acrylamide 30%	BIO-RAD, USA
Albumin, bovine serum (BSA)	Sigma Aldrich, USA
Ammonium persulfate (APS)	BIO-RAD, USA
β-Mercaptoethanol >=99%	Sigma Aldrich, USA
Casy ton	Innovatis, Germany
CCL19	Immunotools, Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, UK
Ethylenediaminetetraacetic acid (EDTA) 0.5 M	Sigma Aldrich, USA
Glycine	BIO-RAD, USA
GM-CSF	Immunotools Germany
Ionomycin	Sigma Aldrich, USA
IL-1β	Strathman Biotec, Germany
IL-4	Immunotools, Germany
IL-6	Immunotools, Germany
Isopropanol prima	Arcus, Norway
Lymphoprep	Axis-Shield, Norway
Methanol	MERCK, Germany
Milk powder	Frema, Germany
NaF	MERCK, Germany
NaOrthovanadat	MERCK, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich, USA
Penicillin/streptomycin	Invitrogen, USA
PGE_2	Sigma Aldrich
PMSF	Roche, Germany
Ponceau S	Sigma Aldrich, USA
Precision Plus Protein Standard	BIO-RAD, USA
RPMI 1640 with ultraglutamine 1	BioWhittaker, Belgium
Sodium dodecyl sulphate (SDS)	BIO RAD, USA

Tetramethylethylenediamine (TEMED)	BIO-RAD, USA
TNFα	Immunotools, Germany
Tris	BIO-RAD, USA
Tween 20	MERCH, Germany
X VIVO 20 Serum free hematopoietic cell medium	Biowhittaker, Belgium

2.6 Kits

Name	Company
BCA™ Protein Assay Kit	Pierce, USA
BD Compbeads	BD Biosciences, USA
ELISA MAX™ Set *Deluxe* Human IL-12p70	BioLegend, USA
Immunstar WesternC™ kit	BIO-RAD, USA
Vybrant CFDA SE, Cell Tracer kit	Invitogen, USA

2.7 FACS Antibodies

Name	Clone	Company
CCR7-PE	150503	R&D Systems, USA
CD1a-PE	NA1/34-HLK	AbD Serotec, Germany
CD14-FITC	UCHM1	AbD Serotec, Germany
CD38-Alexa Fluor 647	AT13/5	AbD Serotec, Germany
CD4 APC	MEM-241	Immunotools, Germany
CD40-FITC	LOB7/6	AbD Serotec, Germany
CD8 PE	LT8	AbD Serotec, Germany
CD80- APC	MEM-233	Immunotools, Germany
CD83-PE	HB15e	AbD Serotec, Germany
CD86-FITC	BU63	AbD Serotec, Germany
CD91-PE	A2Mr alpha-2	AbD Serotec, Germany
HLA-DR-APC	HL-39	AbD Serotec, Germany
IgG APC	PPV-06	Immunotools, Germany
IgG FITC	PPV-06	Immunotools, Germany
IgG PE	PPV-06	Immunotools, Germany

2.8 Western Blot Antibodies

Name	Clone	Company
AKT (Pan)	C67E7	Cell Signaling, USA
Phospho-AKT	D9E	Cell Signaling, USA
P44/42 MAP	137F5	Cell Signaling, USA
Phospho-P44/42 MAP	9101	Cell Signaling, USA
P38 MAPK	9212	Cell Signaling, USA
Phospho-p38 MAPK	3D7	Cell Signaling, USA
SAPK/JNK	56G8	Cell Signaling, USA
Phospho-SAPK/JNK	81E11	Cell Signaling, USA
Goat anti-rabbit (HRP)		BIO-RAD, USA
RelB	SC-226	Santa Cruz
TFIID (TBP)	SC-273	Santa Cru

3 Methods

3.1 Generation of monocyte derived DC

The generation of monocyte derived DC requires several steps. First PBMC were isolated from buffy coat blood. Then the monocytes were isolated from the PBMC by plastic adherence. The monocytes were then cultured and stimulated to become immature DC, before these cells could be stimulated to develop into mature DC.

3.1.1 Isolation of PBMC

The bag containing buffy coat blood was poured into a T75 cell culture flask. The amount of blood from a buffy coat bag is roughly 50ml, and this was diluted approximately 1:4 with RT PBS up to 130 ml. 12 ml lymphoprep were added to four 50 ml tubes, and 33 ml of diluted blood were carefully layered on top of the lymphoprep on each tube. The four tubes were centrifuged for 30 min and 23°C at 800g with no brake. After centrifugation, the PBMC were visible as a white band of cells between the lymphoprep and the plasma. PBMC were transferred from the four tubes into two new 50ml tubes. Cold PBS was added up to 50ml and centrifuged for 5 min and 4°C at 400g. PBMC were then washed 2-3 times with cold PBS and centrifugation for 5 min and 4°C at 400g. The supernatant was removed and pellet was resuspended in 10 ml RP10 medium.

3.1.2 Ex-vivo generation of DC

PBMC were transferred to T75 cell culture flasks, approximately $1x10^8$ cells in each flask and 10 ml in total volume. First RP10 medium was added into the flasks, and then the PBMC were transferred. Flasks were incubated 1 h in a humidified incubator at 37°C, 5% CO_2. After 1 h incubation monocytes had attached to the surface of the flasks. Non-adherent cells were collected in the medium before 10 ml RT PBS were added to each flask to wash and remove non-adherent cells other than monocytes. This wash procedure was repeated 1-2 times until few flowing cells remained. Then there were added 10 ml of RP10 medium containing 20 ng/ml IL-4 and 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) to each flask before they were incubated for 6 days in a humidified incubator at 37°C, 5% CO_2.

This stimulation develops the monocytes into immature DC. Every 2-3 days of incubation new cytokines were added directly to the flasks, at the same concentrations as before.

3.1.3 Maturation of DC

Immature DC were harvested from all T75 flasks on day 6. Cells were detached by pipetting medium up and down a few times before transferred to new tubes. Remaining cells were washed loose with ice-cold PBS w/2 mM EDTA, and this used PBS that contained remaining DC were collected into tubes. Washing steps were repeated 1-2 times until all cells were collected.

When all cells were detached and transferred to tubes, tubes were centrifuged for 5 min and 4°C at 400g. The conditioned RP10 medium was collected for further use and PBS wash supernatant was removed. Pellets were dissolved in cold PBS, collected into one tube and centrifuged again for 5 min and 4°C at 400g. Washing procedure was repeated before pellet was resuspended in 5 ml of the conditioned RP10 medium and counted. Cells were then transferred to six well plates, $1{,}5x10^6$ cells/ well, total volume in each well was 2 ml. To induce maturation of DC, incubation with different cytokines is required. In addition to IL-4 (20 ng/ml) and GM-CSF (100 ng/ml), TNFα (10 ng/ml), IL-1β (10 ng/ml), IL-6 ($1x10^3$ IU/ml) and PGE_2 (1 μg/ ml) were added.

Since I was testing how bromelain affects the maturation of DC, different concentrations of bromelain were added in addition to IL-4 and GM-CSF instead of the cytokine cocktail.

Calculated amount of medium were added to the wells before 1 ml of the cell suspention were transferred to each well. After this, the calculated volume of bromelain and cytokines were added. Wells containing immature cells that were supposed to stay immature only received IL-4 and GM-CSF. The cells were then incubated in a humidified incubator for 24h at 37°C, 5% CO_2.

Table 3.1: **Maturation stimuli of DC**

DC population	*Cell suspension **	*Bromelain ** (Stock 1 mg/ml)*	*Medium ****
Immature DC	$1,5x10^6$		1 ml
Cytokine DC	$1,5x10^6$		1 ml
Bromelain (50 µg/ml)	$1,5x10^6$	100 µl	0,9 ml
Bromelain (25 µg/ml)	$1,5x10^6$	50 µl	0,95 ml
Bromelain (10 µg/ml)	$1,5x10^6$	25 µl	0,975 ml
Bromelain (5 µg/ml)	$1,5x10^6$	5 µl	0,995 ml

* Each well was added $1,5x10^6$ cells in 1 ml. ** The amount of bromelain used. *** Volume of medium added to achieve a 2 ml total of RP10 medium.

3.1.4 Harvesting of DC

The different cell populations were checked and compared for morphological distinctions using a microscope before the cells were harvested and transferred to 15 ml tubes and centrifuged for 5 min and 4°C at 400g. Ice-cold PBS w/ 2 mM EDTA was added to each well and wells were checked in themicroscope for adherent cells. These remaining cells detached as a consequence of the ice-cold PBS w/EDTA repeatedly pipetted.
After centrifugation supernatants from all cell populations were collected, aliquoted and stored at -20°C in eppendorf tubes for later analyses.

Pellet was resuspended using the PBS from the wells, and new ice-cold PBS w/EDTA were added to the wells to collect the last part of remaining cells. This PBS was also collected into the tubes with cells, before all cells were centrifuged for 5 min and 4°C at 400g. After centrifugation the pellet was resuspended in RP10 medium and counted.

3.2 Standard-FACS for DC

Flow cytometry allows to measure properties of individual cells. The machine analyzes one cell at the time as they pass through a laser beam and cause light to scatter and fluorescent dyes to emit light at different frequencies forward scatter channel (FSC) indicates the size of the cells while the side scatter channel (SSC) shows the granularity of the analyzed cells.

These parameters are used to distinguish between different living cells and cellular debris. By staining cells with fluorochrome conjugated antibodies it is among other things possible to investigate the phenotype of the cells. Both molecules on the cell surface and targets in the cytoplasm can be analyzed with this method.

After the cells were harvested and counted, $5x10^5$ cells in 250 µl FACS-buffer were transferred into a 15 ml tube. First FcReceptor block was added to the cells (2,5 µl/$5x10^5$ cells) and incubated at RT while antibodies were prepared in a 96 well plate. Cells used for phenotype analyses were stained with FITC, PE or APC conjugated mAb specific for CD14, CD1a, HLA-DR, CD83, CD80, CD86, CD40, CCR7, CD38 and CD91. As a control FITC, PE and APC conjugated mouse IgG was used. Antibodies were prepared in master mixes and distributed to wells before 50 µl cell suspension ($1x10^5$cells) were added to each well. For the compensation and negative control, 20 µl beads were added. After cell suspension was added, cells and antibodies were incubated for 10 min in the dark at RT. Then 150 µl FACS buffer were added to each well and the plate was centrifuged 5 min and 4°C at 400g. The supernatant was removed and new FACS-buffer was used to dissolve pellets before centrifugation was repeated. The cells were then resuspended in 150 µl FACS buffer and transferred to small FACS tubes for flow cytometry analysis.

3.3 Western blot

Western blot was performed with both protein lysates and nuclear extracts. Remaining cells after the different analyses had been performed were used for protein lysate. Amount of proteins in the lysate was later determined by a BCA assay. For SDS-PAGE a 12% gel was used, and after gel electrophoresis, proteins were transferred onto a membrane by Western blotting. To be able to detect more than one protein of similar size, the membranes were stripped.

3.3.1 Preparation of protein lysate

Cells were washed with PBS and transferred to eppendorf tubes and centrifuged for 10-30s at 12100g. All supernatant was carefully removed and pellet was dissolved in freshly prepared radio-immuno-precipitation assay (RIPA) buffer (approx.100µl/$1x10^6$ cells), followed by 10 min incubation on ice. The lysates were then centrifuged for 5 min and 4°C at 17000g. The supernatant containing the proteins was transferred to a new pre-cooled eppendorf tube on ice,

and 5µl of the lysate was transferred to a second pre-cooled eppendorf tube for future BCA assay analysis. The BCA aliquot was placed at -20°C and the protein lysate was placed at -80°C.

3.3.2 Preparation of nuclear extracts

Cell suspension containing $5x10^5$ cells were transferred to 15 ml tubes and filled with 10 ml Tris buffered saline (TBS). Cells were then centrifuged for 5 min and 4°C at 400g and the pellet was dissolved in 1 ml TBS, transferred to eppendorf tubes and centrifuged for 15 s at 12100g before the supernatant was carefully removed. Nuclear extracts were prepared according to the protocol described by Schreiber et al (43). The pellet was dissolved in 400 µl cold buffer A and incubated 15 min on ice. Then 25 µl 10% NP-40 solution was added and sample was vortexed for 10s before it was centrifuged 30s at 17000g. Supernatants containing cytoplasm and RNA were collected into new pre-cooled tubes and placed at -80°C. Pellet was dissolved in 50 µl cold buffer C and incubated 15 min on ice with frequently flicking of the tubes. A 5 min centrifugation at 17000g and 4°C followed before the supernatant containing the nuclear extracts was transferred to a new pre-cooled tube and placed at -80°C. Preparation of nuclear extracts was kindly performed by members of the group of Silke Appel.

3.3.3 BCA protein Assay

The exact amount of protein in the protein lysates prepared need to be determined. This was done by BCA protein assay. A 5 µl aliquot from each sample of protein lysate were thawed on ice. Standard was prepared from 1,5 mg/ml BSA with the following concentrations; 1,5 mg/ml, 0,750 mg/ml, 0,375 mg/ml, 0,188 mg/ml, 0,094 mg/ml, 0,047 mg/ml, 0,023 mg/ml and a blank containing RIPA buffer. Unknown samples were diluted 1:5 in PBS and vortexed before 10 µl from each sample and standard were transferred to a 96 well plate in duplicates. Working Reagent (WR) was prepared by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B (50:1), and 200 µl of the WR were added to each well. The plate was covered with adherent plastic and placed in a 37°C incubator. After 1hr, the plate was read at 595 nm in a microplate reader where exact amount of proteins were defined. Results were analyzed by Soft Max Pro software.

3.3.4 SDS-PAGE

Table 3.2: **Gels used for SDS-PAGE**

Resolving gel 12%		***Stacking gel 5%***	
dH_2O	3.4 ml	dH_2O	2.85 ml
30% Acrylamide/Bis	4.0 ml	30%Acrylamide/Bis	0.85 ml
1.5M Tris-HCl pH 8.8	2.5 ml	0.5M Tris-HCl pH 6.8	1.25 ml
10% SDS	0.1 ml	10% SDS	50 µl
10% APS	50 µl	10% APS	25 µl
TEMED	10 µl	TEMED	5 µl

A 12 % resolving gel was prepared and pipetted into a MP3 glass cassette system. Isopropanol was layered on top of the resoling gel. After the gel had polymerized for approximately 30 min, the surface was rinsed with distilled water. Excess water was removed with filter paper and the stacking gel was added on top and a comb was inserted. After stacking gel had polymerized, gels were kept moist and cold until loading.

According to BCA assay results, 20 µg of each protein sample were prepared in a volume of 20 µl, diluted with RIPA buffer before 4µl 6x Lämmli buffer was added to each sample. For the nuclear extracts 15 µl of sample were used and 3 µl 6x Lämmli buffer was added to each sample. Samples were then denatured at 100°C for 5 min. Gel cassette was placed in a buffer chamber filled with 1x Running buffer. The comb was removed and wells were cleaned from unpolymerized acrylamide with running buffer using a syringe. Samples were loaded using pipette loading tips, and 6µl marker (Precision Plus Protein Standard, Kaleidoscope) was added to one well. To remaining empty wells, the appropriate amount of 6x Lämmli buffer were added. Gel electrophoresis was done at 150 V until the blue running front had left the gel, approximately 1 hour and 15 minutes.

3.3.5 Western Blot

Proteins were transferred from gel to membrane by Western blot. Wet Whatman filterpaper, gel, nitrocellulose membrane and filterpaper were placed in that order between fiber pads and placed in a gel cassette holder. Everything was kept moist by cold blotting buffer and a Bio-

ice cooling unit was placed in the buffer tank with the gel cassettes. Proteins were transferred at 250 mA for 1h, while the blotting buffer was stirred to ensure equal temperature and ion distribution. After the transfer, the membrane was washed in 1x TBST (0.5%) and proteins were visualized by adding Ponceau S solution. The membrane was then cut to appropriate size and marked before it was incubated 1h in blocking buffer at RT with agitation. This was done to prevent unspecific binding of antibodies. Phospho specific primary antibodies were incubated overnight, 1:1000 in 5 % BSA in 1xTBST (0.1 %) at 4°C with agitation. Non-phospho primary antibodies were incubated 1h, 1:1000 in 5 % milk in 1xTBST (0.5 %) at RT with agitation. Secondary antibodies were incubated 1h, 1:2000 in 5 % milk in 1xTBST (0.5 %) at RT with agitation. Membrane was washed 4 x 5 min with 1xTBST (0.5 %) between incubation with primary and secondary antibody. After 1h incubation with secondary antibody, membrane was washed 4 x 5 min with 1xTBST (0.5 %) followed by 5 min with 1xTBS. Proteins were then visualized by Immunstar™ Western C™ Kit in a Chemi Doc machine and analyzed using Quantity One software.

3.3.6 Stripping of the membrane

Membranes were stripped to remove previously bound antibodies. This enabled detection of other antibodies toward other proteins of similar size. Stripping buffer without β-Mercaptoethanol was prepared and warmed up to 60°C in a water bath. When buffer was warm, β-Mercaptoethanol was added under a fume hood. Membranes were then incubated in the stripping buffer for 30 minutes at 60°C with shaking. After incubation membranes were washed 5 x 5 minutes with 1xTBST (0.5%).

3.4 Chemotaxis

DC ability to migrate by chemotaxis towards chemokine CCL19 was tested. CCL19 binds to chemokine receptor CCR7 expressed on mature DC. A 96 transwellplate with 8 µm pore size was used. 235 µl RP10 + CCL19 (100 ng/ml) were added in the lower chamber, and $5x10^4$ DC in 80 µl RP10 were added to upper part before the plate was incubated for 5 h or 19 h in a humidified incubator at 37°C and 5% CO_2. After incubation, cells that had migrated toward the lower chambers containing CCL19 were collected with the medium from the lower chambers. 235 µl cold PBS were added to each well to collect remaining cells before they were counted using Casy cell counter. In addition to analyzing the number of cells migrated

toward medium containing CCL19, number of cells migrated without CCL19 was analyzed. This was done by using pure RP10 medium not containing CCL19.

3.5 Enzyme-linked immunosorbent assay (ELISA)

ELISA MAX^{TM} Set (Biolegend) was used to measure the cytokine secretion of the generated DC populations. Supernatants tested were collected and frozen down after the 24 h stimulation with bromelain or the cytokine cocktail.

The ELISA was performed according to the protocol of the manufacturer.

An IL-12p70 specific monoclonal capture antibody was coated on a 96-well plate. Next morning plate was washed 4 times with washing buffer. To block non-specific binding and to reduce background, plate was incubated with 1 X Assay Diluent for 1 h at RT with shaking. Plate was then washed 4 times with wash buffer. Samples and standard were then added in duplicates to the plate. Six two-fold serial dilutions of the 1000 pg/ml standard (recombinant human IL-12p70) were used. The plate was then incubated for 2 h at RT with shaking. IL-12p70 present in samples will bind to capture antibody coated onto the plate. The wash procedure was then repeated, and the detection antibody coupled to biotin was added to the plate. The detection antibody will bind to the detected IL-12p70 in the samples during the 1 h incubation in RT with shaking. The wash procedure was repeated and an avidin horseradish peroxidase (av-HRP) was added to the plate and incubated 30 min in RT with shaking. Avidin-HRP amplifies the detection by the fact that several avidin molecules can bind to the biotin coupled to the detection antibody. The wash procedure was then repeated before a 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and incubated in the dark for 15 min. TMB is a visualizing reagent that produces a blue color in proportion to the amount of protein in the sample. This reaction was stopped by adding 1 M H_2SO_4 to the plate, the blue color then turns into yellow and the absorbance was read at 450 nm in a microplate reader. Results were analyzed by Soft Max Pro software.

3.6 Mixed Lymphocyte Reaction (MLR)

A MLR was performed to analyze the ability of the generated DC populations to stimulate T cell proliferation. The system used was allogeneic, where lymphocytes from another donor were used. These cells were collected as non-adherent cells (NAC) when monocytes where isolated from PBMC.

3.6.1 Preservation of NAC in cryotubes

The cell suspension collected from the washing steps when monocytes were isolated was centrifuged for 5 min and 4°C at 400g, before the pellet was dissolved in 5 ml fetal bovine serum. Cryotubes enough for ±5x10^7 cells in each tube were prepared. 10% DMSO was added shortly before the cells were transferred to cryotubes, placed into Mr. Freeze and placed in a -80°C freezer. Mr. Freeze contains isopropanol and makes sure the cells are gradually frozen down to -80°C.

3.6.2 Thawing of NAC

One vial of NAC was taken out of the -80°C freezer and placed in a 37°C water bath. Right before it was completely thawed the vial was removed from the water bath and placed into the sterile bench. A small amount of prewarmed RP10 medium was added to the vial and the whole volume was then transferred to a 15 ml tube containing 9 ml pre-warmed RP10 medium. The vial was washed with approx. 1 ml RP10 medium from the same tube to collect remaining cells. Cells were then centrifuged 5 min and RT at 400g. Supernatant were discarded, pellet was dissolved in 10 ml warm RP10 medium and centrifugation was repeated. After centrifugation cells were resuspended in 5 ml warm RP10 medium and transferred to a T25 flask and placed in humidified incubator 37°C and 5% CO_2 over night.

3.6.3 Labeling NAC with CFDA SE

NAC were transferred from the T25 flask into a 15 ml tube. Cells were then centrifuged 5 min and RT at 400g before resuspended in 10 ml RP10 medium and counted. If cell aggregates were observed, the cells were passed through a cell filter, as it is important that the cell suspension consisted of single cells. The correct number of cells to be used was transferred into a new tube. Cells for labeling were then centrifuged and re-suspended in pre-warmed PBS/0.1% BSA at a concentration of 1x10^6cells/ml. CFDA SE was then added to the cell-suspension, 1µl of 10 mM stock/ml followed by 10 min incubation at 37°C. Five volumes of ice-cold RP10 medium were added to the cells to quench the staining, before cells were incubated 5 min on ice. Cells were then centrifuged twice at 5 min and 4°C at 400g before being re-suspended in fresh RP10 medium at a concentration of 2x10^6 cells/ml RP10 medium.

3.6.4 Co-culturing DC with allogeneic lymphocytes

DC were transferred to a 96 well cellculture plate in the concentration $5x10^4$ DC in 100 µl RP10 medium. The next day $2x10^5$ labeled allogeneic NAC in 100µl RP10 medium were added to the DC. In addition to the wells containing DC, the same amount of labeled NAC were also added to 3 empty wells used for compensation control for FITC, negative and positive control. The negative control is performed to be sure that the T cells do not proliferate without stimulation by allogeneic DC. As a positive control, one of the wells with labeled NAC was added PMA (25 ng/ml) and Ionomycin (1 µg/ml). Unlabelled NAC were also added to 3 wells for compensation controls. Wells with a total volume under 200 µl were added extra RP10 medium up to 200 µl. In addition, wells only containing DC were cultured. These were used to control that the DC populations did not contain too many lymphocytes, as these might show as proliferated cells when analyzed. Cell culture plate was then placed in humidified incubator at 37°C, 5% CO_2 for 6 days for T cell proliferation before antibody staining and flow analysis were done.

Staining with APC and PE mAb specific for CD4 and CD8 was performed as described previously in 3.2.

3.7 Statistical analysis

Statistical analyses were performed using Prism, results were analyzed using the Kruskal-Wallis test. This is a non-parametric one-way ANOVA. This test state if there is a difference between the analyzed groups. A Dunns post-hoc test was then performed to analyze which groups that were different compared to each other. The difference between groups were considered significant if $p<0.05$. Significant differences between immature cells and cytokine DC are not shown in any of the figures, as these results are not relevant for this study.

4 Results

Immature DC were generated from monocytes by stimulation with GM-CSF and IL-4 for 6 days. After the generation of immature DC, cells were matured for 24h. All cells still received IL-4 and GM-CSF. One cell population was matured with a cytokine cocktail containing TNF-α, IL-1β, IL-6 and PGE_2, while others were stimulated with different concentrations of bromelain, to investigate the maturation effect bromelain has on immature DC. Some cells were kept immature by receiving only GM-CSF and IL-4. These are referred to as immature DC, while DC stimulated with the cytokine cocktail are referred to as cytokine DC. Bromelain concentrations investigated were 50µg/ml, 25µg/ml, 10µg/ml and 5µg/ml, and these are referred to by the concentration value.

4.1 Cell viability and size

4.1.1 DC viability is reduced by adding high concentrations of bromelain

After 24h stimulation with different bromelain concentrations, the viability was examined with an automated CASY cell counter and compared to cytokine DC and immature DC. All 6 independent experiments (n=4 with 5µg/ml bromelain) revealed the same trend. Figure 4.1 shows that all cell populations stimulated with bromelain have a lower viability than DC stimulated with the cytokine cocktail. Cells that received the cytokine cocktail had the highest viability, while cells stimulated with 50µg/ml bromelain clearly had the lowest viability.
The bromelain concentration showing the highest viability is 25µg/ml.

4.1.2 Concentration dependent effect of bromelain on size of DC

Cell size after 24h stimulation was also measured. Cells stimulated with 50µg/ml and 5µg/ml bromelain appeared more similar in size to immature cells. These were smaller in size than cells stimulated with the cytokine cocktail. Stimulation with 25µg/ml and 10µg/ml bromelain resulted in DC with similar size to cytokine DC (figure 4.2).

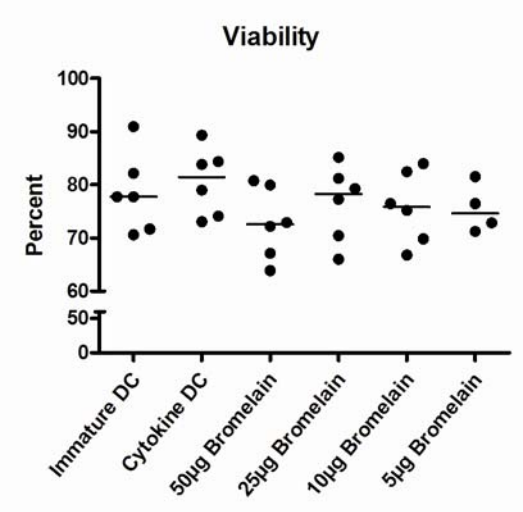

Figure 4.1: **DC stimulated with bromelain have lower viability than DC stimulated with the cytokine cocktail and immature DC**. Monocyte derived DC were incubated for 24h with different concentrations of bromelain and their viability was compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. Viability was determined with an automated CASY cell counter. Six independent experiments are shown (n=4 for 5µg/ml bromelain) and median is indicated by a bar. Cytokine DC are DC matured with the cytokine cocktail. The highest concentration of bromelain (50µg/ml) showed an apparent lower viability compared to the other populations, and all DC receiving bromelain had lower viability than cytokine DC.

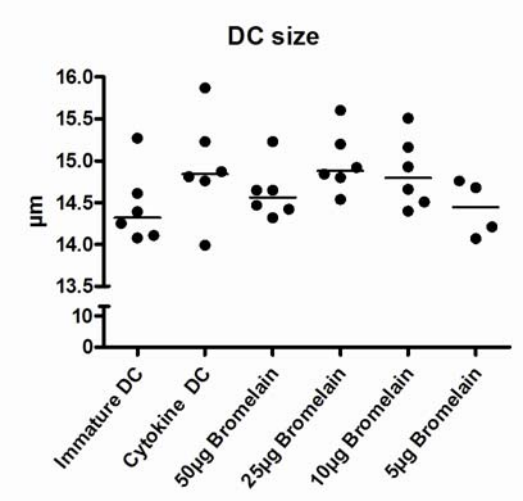

Figure 4.2: **DC stimulated with 25µg/ml and 10µg/ml bromelain have similar size to DC stimulated with the cytokine cocktail, whereas DC stimulated with higher and lower concentrations of bromelain are smaller in size.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and their sizes were compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2 and immature DC. Cell size was measured with an automated CASY cell counter. Six independent experiments are shown (n=4 for 5µg/ml bromelain) and median is indicated by a bar. Cytokine DC are DC stimulated with the cytokine cocktail. DC stimulated with 50µg/ml and 5µg/ml bromelain are smaller in size and thus more alike immature DC

4.2 Phenotype analyses of generated DC

The phenotype of the different DC populations was analyzed by flow cytometry and $2x10^4$ cells were analyzed. In order to exclude lymphocytes and cell debris from the analysis, DC were gated according to size and granularity. Figure 4.3 shows the gating strategy on a representative plot using FlowJo software.

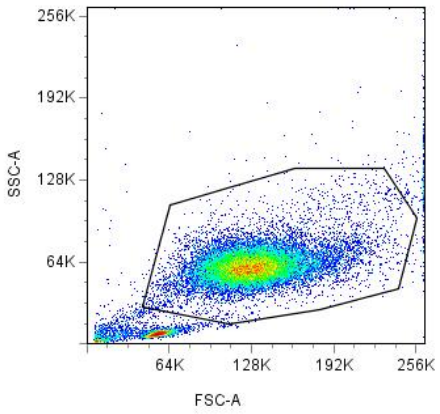

Figure 4.3: **Generated DC were gated according to size and granularity**. Forward scatter channel (FSC) indicates size and side scatter channel (SSC) indicates the granularity of the analyzed cells. When DC are gated after these conditions, lymphocytes and debris are excluded from the analysis, and only DC appear in the calculations.

4.2.1 DC markers indicate successful generation of DC

Staining with antibodies toward surface markers can give an indication of the phenotype of the generated cell populations. I could confirm by flow cytometry that the generation of DC from monocytes had been successful. As expected the DC generated had low expression of CD14 (A) in addition to high CD1a (B) expression as shown in figure 4.4. This indicates that the generated cells are monocyte derived DC.

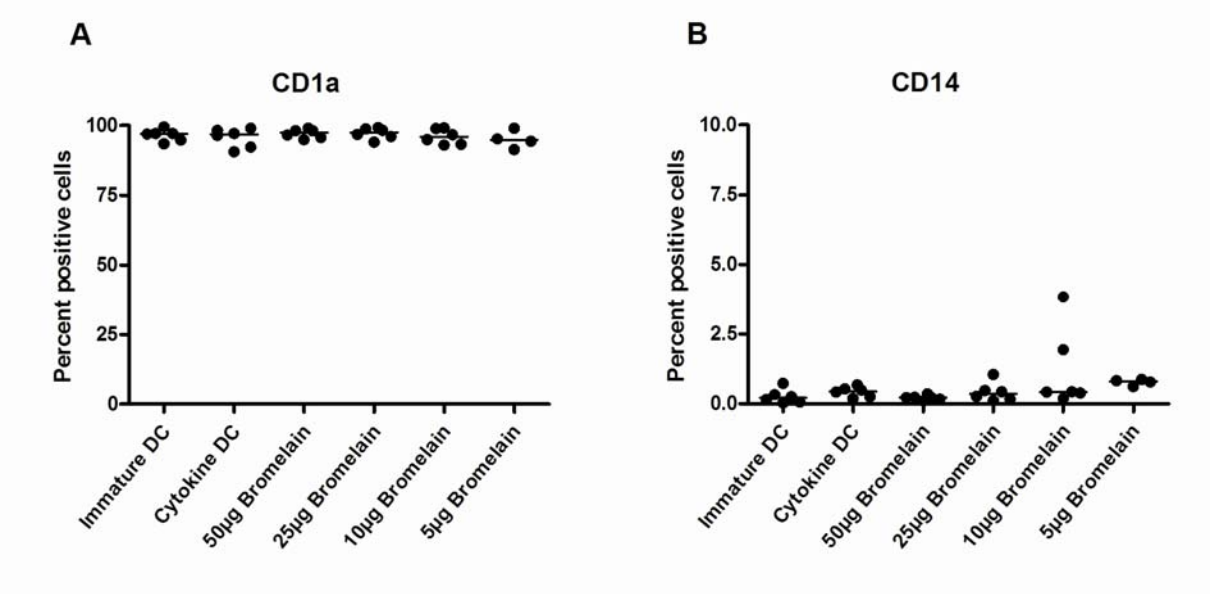

Figure 4.4: **DC stimulated with bromelain express DC marker CD1a, and they do not express CD14.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. Six independent experiments are shown (n=4 for 5µg/ml bromelain) and median is indicated by a bar. Cytokine DC are DC matured with the cytokine cocktail. Percentage of cells expressing CD14 and CD1a were determined by flow cytometry. Results reveal that all cell populations express high amounts of DC markers CD1a (A), in addition to almost no expression of the macrophage/monocyte marker CD14 (B). This indicates that generation of DC from monocytes has been successful.

4.2.2 Concentration dependent upregulation of maturation markers following treatment with bromelain

Surface markers indicating maturation for DC were examined by flow cytometry. Matured DC should have higher expression of HLA-DR and CD83 as well as lower expression of CD91 when compared to immature DC. The expression of these surface markers on bromelain stimulated DC therefore indicates the maturation effect bromelain has on immature DC. Figure 4.5 shows the expression of these surface markers in 6 independent experiments. Cytokine DC had high expression of HLA-DR (A) and CD83 (B), while immature DC showed lower expressions of these surface markers. DC stimulated with bromelain had somewhat lower expression of HLA-DR and CD83 compared to cytokine DC. CD91 expression was high on the immature DC population, while lower on the DC stimulated with the cytokine cocktail. Bromelain stimulated DC showed an even more pronounced downregulation of this marker, and a significant reduction was detected between immature DC and DC stimulated with 50µg/ml bromelain. These results indicate a somewhat less mature phenotype for bromelain treated DC compared to DC stimulated with the cytokine cocktail.

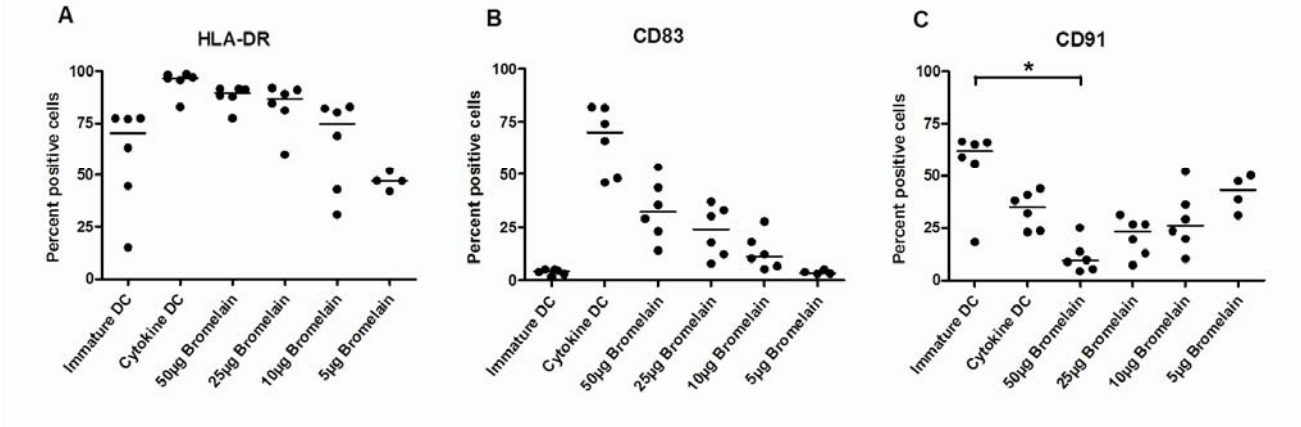

Figure 4.5: **Concentration dependent upregulation of maturation markers following treatment with bromelain** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. Six independent experiments are shown (n=4 for 5µg/ml bromelain) and median is indicated. Cytokine DC are DC matured with a cytokine cocktail. Percentage of cells expressing HLA-DR, CD83 and CD91 were determined by flow cytometry. Cytokine DC show high expression of HLA-DR (A) and CD83 (B), while immature DC show low expression of these markers. Bromelain stimulated DC expressed less for these maturation markers compared to cytokine DC, although an upregulation was detected when bromelain stimulated cells were compared to immature cells. Percentage of CD91 positive DC was lower for all populations compared to their origin, immature DC. However, higher concentrations of bromelain showed a greater decrease than the other cell populations, and 50µg/ml bromelain DC had a significant downregulation of CD91 compared to immature DC (C). Kruskal-Wallis and Dunns post-hoc test were used for statistical analyses, and * indicates $p< 0.01$.

4.2.3 Co-stimulatory molecules are expressed on bromelain stimulated DC

Proliferation and differentiation of naive T cells require an extra signal in addition to the antigen induced signal. The second signal is provided by co-stimulatory molecules present on DC and their ligands on T cells. CD40, CD80 and CD86 are all co-stimulatory molecules on DC. The expression of these surface molecules are expected to be upregulated on matured DC compared to immature DC. Results from these markers are shown in figure 4.6. In (A) the expression of CD80 is vizualized. The DC population with the highest percentage of positive cells for this marker is the one stimulated with 50µg/ml bromelain. The other concentrations of bromelain stimulated cells had CD80 expressions more like immature DC. CD86 is clearly upregulated DC treated with bromelain when compared to immature cells, although cytokine DC have an even higher expression (B). All DC populations show high percentages of cells positive for CD40 (C). While percentage of positive cells shows the number of cells expressing the molecule, the median fluorescence intensity (MFI) indicates the quantity of the

molecule on each cell. MFI measured for CD40 reveals that treatment with bromelain results in higher quantity of CD40 on the cells surface (D).

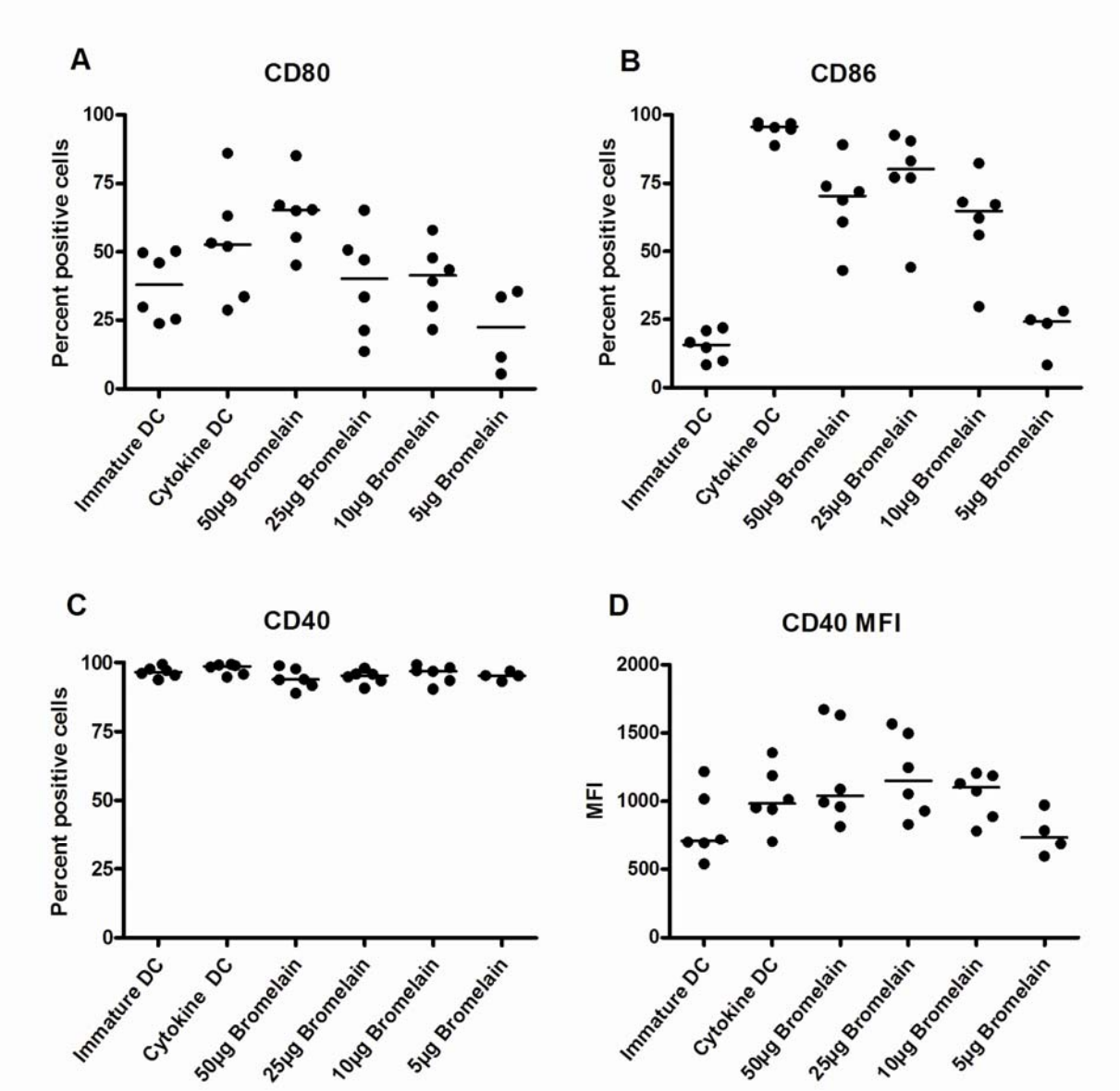

Figure 4.6: **Bromelain stimulated DC express co-stimulatory molecules, although less than cytokine DC.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. Six independent experiments are shown (n=4 for 5µg/ml bromelain) and median is indicated by a bar. Cytokine DC are DC matured with the cytokine cocktail. Percentage of cells expressing CD80, CD86 and CD40 and the MFI were determined by flow cytometry. High concentrations of bromelain stimulation lead to upregulation of CD80, lower concentrations show no upregulation compared to immature cells expression (A). CD86 is clearly upregulated on bromelain DC compared to the expression on immature cells (B). All cell populations generated show high percentages of cells positive for CD40 (C), and MFI measured indicate higher amount of CD40 on cells stimulated with bromelain (D).

4.2.4 Migration markers show low CCR7 and high CD38 expression on bromelain stimulated DC

In addition to DC and maturation and co-stimulatory markers, surface molecules known to affect migration of DC were analyzed. The ability to migrate to the lymph nodes after encounter with an antigen is important for immune responses since DC need to encounter with naïve T cells, and these have their residence in the lymph nodes. CCR7 is a chemokine receptor that drives the DC to the lymph nodes where its ligands, CCL19 and CCL21 is expressed at high concentrations. Another molecule shown to affect the migration of DC is CD38. CD38 is a 45 kD multifunctional type II transmembrane glycoprotein, and has shown to function as an ectoenzyme, regulating immune cells (44). Its expression is upregulated on activated cells and therefore proposed to be a marker for cell activation (45), but not much is known concerning its functions on DC. Figure 4.7 shows that DC stimulated with the cytokine cocktail had upregulated expression of the chemokine receptor CCR7, while only few DC stimulated with bromelain and immature DC expressed this molecule (A). CD38 expression was clearly upregulated on all populations treated with bromelain, whereas CD38 expression on cytokine DC was downregulated. Cell populations stimulated with 50µg/ml and 25µg/ml bromelain had significantly higher percentage positive cells compared to cytokine DC (B).

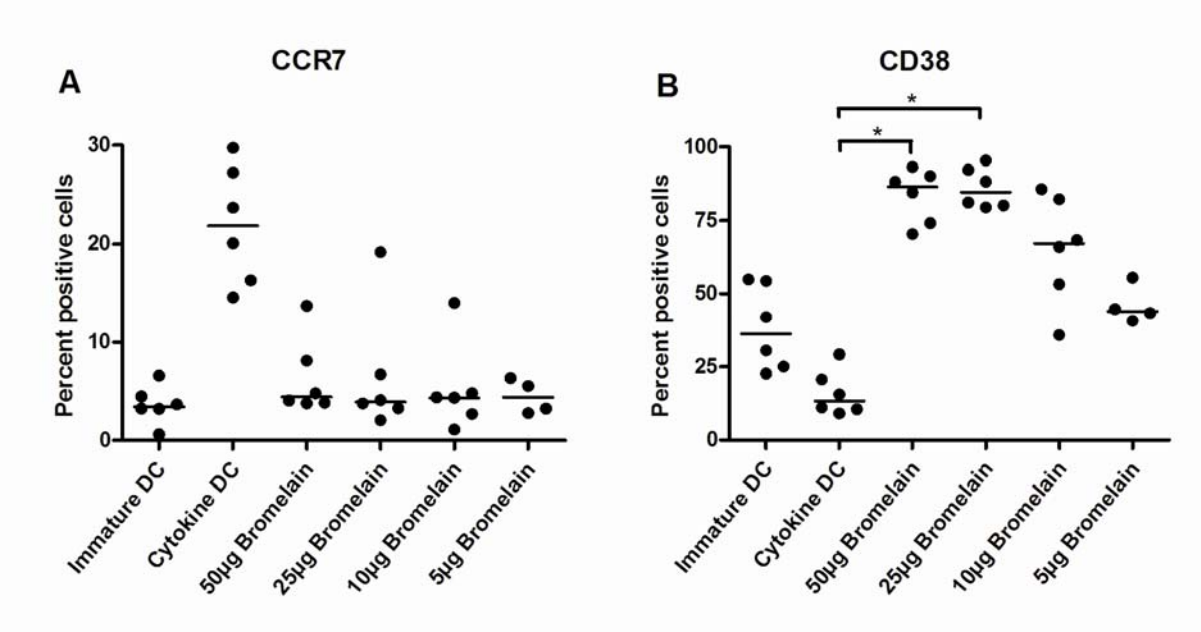

Figure 4.7: **Bromelain stimulated DC have low CCR7 and upregulated CD38 expression.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. Six independent experiments are shown (n=4 for 5µg/ml bromelain) and median is indicated by a bar. Cytokine DC are DC matured with the cytokine cocktail. Percentage of cells expressing CCR7 and CD38 were determined by flow cytometry. Bromelain treated and immature DC show low percentages of cells positive for CCR7 compared to cytokine DC(A). CD38 expression on bromelain stimulated DC was clearly increased compared to immature cells, Cells stimulated with 50µg/ml and 25µg/ml bromelain had significantly higher CD38 expression compared to cytokine DC. These showed the opposite result, where CD38 was downregulated (B). Kruskal-Wallis and Dunns post-hoc test were used for statistical analyses, and * indicates $p< 0.001$.

4.3 Phosphorylation pattern of cellular proteins

Phosphorylated and non-phosphorylated MAP kinase proteins and Akt/PKB from protein lysates were examined by Western blotting. The results were analyzed using ChemiDoc software. The band intensity for cytokine DC was set as 100% and the band intensities of the other cell populations were compared relative to this. Ratio between phosphorylated and non-phosphorylated proteins was calculated by dividing the value for the phosphorylated band by the value obtained for the total amount of the protein. Six independent experiments (n= 4 for 5µg/ml bromelain) were analyzed.

4.3.1 DC stimulated with bromelain have higher values of phosphorylated ERK and p38 and less phosphorylated JNK compared to DC stimulated with the cytokine cocktail

A representative Western blot and ratio between phosphorylated ERK (p-ERK) and total ERK are illustrated in figure 4.8. These clearly indicate that cytokine DC had lower amounts of phosphorylated ERK. In addition to DC stimulated with bromelain, immature DC also showed high values of p-ERK.

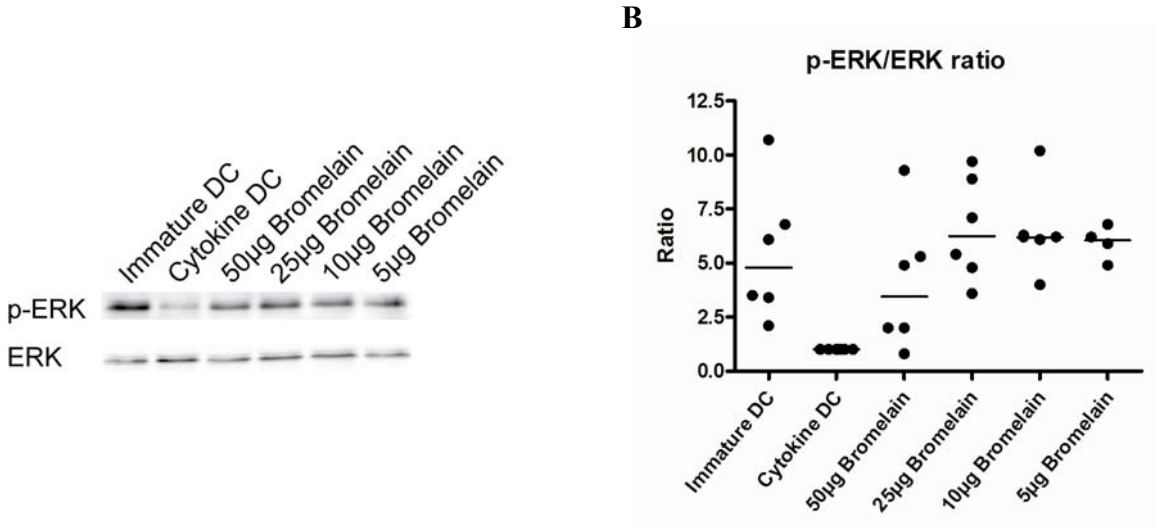

Figure 4.8: **Bromelain stimulated DC show more phosphorylated ERK than cytokine DC.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. A representative Western blot of phosphorylated ERK (p-ERK) and total ERK is shown (A), where a 12% resolving gel was used. The band density for cytokine DC was set as 100%. The ratios between p-ERK and ERK from all 6 independent experiments are shown in B. Median is indicated by a bar. All experiments showed similar results, indicating that cytokine DC had clearly lower amounts of p-ERK than immature and bromelain stimulated DC.

Results from analyses of phosphorylated p38 (p-p38) and total p38 on DC stimulated with bromelain are shown in figure 4.9. When compared to DC stimulated with the cytokine cocktail, the bromelain treated cells had more phosphorylated p38, although not clearly higher values. DC populations expressing the highest phosphorylation level of p38 is 25µg/ml and 10ml/µg bromelain.

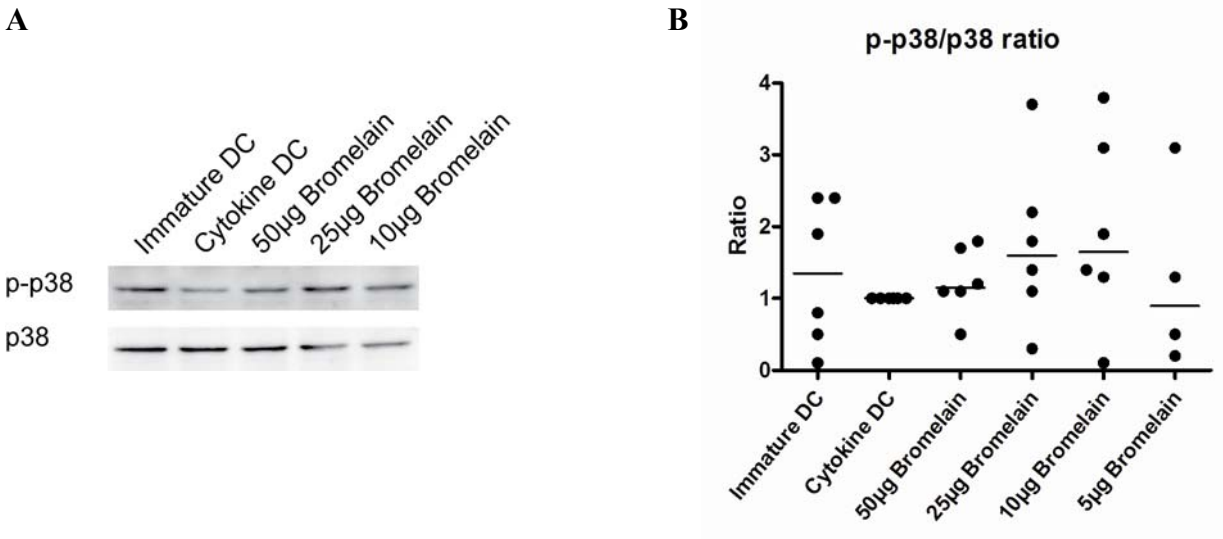

Figure 4.9: **Bromelain stimulated DC show more phosphorylated p38 than Cytokine DC.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. A representative Western Blot of phosphorylated p38 (p-p38) and total p38 is shown (A), where a 12% resolving gel was used. The band density for cytokine DC was set as 100%. The ratios between p-p38 band and p38 band from all 6 independent experiments are shown in B. Median is indicated by a bar. 25µg/ml and 10µg/ml bromelain had the highest values of phosphorylated p38, while 50µg/ml and 5µg/ml had a lower median value, closer to the median value for cytokine DC

JNK phosphorylation was also examined. As shown in figure 4.10, bromelain stimulated DC had less JNK phosphorylation than cytokine DC. This was the case for all concentrations of bromelain treated cells, and immature DC had even lower phosphorylation values. Figure 4.10 (A) shows a representative Western blot. As the figure indicates, the level of phosphorylated JNK is very low for immature DC and for the lowest concentrations of bromelain stimulated cells.

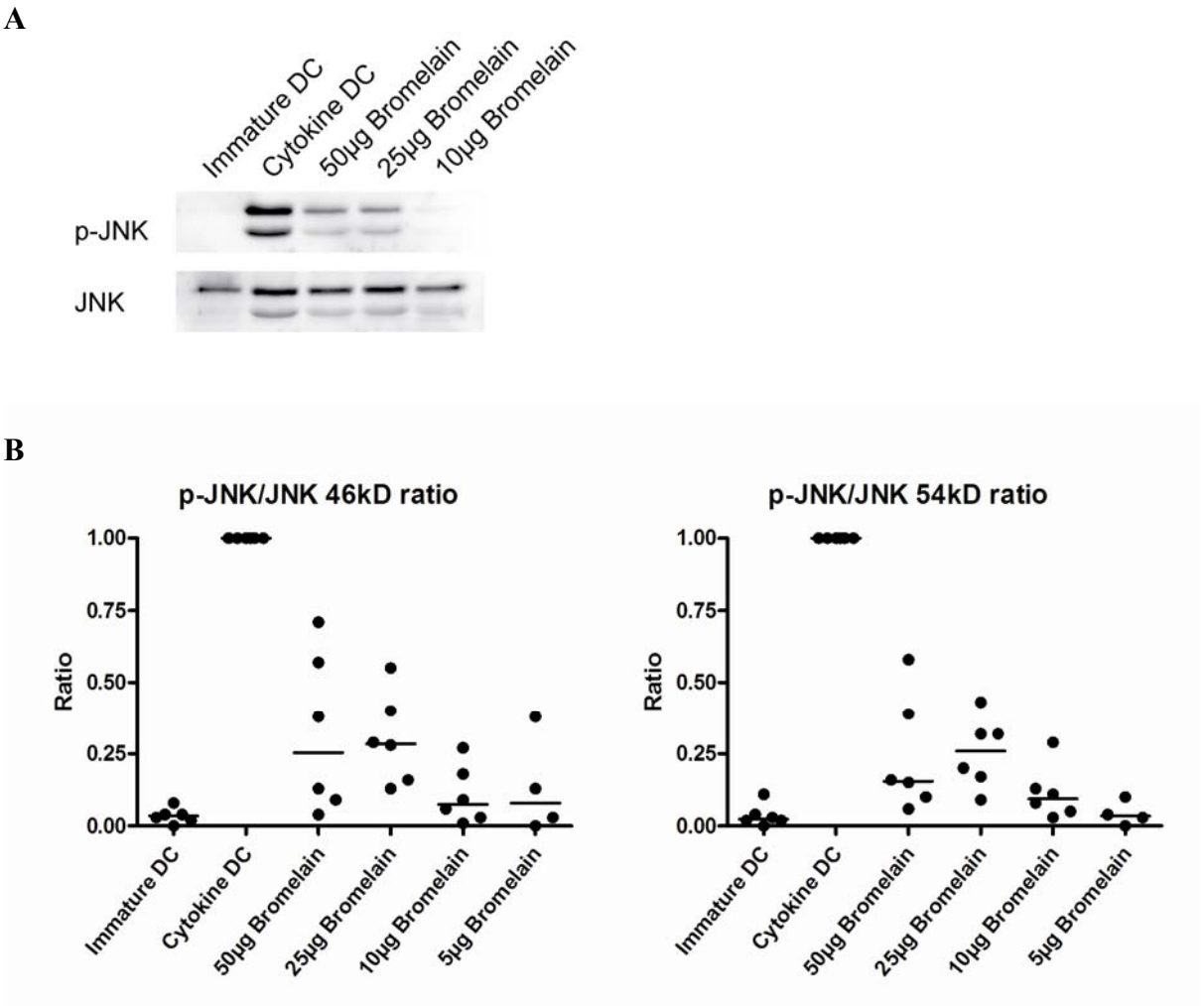

Figure 4.10: **Bromelain stimulated DC show less phosphorylated JNK than Cytokine DC.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. A representative Western blot of phosphorylated JNK (p-JNK) and total JNK is shown (A), where a 12% resolving gel was used. The band density for cytokine DC was set as 100%. The ratios between p-JNK bands and JNK bands from all 6 independent experiments are shown in B. Median is indicated by a bar. Immature DC, in addition to all concentrations of bromelain stimulated DC showed considerably lower values of p-JNK than DC stimulated with the cytokine cocktail.

4.3.2 Three experiments with similar results indicate that cytokine DC had less amount of p-AKT compared to bromelain treated cells.

Figure 4.11 (A) shows a representative Western blot of phosphorylated AKT (p-AKT) and total AKT, and (B) shows the ratio between p-AKT and AKT for 5 independent experiments. One of the six experiments had no phosphorylated AKT. Out of the five experiments with detectable p-AKT, one showed very high values for the bromelain stimulated DC while another had lower values for the same DC populations. The three experiments with similar

results indicate that cytokine DC had less amount of p-AKT compared to bromelain treated cells.

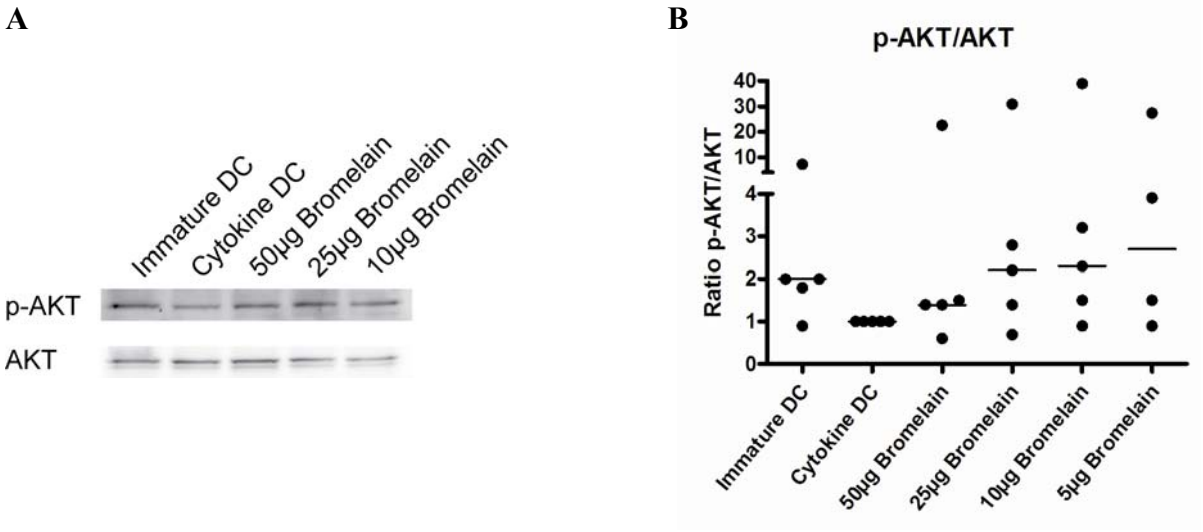

Figure 4.11: **Bromelain stimulated DC show more phosphorylated AKT than Cytokine DC.** Monocyte derived DC were incubated for 24 h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. A representative Western blot of p-AKT and total AKT is shown (A), where A 12% resolving gel was used. The band density for cytokine DC was set as 100%. The ratio between p-AKT band and AKT band from all 5 independent experiments is shown in B. Median is indicated by a bar. Out of 6 experiments, 3 had similar results, where there was detected more p-AKT in bromelain stimulated DC compared to cytokine DC. One experiment had very high values of p-AKT in the bromelain treated DC populations, another showed lower p-AKT values for these DC populations, while the last experiment had no measurable p-AKT levels.

4.4 Bromelain stimulated DC have upregulated nuclear RelB expression compared to immature DC, but they do not express as much nuclear RelB as cytokine DC

Nuclear extracts were examined and analyzed for RelB expression by Western blot. RelB is a subunit of the NF-κB transcription factor family and studies implicate RelB to be an important regulator in the differentiation of DC (46). To make sure all wells had equal loading, the blot was reprobed with an antibody against TATA binding protein (TBP). 4 independent experiments (n=3 for immature DC and 10µg/ml bromelain, and n=0 for 5µg/ml bromelain) are included in the analysis of nuclear RelB. Amounts of nuclear RelB present in the different DC populations are shown in figure 4.12.

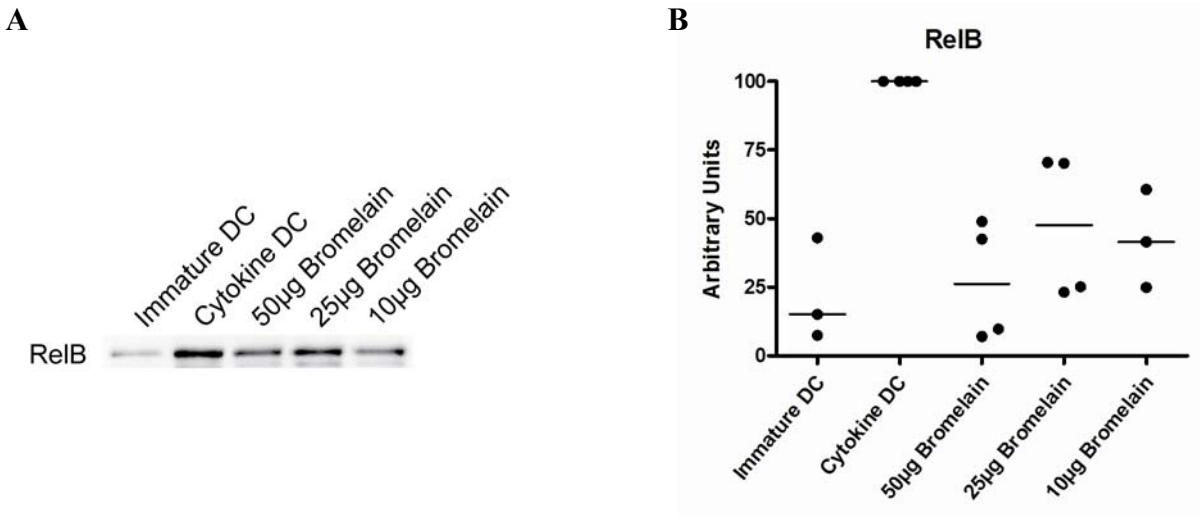

Figure 4.12: **Bromelain stimulated DC have less nuclear RelB expression compared to Cytokine DC.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. A representative Western blot of nuclear RelB expression is shown (A), where a 12% resolving gel was used. The band density for cytokine DC was set as 100%. Nuclear RelB expression for 4 independent experiments (n=3 for immature and 10µg/ml bromelain and n=0 for 5µg/ml bromelain) is shown in B, median is indicated by a bar. Compared to DC stimulated with the cytokine cocktail bromelain treated DC had less nuclear RelB. However they expressed more nuclear RelB than immature DC.

4.5 DC stimulated with bromelain produce more IL-12p70 than cytokine DC

After 24h of stimulation with bromelain and the cytokine cocktail, respectively, supernatants from all different DC populations were collected. The amount of the active form of IL-12, IL-12p70 produced by the different DC populations was analyzed by ELISA. The minimum detectable concentration of IL-12p70 was 4pg/ml. Although overall low amounts of IL-12p70 were produced, the result revealed that in 4 out of 5 experiments, bromelain stimulated DC produced more IL-12p70 than DC matured with the cytokine cocktail. Immature DC did not produce measurable amounts of IL-12. This was also the case for DC stimulated with only 5µg/ml bromelain; here in 3 of 4 experiments no secretion of IL-12 could be detected. DC stimulated with 25µg/ml bromelain had a clearly higher production of IL-12p70 compared to the other DC populations.

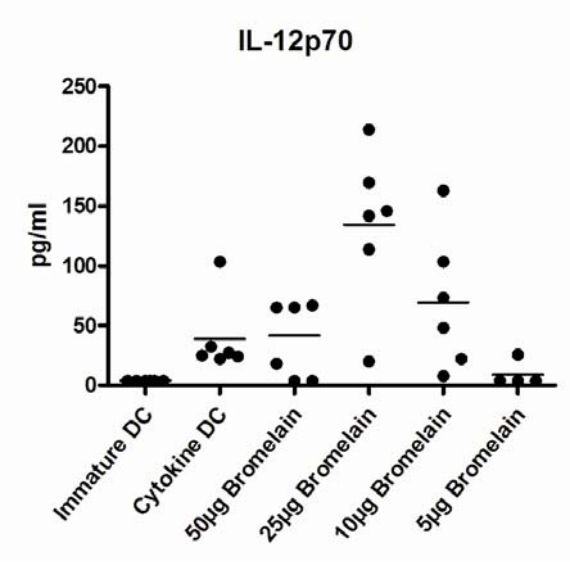

Figure 4.13: **Bromelain stimulated DC produce more IL-12p70 than DC matured with cytokine cocktail.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and compared to DC stimulated with a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2) and immature DC. Supernatants were collected after 24h stimulation and stored at -20°C. DC stimulated with bromelain produced more IL-12p70 than cytokine DC in four out of 5 experiments. DC stimulated with 25µg/ml bromelain showed a higher production compared to the other DC populations. Immature DC did not produce measurable amounts of IL-12p70. DC stimulated with 5µg/ml bromelain only had one value that could be measured while the others were under the limit (4pg/ml) for detection of IL-12p70. Median is indicated by a bar in the figure.

4.6 Chemotaxis

The migratory capacity of the generated DC populations was analyzed in a chemotaxis assay towards CCL19. Mature DC express CCR7 which is the receptor for CCL19. Because of this, mature DC are expected to migrate toward a gradient containing CCL19. Migration was performed for 5 hours and 19 hours. To control random migration, DC migrating without a gradient of CCL19 were also examined. Figure 4.14 shows two experiments performed with only 5 h incubation (A), and two experiments performed with 19 h incubation (B). The 4 independent experiments are shown separately in individual figures due to large variations. Overall, few DC migrated, however, the 19 h incubations indicate somewhat more migrated cells than 5 h incubations. The number of DC migrating toward medium without CCL19 is approximately the same as DC migrating toward CCL19, and no clear difference was discovered between the different DC populations except for less migrated immature DC after 19 h incubation (B).

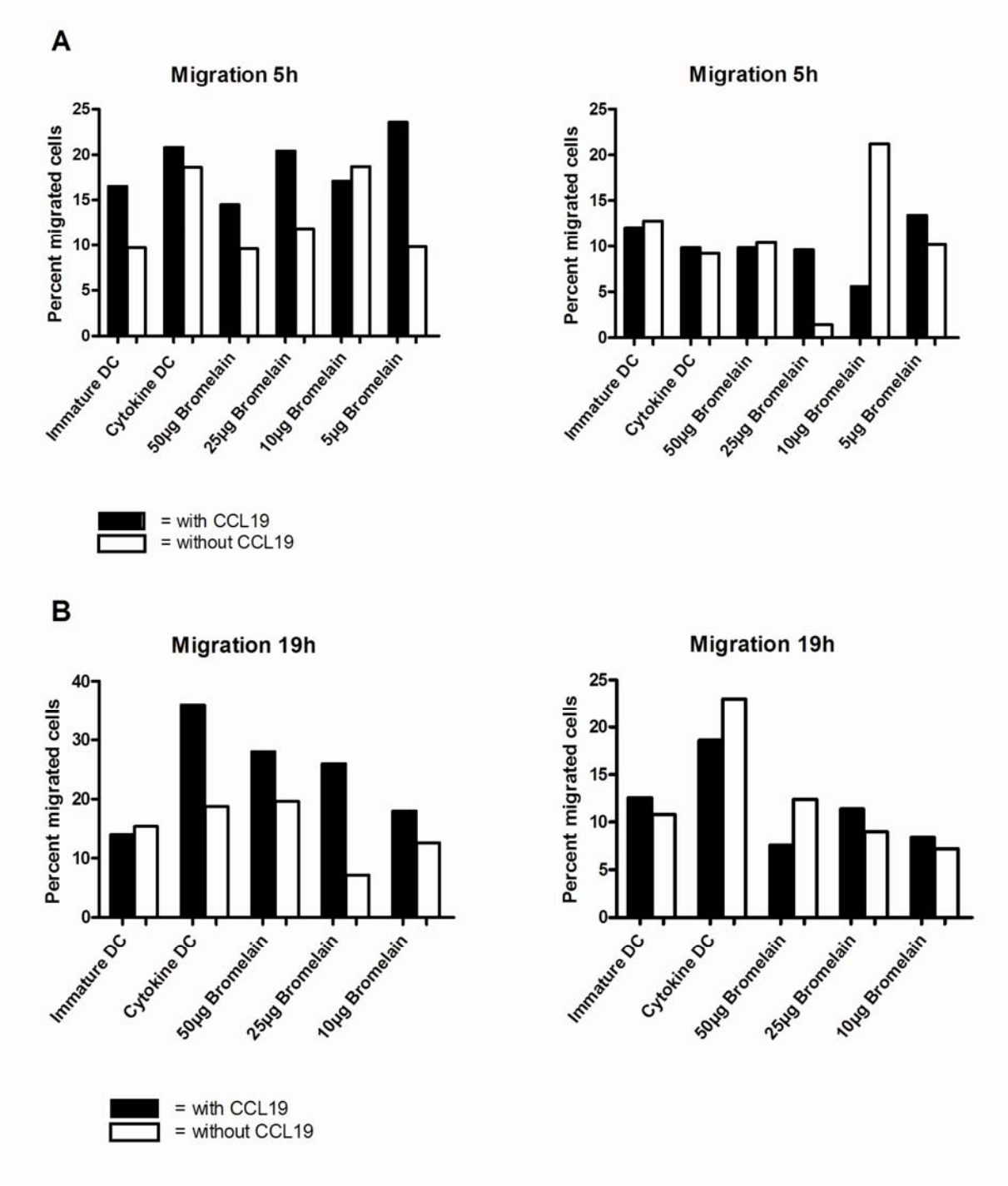

Figure 4.14: **Similar migratory capacity of the different DC populations.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain, a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2), or kept immature. After ended incubation, cells were harvested and $5x10^4$ DC were transferred to the upper chambers on a 96 transwellplate with 8 µm pore size. RP10 medium with and without CCL19 was added to the lower chambers. After 5 h or 19 h migrated DC were collected from the lower chambers and counted with an automated CASY cell counter

4.7 MLR

After 24 h of stimulation with the cytokine cocktail or bromelain, respectively, $5x10^4$ DC from each cell population were transferred to a 96 well plate. The next day $2x10^5$ NAC labeled with CFDA SE were added to each well. Cells convert CFDA SE into CFSE that can be detected. For each cell division, the fluorescence intensity of CFSE is decreased by half. In this way we can detect proliferating cells. DC and NAC were co-cultured for 6 days before the cells were harvested and their proliferation potential was analyzed by flow cytometry. Figure 4.15 shows how the proliferated lymphocytes look in a light microscope.

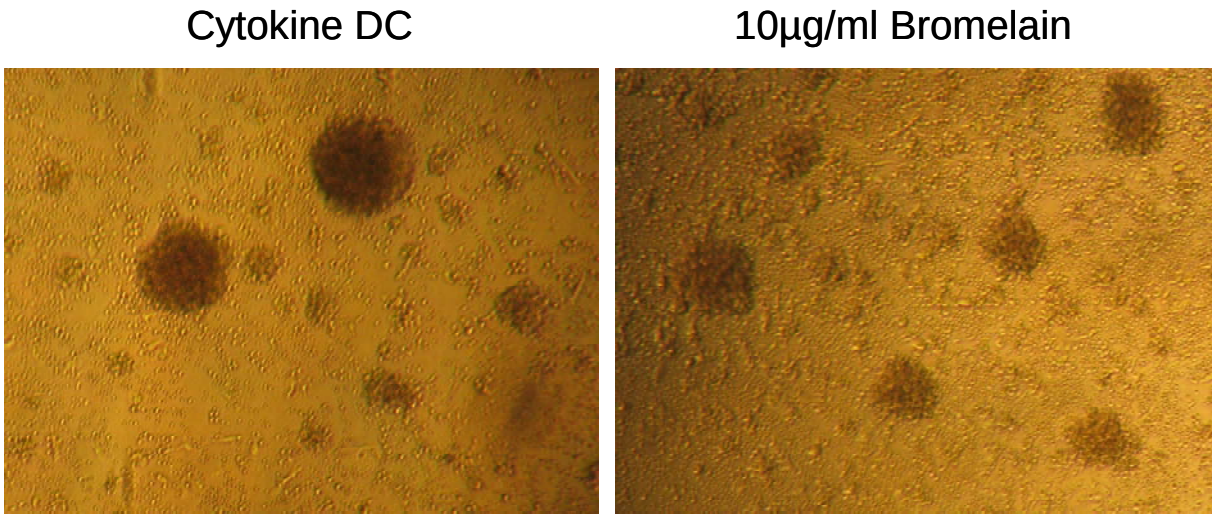

Figure 4.15: **Stimulated DC induce proliferation of lymphocytes.** Picture taken before cells were harvested after 6 days co-culture of $2x10^5$ allogeneic NAC and $5x10^4$ DC from each cell population. Distinct clustering of proliferated lymphocytes could be visualized in the light microscope (40x), here represented by NAC co-cultured with cytokine DC and DC stimulated with 10µg/ml bromelain, respectively. Both DC populations induce proliferation of allogeneic lymphocytes.

4.7.1 DC stimulated with bromelain show similar abilities to activate allogeneic lymphocytes as cytokine DC

Lymphocytes were gated according to size and granularity, this is shown in the upper panel of figure 4.16 (A) together with a control group containing CFSE labeled NAC in the lower panel. This control consists of only NAC without additional DC. Its function is to eliminate suspicion of lymphocyte proliferation induced by DC present in the NAC population, as proliferated cells would be visualized by reduced amount of CFSE in the histogram. Only one

MLR was performed, and these results are shown. Numbers of proliferated lymphocytes are shown in figure 4.16 (B). All different DC populations have the ability to stimulate lymphocyte proliferation, even immature DC. This experiment indicates that DC stimulated with bromelain have the same, if not an enhanced ability to activate allogeneic lymphocyte proliferation compared to DC stimulated with the cytokine cocktail.

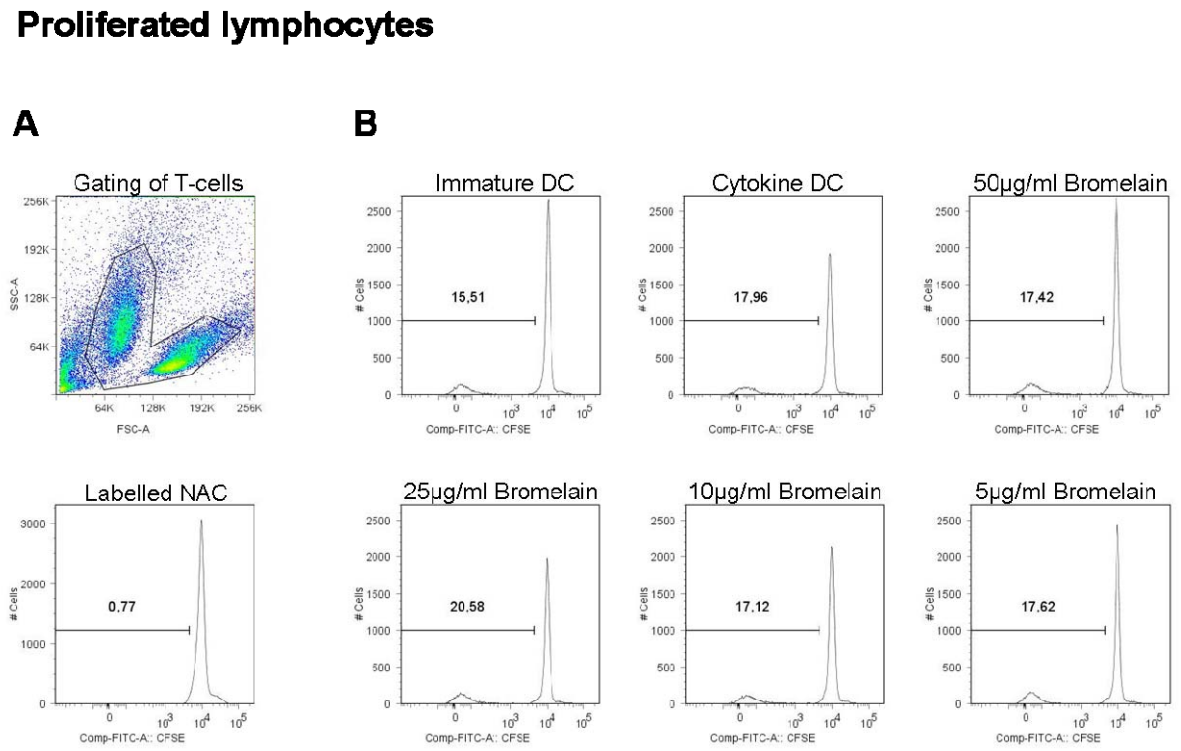

Figure 4.16: **Bromelain stimulated DC have the ability to activate proliferation of lymphocytes.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2), in addition one population of DC was kept immature. DC were then incubated with NAC/T-cells for 6 days before harvested and analyzed by flow cytometry. Figure A upper panel shows how $CD4^+$ and $CD8^+$ T lymphocytes are gated according to size (FSC) and granularity (SSC), while lower panel shows the negative control containing only labeled NAC. Figure B shows the percent proliferated lymphocytes, stimulated by the different DC populations. Numbers of proliferated cells are represented on the Y axis and the amounts of CFSE detected are shown on the X axis. The different DC populations show approximately the same ability to induce proliferation of allogeneic lymphocytes.

Amount of $CD4^+$ and $CD8^+$ T lymphocytes was also detected separately by flow cytometry in order to see which subset of T cells that proliferated. Proliferated lymphocytes were stained with CD4 and C8 antibodies and number of cells belonging to each cell population was determined. Figure 4.17 show the quantity of proliferated $CD4^+$ T lymphocytes stimulated by the different DC populations. Bromelain stimulated DC, with the exception of 50µg/ml, induces more $CD4^+$ T cell proliferation than cells stimulated with the cytokine cocktail.

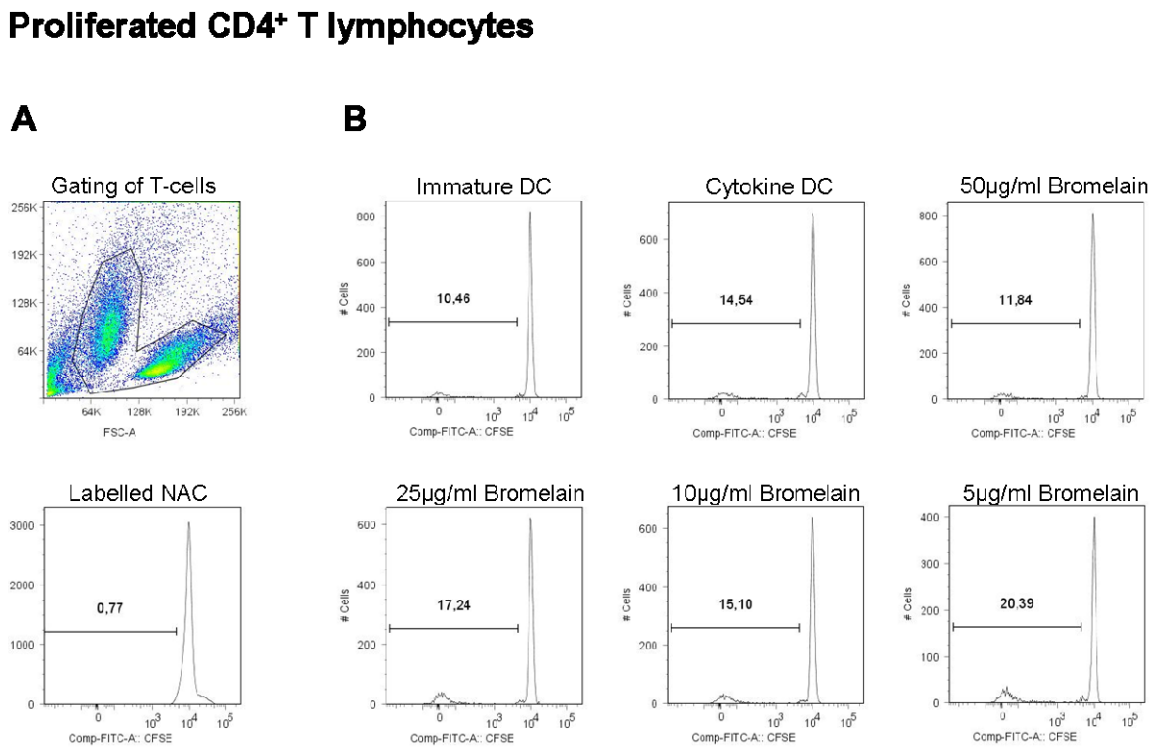

Figure 4.17: **Bromelain stimulated DC have the ability to activate proliferation of $CD4^+$ T-lymphocytes.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2), in addition one population of DC was kept immature. DC were then incubated with NAC/T-cells for 6 days before harvested and analyzed by flow cytometry. Figure A upper panel shows how $CD4^+$ and $CD8^+$ T lymphocytes are gated according to size (FSC) and granularity (SSC), while lower panel shows the negative control containing only labeled NAC. Figure B shows the percent proliferated $CD4^+$ T-lymphocytes stimulated by the different DC populations. Numbers of proliferated cells are represented on the Y axis and the amounts of CFSE detected are shown on the X axis. Bromelain stimulated DC seem to be more potent in inducing proliferation of $CD4^+$ T-lymphocytes.

Figure 4.18 show the quantity of proliferated $CD8^+$ T lymphocytes stimulated by the different DC populations. The different DC populations generated show approximately the same potential for inducing $CD8^+$ T lymphocytes proliferation.

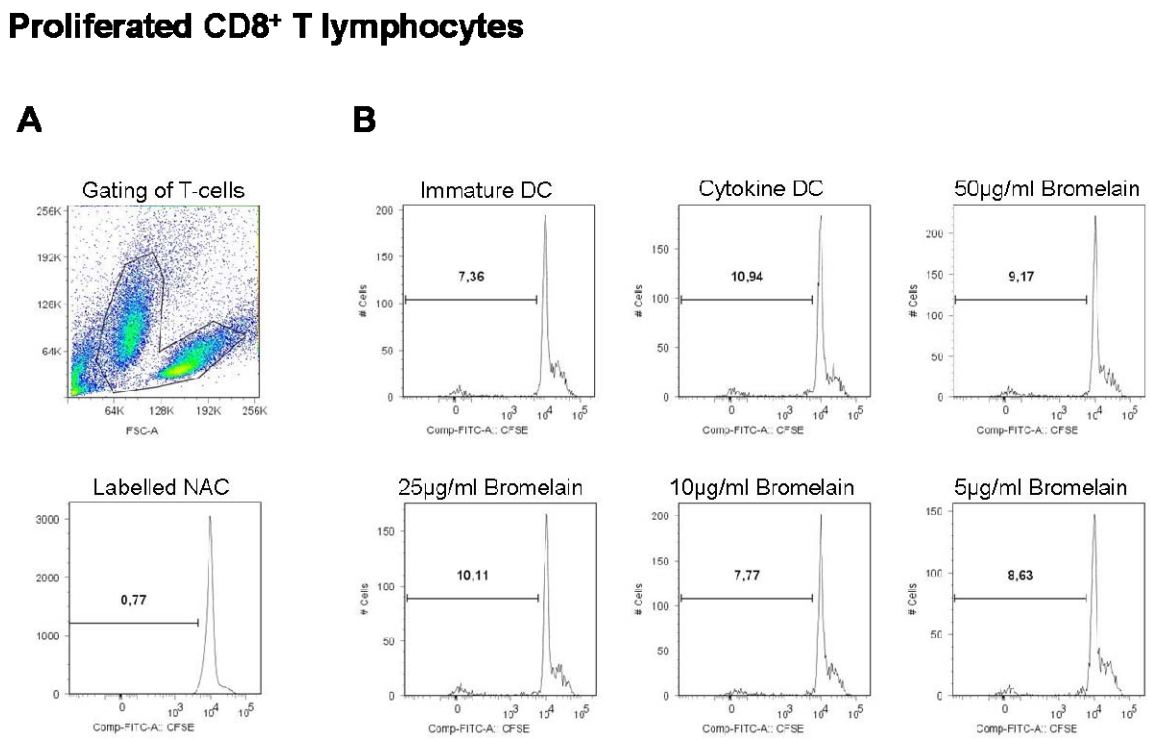

Figure 4.18: **Bromelain stimulated DC have the ability to activate proliferation of $CD8^+$ T-lymphocytes.** Monocyte derived DC were incubated for 24h with different concentrations of bromelain and a cytokine cocktail (TNF-α, IL-1β, IL-6 and PGE_2), in addition one population of DC was kept immature. DC were then incubated with NAC/T-cells for 6 days before harvested and analyzed by flow cytometry. Figure A upper panel shows how $CD4^+$ and $CD8^+$ T lymphocytes are gated according to size (FSC) and granularity (SSC), while lower panel shows the negative control containing only labeled NAC. Figure B shows the percent proliferated $CD4^+$ T-lymphocytes stimulated by the different DC populations. Numbers of proliferated cells are represented on the Y axis and the amounts of CFSE detected are shown on the X axis.

5 Discussion

Several clinical applications have shown that the maturation status of DC have an important role in initiating and directing immune responses (47). The discovery of new reagents for the promotion of DC maturation is therefore of interest for many scientists. The purpose behind this study was to see the effect of bromelain on DC maturation. Immature DC were generated from monocytes isolated from human PBMC. They were stimulated for 24 hours with various concentrations of bromelain, before being analyzed and compared to DC stimulated with the "gold standard", a cytokine cocktail consisting of TNF-α, IL-1β, IL-6 and PGE_2. Since the influence of bromelain on DC has not been investigated earlier, several different outcomes could be expected.

Previous experiments performed with bromelain on glioma cells had shown that bromelain did not produce any cellular toxicity (42). This was confirmed also during my experiments. However, cells treated with the highest concentration of bromelain, 50µg/ml, showed the lowest viability in all 6 independent experiments. In one or another way, high concentration of bromelain slightly affects DC viability, even though glioma cells were not notably affected by this.

After 24 h of stimulation with different concentrations of bromelain, the phenotype of the different cell populations was analyzed by flow cytometry. These results revealed that treatment with bromelain on immature cells did induce a signal that shifted the immature DC towards a mature DC phenotype. Although they clearly had been altered, the results indicated that they had a less mature phenotype than DC stimulated with the cytokine cocktail. Analyses of phosphorylated MAP kinase proteins and AKT in bromelain treated DC imply a phosphorylation pattern more similar to immature DC than cytokine DC. Nuclear RelB expression was lower than in cytokine DC, however it was still higher than for immature cells. Bromelain stimulated DC show a little less migratory capacity compared to cytokine DC, but the results show substantial variation and any clear conclusion is difficult to reach before more experiments have been performed. The mixed lymphocyte reaction performed indicated that bromelain DC and cytokine DC had approximately the same potential for activating allogeneic lymphocytes. Cell culture supernatants were analyzed for IL-12p70

content to investigate the different DC population's cytokine secretion. Bromelain treated DC revealed a clearly higher IL-12p70 production when compared to cytokine DC.
The results indicate a clear concentration dependent effect caused by bromelain. DC treated with the lowest concentration, 5µg/ml bromelain displayed few signs indicating any cell maturation. These cells had low upregulation of co-stimulatory and MHC class II molecules as well as low IL-12 secretion. They also showed the lowest level of nuclear RelB. In the discussion this concentration (5µg/ml) of bromelain will not be mentioned in context with the other concentrations as a maturation inducing stimuli.

The bromelain concentration that showed the most promising results was 25µg/ml bromelain. Nuclear RelB levels were most pronounced in cells stimulated with 25µg/ml bromelain, compared to the other concentrations. The most exciting result was the amount of secreted IL-12p70, where bromelain stimulated DC showed higher production than cytokine DC, and also here, 25µg/ml showed the best result. Studies have revealed that lack of IL-12p70 production is one of the most important deficiencies of the cytokine cocktail (48). Immune responses toward tumors demand strong CTL activation to be able to fight the tumor cells. IL-12 has a pivotal role concerning CTL activation, by stimulating Th1 cells that help inducing even stronger CTL responses by activating these killer cells (18). As revealed by recent studies, the use of PGE_2 in the cytokine cocktail is responsible for inhibiting IL-12 production in DC (49). However, PGE_2 does possess other good qualities, and therefore it is still used in the "gold standard" cytokine cocktail. One of the positive effects caused by PGE2 is the induction of survivin expression in DC. This has shown to increase DC viability by conferring protection from apoptosis (50). In addition studies have shown that PGE_2 stimulation of DC is central concerning their CCR7 surface expression (51).

The DC homing receptor CCR7 has been shown to be important for the ability of DC to migrate toward lymph nodes where they interact with lymphocytes and initiate immune responses (52). CCR7 was not notably upregulated on any DC population stimulated with different concentrations of bromelain, but these cells showed the same level of CCR7 expression as immature cells. Since the cytokine cocktail used contains PGE_2, it was expected that DC stimulated with the cocktail expressed higher levels CCR7. CCL19 and CCL21, which are ligands for CCR7, are expressed in lymphatic vessels and in the T cell areas of the lymph nodes. This chemokine gradient are supposed to affect the DC, and make them migrate to these areas where they meet and interact with T cells (51). However, it was discovered that

the migratory ability of the generated DC did not seem to be affected by the gradient of CCL19. Nearly the same number of cells migrated without this chemokine gradient. This was noticed since we analyzed migration toward pure medium as a control to detect any unspecific migration. Migration of DC is a complex chain of events, and obviously other factors also control and affect the movement of these cells. In the migration assay we used RP10 medium. Included in this medium is FBS that contains several unknown factors. Since these factors are unidentified it might for all we know also include CCL19, and thereby the produced gradient is eradicated. One possible solution is to use serum free medium like X-VIVO for future migration assays.

Another surface receptor on DC, CD38, has also been shown to contribute to migration of DC (53). CD38 is known to activate signals of immune cells, and induce their cytokine secretion. Some years ago it was shown that inhibition of CD38 expression reduced CCL21 driven chemotaxis in monocyte derived DC (53). CD38 is involved in activation processes of different cell types, therefore the expression of CD38 during differentiation of human monocytes into immature and mature DC was analyzed by Fedele and co-workers (45). In this study it was shown that human monocytes are $CD38^+$, and that the expression is lost during the development into immature DC, which are $CD38^-$. When these immature cells were stimulated with LPS for 48 hours, a rapid upregulation of CD38 was induced, but the effect of the cytokine cocktail was not investigated (45). Results from my experiments show that DC treated with the cytokine cocktail had low expression of CD38. Immature DC had more CD38 expressed than cytokine DC, while bromelain stimulated DC expressed even higher percentages of cells positive for CD38 (shown in figure 4.7 B). These results were obviously concentration dependent, where the highest concentrations of bromelain had the highest percentage of positive cells, and the other concentrations follow with reduced values of $CD38^+$ cells. Bromelain induces CD38 expression on DC, while the cytokine cocktail restrains this. It was recently reported that INFα induces upregulation of CD38 on DC when used as an adjuvant in combination with the cytokine cocktail (54).

CD91 is a receptor for heat shock proteins and involved in endocytosis in human cells (31). Since immature DC have the ability to endocytose while mature DC have lost this characteristic, it was expected that immature DC express more CD91 than mature cells. Figure 4.5 C shows that this is the case in my experiments. Immature DC have the highest percentage of cells positive for CD91, Cytokine DC have clearly lower values while

bromelain treated DC express even less CD91. This indicates that bromelain treated DC have poorer ability to endocytose, and therefore their action is possibly shifted toward performing tasks of mature DC such as antigen presentation instead.

Another indicator of DC maturation is the expression of nuclear RelB which is a member of the NF-κB transcription factor family. Mouse models and human studies implicate RelB to be an important regulator during the differentiation and maturation of DC (46, 55) RelB knockout mice show reduced amounts of DC, while in human cells expression is correlated with activation of DC, and RelB mRNA was only detected in the later stages of DC differentiation. (46, 55). Bromelain stimulated DC expressed various amounts of nuclear RelB, dependent on the concentration of bromelain used. Cells stimulated with 25µg/ml bromelain showed more nuclear RelB than cells stimulated with the other concentrations of bromelain. This is in coherence with the rest of my results, indicating 25µg/ml to be the bromelain concentration leading to the most matured DC population. Only 4 of my 6 experiments of nuclear RelB expression could be included in the results. When the TBP control was analyzed, two of the blots showed uneven loading of proteins, and therefore could not be used. Since a BCA assay was not performed on nuclear extracts due to incompatibility of EGTA with the assay, the exact amounts of proteins in each sample was not determined. Even though we always used $5x10^5$ cells to prepare nuclear extracts, this is not as accurate as a BCA assay.

When phosphorylation of MAP kinase proteins and AKT from my experiments was examined, the results revealed an unexpected pattern. DC stimulated with the cytokine cocktail had lower amounts of phosphorylated proteins compared to immature and bromelain treated DC. Previous analyses has shown that MAP kinase proteins activity are affected by maturation and activation of DC (19). For that reason we expected to see a variation among phosphorylated MAP kinase proteins in the different DC populations, due to different stimulations and thereby altered maturation status and signaling by the cells. Since DC stimulated with the cytokine cocktail are known to be activated, the expected result was at least that these DC would have high amounts of the phosphorylated proteins. Since other results confirm that cytokine DC have been activated and matured, it leads us to the assumption that stimulation for 24 hours may be to extensive to detect high phosphorylation values. Some proteins have their highest phosphorylation values probably a short time after stimulation. When I harvested cells and prepared protein lysates after 24 hours,

phosphorylation of MAPK proteins for the cytokine DC population probably already had passed the maximum value of phosphorylated proteins. This results in lower detected levels of phosphorylated proteins since the phosphorylation are removed after activation of the kinases and their activation of downstream proteins. If assuming this to be a possible reason for low values for cytokine DC, it is also plausible that phosphorylation values for bromelain stimulated DC also is not optimal. Therefore it is desirable to perform phospho protein analyses after shorter stimulation periods. The stimulation period was decided to be 24 h in my experiments since phenotypic analyses by flow cytometry, as well as cytokine secretion by ELISA are not measurable after shorter stimulation periods. The DC need some time to change the expression of surface molecules, in addition to translate and secrete cytokines.

In view of the fact that only one MLR was performed, more experiments are required before anything can be stated conclusively. However, these preliminary results indicate that DC stimulated with bromelain do hold a potential for stimulating lymphocytes and initiating immune responses. We did not isolate $CD3^+$ cells from NAC for this experiment, therefore it is expected that proliferated B cells also are present in the number of proliferated lymphocytes measured. However, the amount of $CD4^+$ and $CD8^+$ T lymphocytes that had proliferated were accurately quantified since these cells were detected by CD4 and CD8 specific antibodies. The DC populations used in these types of experiments could be contaminated by some lymphocytes not washed away when monocytes were isolated from PBMC. This problem could be reduced if $CD14^+$ cells were isolated from PBMC, and cultured. A purer DC population will then be generated, and the possible effect of the lymphocytes present in the culture can be eliminated. It is likely that I had some lymphocytes in my DC populations, however, my results do show differences in the number of proliferated cells stimulated by the various DC populations. This indicates that the proliferation of the lymphocytes is due to different stimulations performed by the DC populations, or else we would expect similar proliferation in all populations.

5.1 Conclusion

Although the results show that bromelain stimulated DC are less mature than cytokine DC, demonstrated by phenotype analyses, nuclear RelB expression and phosphorylation of MAP kinase proteins, these cells clearly have been affected by the treatment with bromelain. These

DC have upregulated levels of maturation markers and co-stimulatory molecules when compared to immature cells, and parts of their RelB molecules have been re-localized to the nucleus, but most importantly, they do secrete more IL-12p70 than cytokine DC. These results indicate that bromelain might be an interesting substance that could be used as an additional stimulus during generation of DC, in the further search of the optimal DC population for use in immunotherapy.

5.2 Future perspectives

This study was the first to investigate the effect of bromelain on human monocyte derived dendritic cells. I have discovered that DC are stimulated and matured after treatment with this plant substrate. The mechanisms behind these effects of bromelain on the cells are still unknown. One possibility way could be via toll like receptors, and it might be interesting to examine if TLR are involved in the effect of bromelain on DC. Since bromelain is a crude extract we do not know exactly what it contains and how the different cells respond to this. Some of my experiments need to be repeated, this include additional migration/chemotaxis analyses, as well as further MLR to see if there exist a pattern. For future migration analyses we might try to use serum free X-VIVO medium to eliminate any possible migratory abilities caused by substances in serum. In addition to these allogeneic analyses, it would be interesting to investigate the T cell stimulatory capacity in an autologous setting using tumor specific antigens, since this is the T cell stimulatory capacity that is most important in the light of immunotherapy. It could be possible to analyze phosphorylation patterns for the MAP kinase proteins for several different time points and try to locate the optimal stimulation period for maximal phosphorylation values of these proteins.

As a result from the experiments performed, 25µg/ml bromelain seems to be the most promising concentration for further use. The next step may be to analyze the effect of this concentration of bromelain in combination with the cytokine cocktail or other promising agents/substances, to see if there are any synergistic effects when combined with other stimulatory agents. One of the problems working with DC is the limited number of cells available for each experiment since these cells are not proliferating, only differentiating. Since the focus would be on 25µg/ml bromelain it could be possible to analyze phosphorylation patterns for the MAP kinase proteins at several different time points.

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