Bioprocess Development for the Generation of Monocyte-derived Dendritic Cells: Applicability in Breast Cancer Immunotherapy

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelmsuniversität Bonn

vorgelegt von

Hermann Richard Bohnenkamp

aus

Sankt Augustin

Bonn 2004

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelmsuniversität Bonn.

1. Referent: Prof. Dr. C. Wandrey
2. Referent: Prof. Dr. V. Herzog

Tag der Promotion: 24.06.2004

Acknowledgment

- Herrn Prof. Dr. Christian Wandrey für die excellenten Arbeitsbedingungen am Institut und die Übernahme des Hauptreferates.
- Herrn Prof. Dr. Volker Herzog für die Übernahme des Korreferates und das Interesse an dieser Arbeit.
- Herrn Dr. Thomas Noll für die interessante Themenstellung dieser Arbeit, die excellente Möglichkeit der Kooperation mit anderen Gruppen des EU Konsortiums, die offene Bereitschaft zur Diskussion und das Schaffen einer angenehmen und freundlichen Arbeitsatmosphäre.
- I like to thank very much Prof. Dr. Taylor-Papadimitriou and Dr. Joy Burchell for the excellent cooperation and the great possibilities during my time at Guy's Hospital.
- To Dr. Konstantinos Papazisis: for a wonderful time in London, lots of discussions, many help in the laboratory and the delicious Greek food I am very grateful. It made my time in London something special.
- Frau Bianca Schwartzkopff für eine Menge helfende Hände und eine gute Arbeitsatmosphäre.
- I like to thank very much Angela Barret, Julia Coleman and Lucienne Cooper for lots of help in molecular biology and immunology and a wonderful time in London.
- Herrn Anthony Kurumundayil für sein großes Engagement bei der Durchführung seiner Diplomarbeit.
- Allen Blutspendern und insbesondere Michele Canzoneri, Ralf Herbold, Dr. Thomas Noll und Anthony Kurumundayil möchte ich für die Blutspenden danken, ohne die diese Arbeit nicht möglich gewesen wäre.

- I am very thankful to the Breast Cancer Biology Group for many help and fun during my time in London.
- Der ganzen Zellyfamily für die Hilfe und den Spaß in Jülich.
- Ich danke meinen Eltern für die Unterstützung und ihr Interesse in der Zeit meines Studiums.
- Vor allem möchte ich Dir, Janina, danken, für die Liebe, das Verständis und die Hilfe.

Parts of this thesis were published previously:

- *Bohnenkamp H.R.*, Noll T (2003) Process development for standardized generation of monocyte-derived dendritic cells: Applicability in breast cancer immunotherapy, in: Godia F. (ed): Animal Cell Technology meets Genomics, Kluwer Academic Publishers, Dordrecht, in press.

- *Bohnenkamp H.R.* and Noll, T. (2003) Development of a standardized protocol for reproducible generation of matured monocyte-derived dendritic cells suitable for clinical application. Cytotechnology 42, 121-31.

- *Bohnenkamp H.R.*, Burchell J., Taylor-Papadimitriou J. and Noll T. Apoptosis of monocytes and the influence on yield of monocyte-derived dendritic cells. Submitted.

- *Bohnenkamp H.R.*, Coleman J., Burchell J., Taylor-Papadimitriou J. and Noll T. Breast carcinoma cell lysate-pulsed dendritic cells cross-prime MUC1 specific cytotoxic T-cells identified by peptide-MHC-class-I tetramers. In preparation.

Lecture

- *Bohnenkamp H.R.* and Noll, T. (2003) Process development for standardized generation of monocyte-derived dendritic cells: Applicability in breast cancer immunotherapy. ESACT – European society for animal cell technology, May 2003, Granada / Spain.

Poster

- *Bohnenkamp H.R.*, Burchell J., Taylor-Papadimitriou J. and Noll T. (2003) Induction of $CD4^{+}$ and $CD8^{+}$ T-cell response by breast carcinoma cell lysate-pulsed monocyte-derived dendritic cells. 2^{nd} journées des Cordeliers - latest advances in immunotherapy, October 2003, Paris / France.

Abbreviations

APC	*Antigen presenting cell*
ATCC	*American type culture collection*
AV	*Annexin V*
BCA	*Bicinchoninic acid*
BrdU	*Bromodeoxyuridine*
CBA	*Cytometric bead array*
CD	*Clusters of differentiation*
CLIP	*Class II invariant chain peptide*
CpG DNA	*Oligodeoxynucleotides rich in unmethylated guanine dinucleotides*
CT	*Cytoplasmic tail*
CTL	*Cytotoxic T cell*
d	*Days*
DC	*Dendritic cell*
DMSO	*Dimethyl sulfoxide*
dsRNA	*Double-stranded RNA*
ECD	*PE-Texas Red*
EDTA	*Ethylene diamine tetraacetetate*
ELISA	*Enzyme linked immunosorbent assay*
ER	*Endoplasmatic reticulum*
FACS	*Fluorescence activated cell sorter*
FCS	*Fetal calf serum*
FITC	*Fluorescein isothiocyanate*
FLU M1	*Influenza virus matrix protein 1*
FSC	*Forward scatter*
G-CSF	*Granulocyte-colony stimulating factor*
GM-CSF	*Granulocyte macrophage-colony stimulating factor*
GMP	*Good manufacturing practice*
GALT	*Gut-associated lymphoid tissue*
h	*Hour*
HLA	*Human leukocyte antigen*
HMFG	*Human milk fat globulin*
HPLC	*High performance liquid chromatography*
ICAM-1	*Intercellular adhesion molecule 1*
IFN-γ	*Interferon-gamma*
Ig	*Immunglobulin*
Ii	*Invariant chain*
IL	*Interleukin*
LBP	*LPS binding protein*
LPS	*Lipopolysaccharide*
m	*Milli*
μ	*Micro*

mAb	*Monoclonal antibody*
MACS	*Magnetic cell sorter*
MALT	*Mucosa associated lymphoid tissue*
MHC	*Major histocompatibility complex*
MIP-3β	*Macrophage inflammatory protein 3 beta*
MLR	*Mixed leukocyte reaction*
MUC1	*Mucin 1*
n	*Nano or number of experiments*
NK	*Natural killer cell*
PAGE	*Polyacrylamide gel electrophoresis*
PAMP	*Pathogen associated molecular pattern*
PBMC	*Peripheral blood mononuclear cell*
PBS	*Phosphate buffered saline*
PCR	*Polymerase chain reaction*
PC5	*PE-cyanin 5.1*
PE	*R-Phycoerythrin*
PGE_2	*Prostaglandin E_2*
PI	*Propidiumiodide*
PRR	*Pattern recognition receptors*
PS	*Phosphatidylserine*
rhu	*Recombinant human*
SSC	*Sideward scatter*
SD	*Standard deviation*
SDF1-α	*Stem derived factor 1 alpha (short form)*
SDS	*Sodium dodecyl sulfate*
SEM	*Scanning electron microscopy*
SM	*Stripped mucin*
TAA	*Tumor associated antigen*
TAP	*Transporter for antigen processing*
TCR	*T cell receptor*
TF	*Tissue factor*
TGF-β	*Transforming growth factor-beta*
Th	*T-helper*
TNF-α	*Tumor necrosis factor-alpha*
TR	*Tandem repeat*
U	*Unit*

List of Figures

List of Tables

Contents

Chapter 1

Introduction

Dendritic cells play a fundamental role in the initiation of effective immune responses against microorganisms and tumors. Therefore, these unique cells are very promising therapeutic tools in order to manipulate the immune system. In cell based immunotherapeutical vaccine strategies dendritic cells are thought to enhance, control and regulate the immune system to overcome tumor tolerance to combat cancer.

1.1 Objectives and Concept

This thesis is embedded into a project of the european commission of the 5^{th} frame project (Project QLK3-2002-01980): "Development of an immunotherapy for breast cancer based on dendritic cells by developing and comparing different types of tumor-specific immunogens (Cancer Immunotherapy)". In this context, the goals of this thesis are specified below:

- The development and optimization of a protocol for the standardized and reproducible generation of fully functional dendritic cells in a clinical scale.
- The transfer of the developed protocol to a fully closed system that is in accordance to GMP (Good Manufacturing Practice) requirements.
- The production of an effective immunogen based on whole tumor cell information.
- Delivery of the whole tumor cell information to dendritic cells and the evaluation in vitro using autologous T cell stimulation assays.

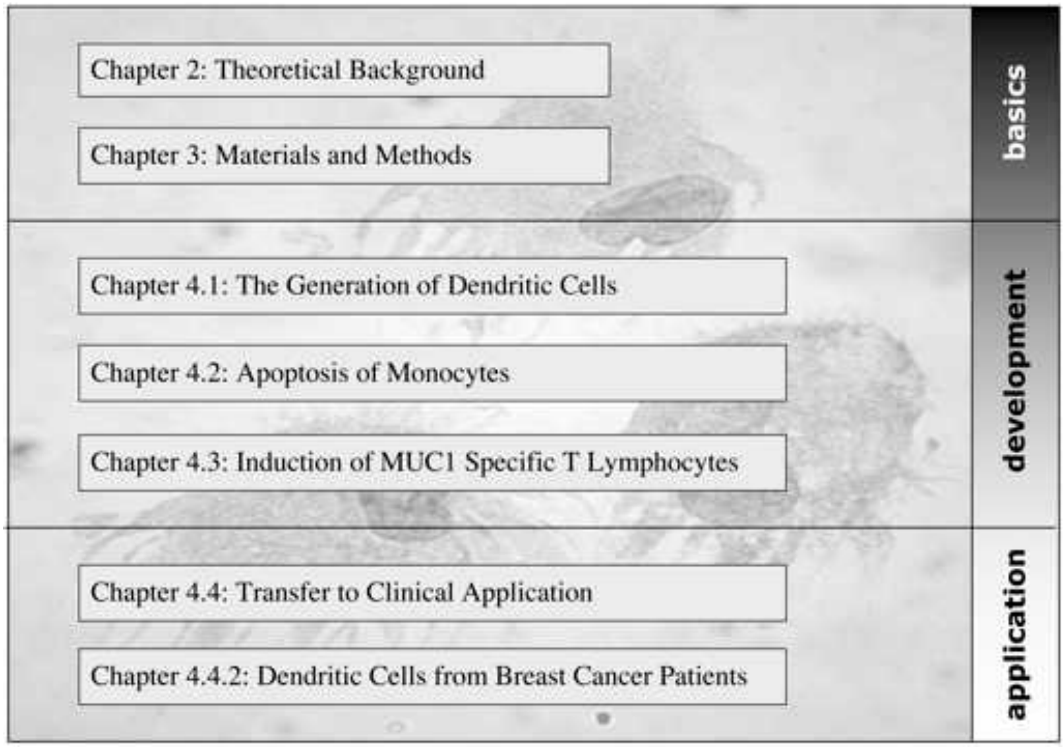


Figure 1.1: The concept of this thesis

- The applicability of the developed system needs to be proven by the comparison of the dendritic cell protocol using blood from healthy donors with dendritic cells generated from blood from breast cancer patients.

The concept of this thesis is illustrated in figure 1.1. First of all, the theoretical background is explained in chapter 2 on page 3 followed by the illustration of the generation of dendritic cells in chapter 4.1 on page 49. The chapter 4.2 on page 70 is dealing with the apoptosis of monocytes and their fate during differentiation to dendritic cells. Furthermore, the evaluation of dendritic cells loaded with whole tumor cell information is described in chapter 4.3 on page 75 by using the tumor-associated antigen MUC1 as model system. Additionally, the applicability in breast cancer immunotherapy with the development of a fully closed system and the comparison of dendritic cells from either blood from healthy donors or breast cancer patients' blood is illustrated in chapter 4.4 on page 104.

Indeed, most of the experiments were made possible by cooperation with one partner of the project, the Breast Cancer Biology Group (Cancer Research UK) at Guy's Hospital in London, UK.

Chapter 2

Theoretical Background

2.1 The Components of the Immune System

The immune system, which may be seen as an organ distributed throughout the body, protects the host against pathogens wherever these may enter. Microorganisms that cause pathology can be categorized in viruses, bacteria, parasites and fungi. In recognition and elimination of malignant transformed cells immunity also plays an important role.

The cellular and soluble components of the immune system, which are essential in protection, recognition and defense to infection, can be divided into four main parts. The first line of an effective barrier against most microorganisms form the epithelial surfaces (1) of the body, namely the skin and the mucous membranes. However, microorganisms that do succeed in crossing the epithelial surfaces have to be recognized by the body. These immune responses are mediated by leukocytes (2), the white cells of the blood, and soluble components (3) like antibodies and the complement system. The effective response is launched in the peripheral lymphoid organs (4), which are distributed throughout the body (Janeway et al., 2001).

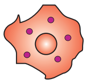
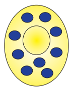
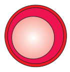
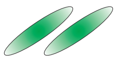
The leukocytes (white blood cells) as well as the two other cellular constituents of blood, the erythrocytes (red blood cells), that transport oxygen, and the thrombocytes (blood platelets), that trigger blood-clotting in damaged endothelium, derive from the same progenitor cells: the hematopoietic stem cells in the bone marrow. This hierarchical system of the hematopoiesis, the blood formation, is a continuous process by which all blood cells are replenished as needed. Additionally, the equilibrium between produced cells and removal of aging cells is precisely maintained. From the pluripotent stem cells two more specialized types of stem cells derive, the common myeloid and the common lymphoid progenitor cells (Encyclopedia Britannica, 2004; Kondo et al., 2003).

These two specific progenitor lines give rise to different types of leukocytes. The myeloid lineage develops completely in the bone marrow and can differentiate, accompanied by growth factors, to erythrocytes, thrombocytes, mast cells, granulocytes (neutrophil, eosinophil and basophil), monocytes/macrophages and dendritic cells. The lymphoid lineage gives rise to the lymphocytes, which are distinguished by their sites of differentiation: B lymphocytes mature in the bone marrow and T lymphocytes and natural killer (NK) cells in the thymus. The components of the immune system and their function is shown in figure 2.1 on page 5.

Functionally, a natural, innate response and a specific, adaptive immune response can be distinguished. The innate immunity depends on germline-encoded receptors, which are expressed on all cells of a particular type, e.g. macrophages or granulocytes, and recognize broad classes of pathogens to trigger an immediate response. On the other hand, the adaptive immune system uses a large repertoire of receptors encoded by rearranging genes to recognize a wide variety of molecular structures. As a consequence, adaptive immunity occurs delayed, because few B and T lymphocytes specific for the pathogen must undergo clonal expansion and become effector cells to remove the infectious agent. Thus, the immediate and early immune responses are mediated by the innate immunity, whereas the late and specific reaction is induced by the adaptive immunity (Michal, 1999).

The lymphoid organs are organized tissues with large numbers of lymphocytes and can be divided into the central or primary lymphoid organs, where lymphocytes are generated, and the peripheral or secondary lymphoid organs, where the adaptive immune response is initiated. The bone marrow and the thymus are the central lymphoid organs. Within the peripheral lymphoid organs, a series of distinct compartments can be distinguished: The peripheral lymph nodes and spleen respond to antigens that have entered the tissues or spread into the blood, the mucosal immune system (MALT - mucosal associated lymphoid tissue) including the gut-associated lymphoid tissues (GALT) and specialized structures called Peyer's patches, the body cavities and the skin. In each of these compartments specially adapted responses to pathogens are generated to a particular set of body tissues. Thus, naive lymphocytes are continually circulating between blood and secondary lymphoid organs until they have encountered their specific antigen and become activated (Janeway et al., 2001).

Innate Immunity

Component		Activated Function
Epithelial Surfaces		Physical barrier between internal milieu and external world
Macrophage		Phagocytosis Activation of bactericidal mechanism Antigen presentation
Neutrophil		Phagocytosis Activation of bactericidal mechanism
Eosinophil		Killing of antibody-coated parasites
Basophil		Response to parasitic infection
Mast Cell		Release of granules containing histamine and other active reagents
Natural Killer (NK) Cell		Detection and attacking of certain virus infected cells Triggered by invariant receptors
Complement System		Recruitment of inflammatory cells Opsonization of pathogens Killing of pathogens
Cytokines		Released by cells to an activating stimulus Induce responses by binding to specific receptors

Adaptive Immunity

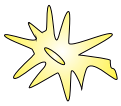
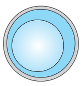
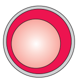
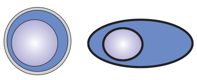
Component		Activated Function
Dendritic Cell		Antigen uptake in pheripheral tissue Antigen presentation in lymph nodes
T-helper Lymphocyte		Regulation of adaptive immune response Th1 cells activate Macrophages, NK cells and cytotoxic T cells Th2 cells activate B cells Providing immunological memory
Cytotoxic T Lymphocyte		Induction of apoptosis in cells they recognize Providing immunological memory
B Lymphocyte/Plasma Cell		Release of antibodies Providing immunological memory
Antibodies		Neutralization, opsonization, complement activation
Cytokines		Activation, regulation, inhibition

Figure 2.1: The components of the immune system and their activated function

2.2 Concepts in Immunology

2.2.1 The Adaptive Immune Response

Naive T lymphocytes, mature cells that have not encountered their antigens, are recirculating through the bloodstream and the peripheral organs, until they are induced to clonal expansion by a matching antigen. Unlike B cells, T cells do not recognize native, soluble proteins (Koch and Stockinger, 1991). Instead they are activated by their specific antigen in the form of peptide:MHC complexes on the surface of professional antigen presenting cells (APCs). However, the peptide:MHC complexes are not sufficient, on their own, for activation of naive T cells. The stimulation requires the simultaneous delivery of a co-stimulatory signal by the APCs. Thus, the first signal is mediated through the ligation of the peptide:MHC complex to the T cell receptor (TCR) and the second subset of co-stimuli are delivered by counter receptors expressed by the APCs (Acuto and Michel, 2003). The MHC restricted antigen recognition is illustrated in figure 2.2. Effector T cells can be divided into three functional classes: Cytotoxic T cells that are activated by MHC class I molecules, T-helper cells type 1 (Th1) and T-helper cells type 2 (Th2) that are engaged by MHC class II molecules. As only dendritic cells, macrophages and B cells are able to express both classes of MHC complexes and co-stimulatory signals on their surface, these cells are known as professional antigen presenting cells. However, the most potent activators of naive T lymphocytes are mature dendritic cells and thought to induce all primary T cell responses in vivo (Janeway, 1999). Furthermore they play a crucial role in the control of immunity, tolerance of self antigens and induction of long-lasting memory, one of the most important features of the adoptive immunity (Banchereau and Steinman, 1998; Sallusto and Lanzavecchia, 2002).

Antigen Recognition by T cells

T lymphocytes are recognizing antigen:MHC complexes with their T cell receptors (TCRs) accompanied by a co-receptor, namely CD4 on T-helper cells and CD8 on cytotoxic T cells. With these co-receptors T lymphocytes are able to distinguish between MHC class I with CD8 and MHC class II molecules with CD4. The TCR is a heterodimer, which is composed of an α- and a β-chain. A small subpopulation of T cells consists of a γ- and a δ-chain. Thus, two T cell populations can be divided by their different TCRs: The $\alpha\beta$ and the $\gamma\delta$ T cells. Every chain is build of a variable domain, which determines the specificity of the TCR, and a constant domain

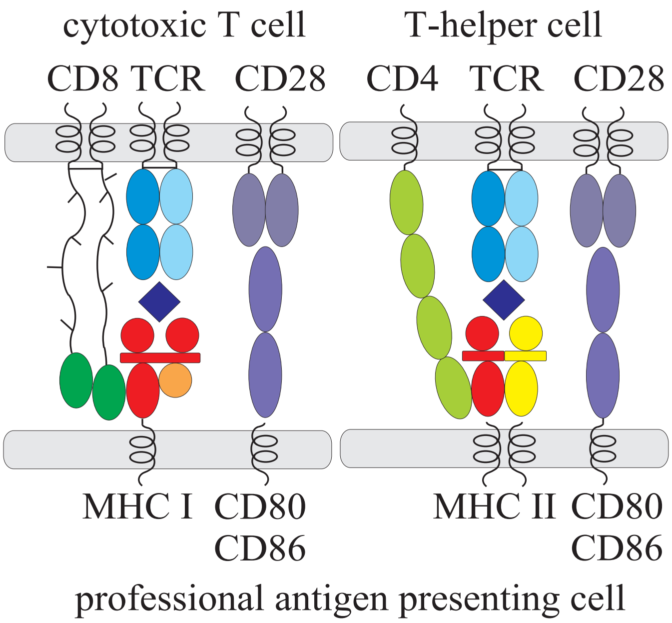


Figure 2.2: T cell recognition of antigens is MHC restricted. The specific T cell receptor (TCR) recognizes a MHC:antigen complex. Both MHC:peptide complex and co-stimulatory signal is required for activation of T cells. Cytotoxic T cells (CD8) bind to MHC class I molecules, T-helper cells (CD4) bind to MHC class II.

near the membrane (figure 2.2). The huge repertoire of T lymphocyte receptors, the variable receptor chains, are encoded in several pieces and assembled in the developing lymphocyte by DNA recombination, a mechanism known as gene rearrangement.

The MHC Molecules

Functionally, MHC molecules present antigen derived peptide fragments on their cell surface for recognition by T cells. Two important properties are essential to make it difficult for pathogens to evade: The MHC molecules are polygenic, caused by the different MHC class I and class II genes for different sets of peptide specificity and they are polymorphic, originated by different variants of each gene (alleles) within the population. This effects directly the range of bound peptides, the confirmation of the bound peptide and the direct interaction of the peptide:MHC complex with the TCR (Janeway, 2001).

The MHC class I molecule is a heterodimer of a membrane-spanning α chain, which folds into three domains (α1, α2 and α3), bound noncovalently to β2-microglobulin. The genes encoding for the MHC molecules are located in the major histocompatibility complex on chromosome 6. In humans the MHC molecules are called Human Leukocyte Antigen (HLA). The class I region contains the genes encoding for the α-chain of the MHC class I molecules HLA-A, HLA-B and HLA-C. The genes for the β2-microglobulin are located on chromosome 15 (Michal, 1999).

Two transmembrane chains, α and β, are characteristic for the MHC class II molecule, whereby each chain forms two additional domains, $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$. The subregions DP, DQ and DR in the class II region of the major histocompatibility complex contain the genes for the α- and β-chains of the HLA-DP, HLA-DQ and HLA-DR molecules.

The generation of peptide:MHC class I complexes, which are expressed by all nucleated cells of the organism, results from a multi-step process, which is illustrated in figure 2.3 on page 9. Generally, proteasomes freely diffuse in the cytoplasm and degrade proteins to peptides that range in size from 4 to 20 amino acids. These peptides are transported by an transporter for antigen processing (TAP), that is resident in the endoplasmatic reticulum (ER), where the MHC class I molecules are directly loaded. Almost all of the MHC class I associated peptides are 8-11 amino acids in length, typically 9 residues, that fit into the binding groove of the complex. The chaperones tapasin, calreticulin and ERp60 directly interact with the MHC molecule and are required for optimal peptide loading. After a successful peptide binding, the MHC class I molecules are released to the cell surface. Thus, the peptide:MHC class I complex presents peptides derived from intracellular proteins on the cell surface, which is important for detection of virus-infected and malignant transformed cells (Yewdell et al., 2003).

MHC class II molecules are synthesized, like MHC class I molecules, in the endoplasmatic reticulum. To prevent aggregation with peptides in the ER, the invariant chain (Ii) binds to the peptide-binding groove of MHC class II heterodimers and escorts them from the ER and Golgi into a vesicle that becomes part of the endosomal pathway (shown in figure 2.4 on page 9). Within this pathway, the Ii is degraded by proteases, leaving the CLIP (class II associated invariant chain peptide) fragment of Ii in the binding groove of the MHC class II complex. Engulfed antigens also enter endosomes and are digested into peptides by proteases in acidic vesicles. These peptides containing vesicles intersect with MHC class II molecules bearing vesicles to create the MHC class II compartment. The MHC encoded HLA-DM heterodimer catalyzes the release of CLIP and the binding of degraded antigens, whereby the length of the bound peptides is not constrained caused by an open binding groove of the MHC class II molecules. Finally, these peptide:MHC class II molecules are presented on the cell surface. In

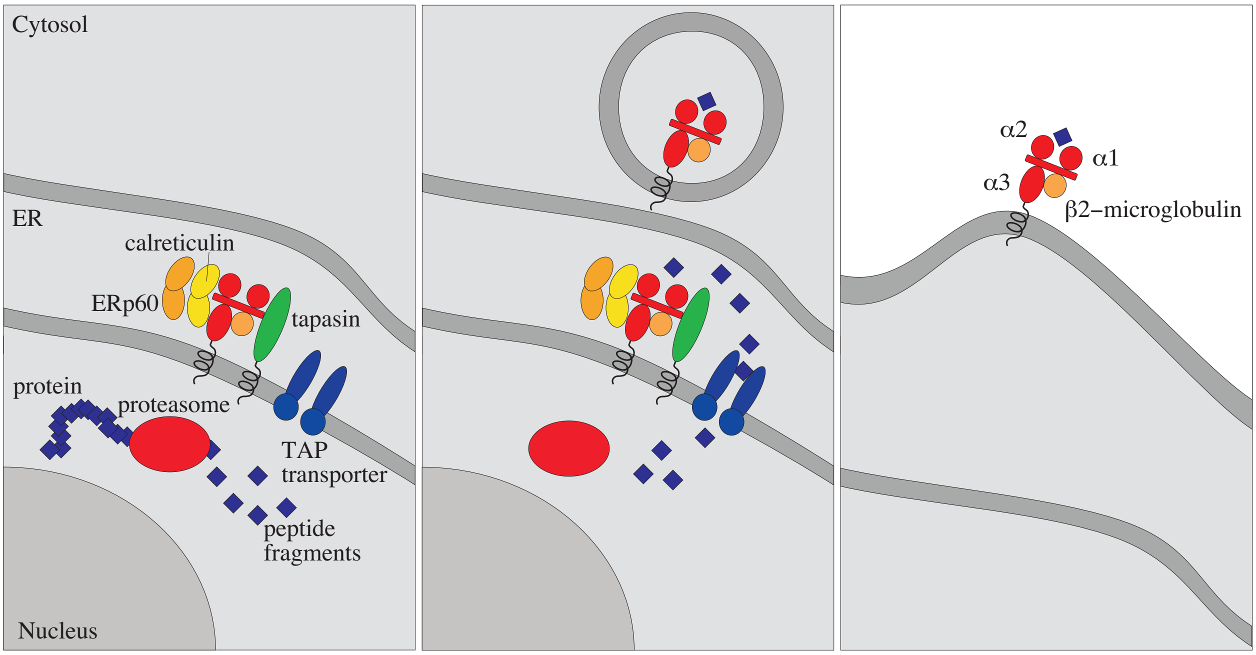


Figure 2.3: MHC class I molecules bind peptides in the endoplasmatic reticulum (ER)

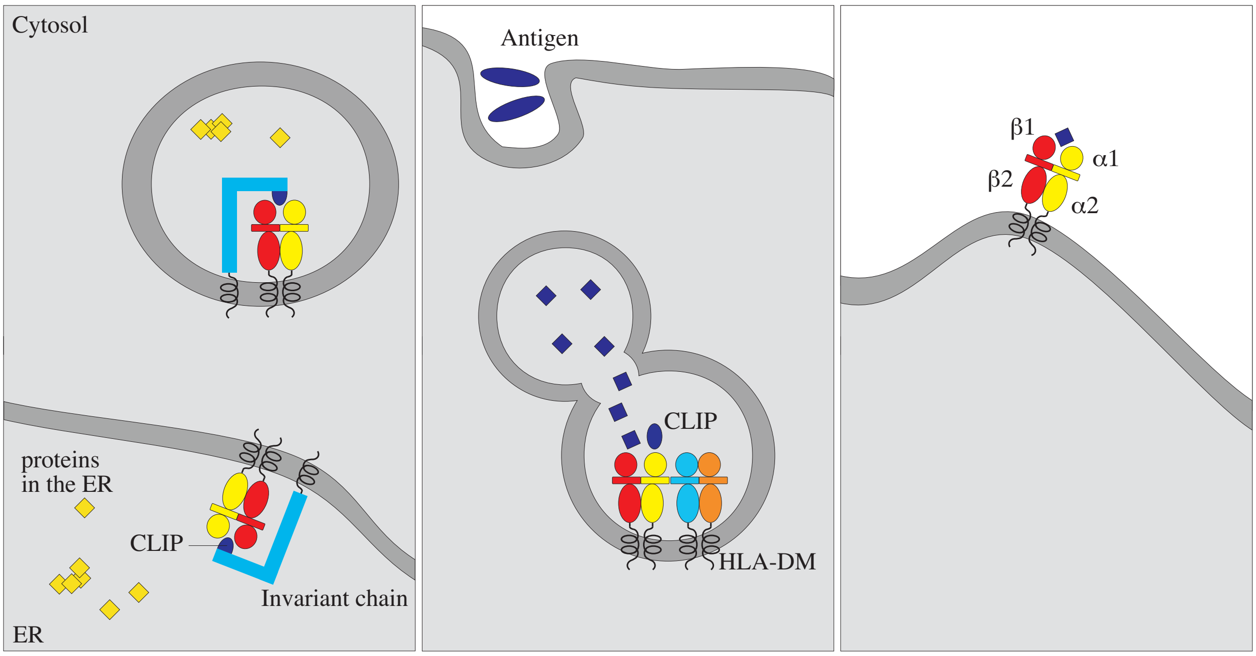


Figure 2.4: MHC class II molecules bind peptides generated in acidified endocytic vesicles. In these vesicles, HLA-DM binds to HLA class II:CLIP complexes for catalyzing the CLIP release and binding antigenic peptides

conclusion, the MHC class II pathway presents peptides derived from exogenous antigens on the surface of APCs (Bryant and Ploegh, 2004; Koch et al., 2000).

The Co-stimulatory Signal

The clonal expansion and acquisition of effector function of naive T cells depend on the strength of signals received by the T cell receptor and by an array of co-stimulatory signals. The most prominent receptors on T cells are CD28, which interacts with CD80 and CD86 on APCs, and CD40 ligand, which can be engaged by CD40. Triggering of the constitutively expressed CD28 on naive T cells by either CD80 or CD86 on APCs provides a potent co-stimulatory signal, which results in induction of Interleukin-2 (IL-2, the proliferation factor of T cells), expression of the high affinity α-chain of the IL-2 receptor (CD25) and entry into the cell cycle (Carreno and Collins, 2002). Induction of CD28 is also critical in delivering survival signals. Furthermore induction of CD28 enhances the expression of 'second wave' co-stimulatory receptors, e.g. CD40 ligand, which is critical in class-switched antibody responses and activation of dendritic cells (Acuto and Michel, 2003).

Surface molecules on cells are grouped systematically in a list named clusters of differentiation (CD). The cell-surface molecule is designated CD followed by a number. However, the number does not refer to a function or structural element of the corresponding surface protein. The discussed co-stimulatory molecules belong to different families, e.g. CD28, CD80 and CD86 to the Immunglobulin Superfamily and CD40 and CD40 ligand to the TNF (tumor necrosis factor) receptor family.

2.2.2 Dendritic Cells

Dendritic cells (DCs) were firstly described by Langerhans in 1868, who found these unique cells with their long veils in the skin. Nevertheless, it was long speculated about their function in the immune system. The identification of mouse spleen DCs by Steinman and Cohn not before 1973 established the knowledge that DCs are the initiators and modulators of the immune response (Banchereau and Steinmann, 1998; Hart, 1997). As the sentinels in vivo, they capture antigen efficiently, migrate to the lymphoid organs and stimulate naive T lymphocytes for clonal expansion, which subsequently migrate to the site of initial infection to eliminate the infectious agent (figure 2.5). In figure 2.6 on page 11 the activation of $CD8^+$ and $CD4^+$ T cells by dendritic cells and the involved receptor molecules are illustrated.

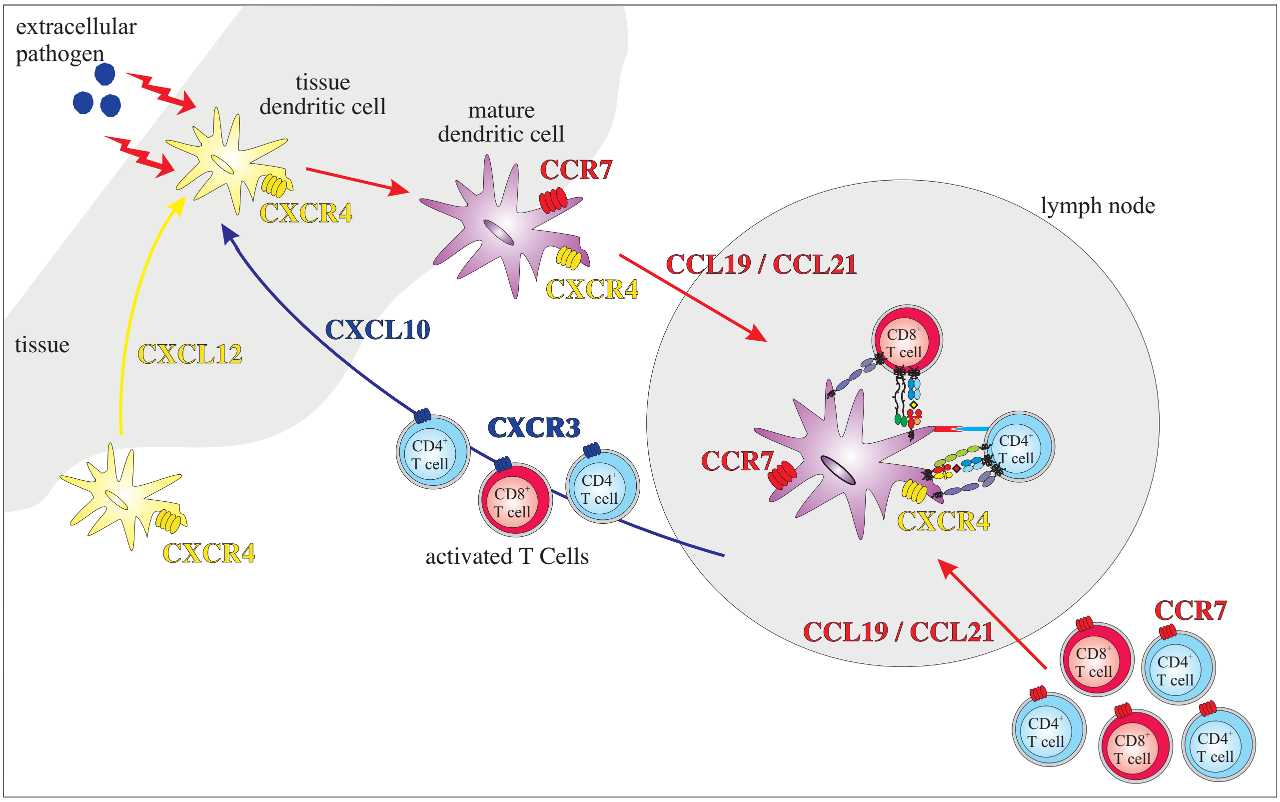


Figure 2.5: In tissue resident dendritic cells efficiently take up antigen and migrate towards lymph nodes. During this time they get matured and activate T cells in draining lymph nodes.

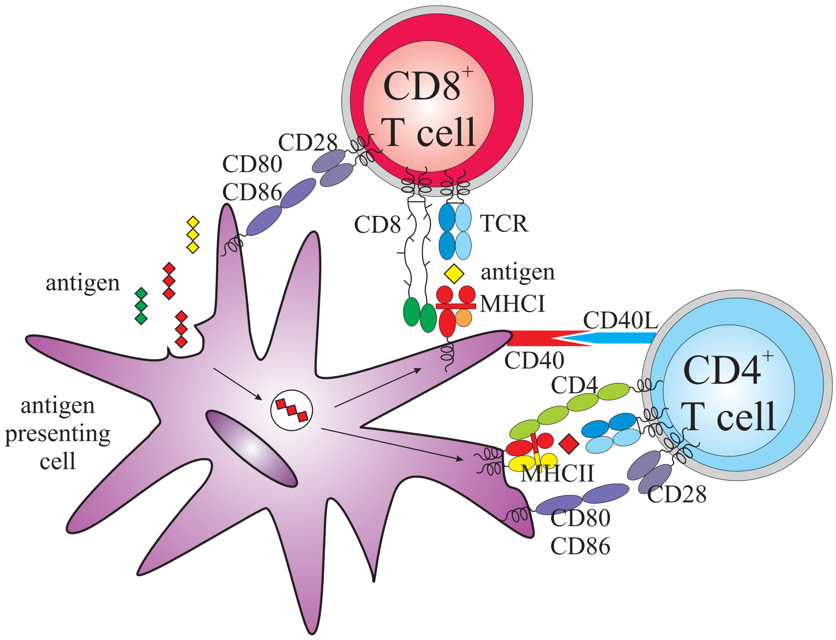


Figure 2.6: Dendritic cell stimulation of naive T cells is required for clonal expansion

The unusual shape gives dendritic cells their name: Many fine veils or dendrites (Greek: $\delta\epsilon\nu\delta\rho$o, dendro: tree) are displayed in isolated cells. This suits the function of efficient uptake of antigen and presentation to lymphocytes by a high amplification of the surface (Bell et al., 1999).

Antigen uptake and presentation are the main functions of dendritic cells. For this reason, two defined phenotypes can be distinguished: the immature and the matured. Immature dendritic cells can efficiently endocytose solid antigens or apoptotic cells by receptor-mediated phagocytosis (Greek: $\phi\alpha\gamma\epsilon\iota\nu$, phagein: eating) or soluble antigens by receptor-independent macropinocytosis (Greek: $\pi\iota\nu\epsilon\iota\nu$, pinein: drinking). With both pathways dendritic cells can present the exogenous antigen not only on MHC class II molecules, but also in the context of MHC class I. This unique feature of DCs is called cross-presentation and consequently the stimulation of $CD8^+$ cytotoxic T cells cross-priming (Guermonprez et al., 2002). It was shown on antigens bound to latex beads that these can be processed by both phagocytosis and macropinocytosis and cross-presented on dendritic cells (Shen et al, 1997; Reis e Sousa and Germain, 1995). Recently it was suggested that cross-presentation is controlled by the existence of phagosomal compartments, generated through fusion of the endoplasmatic reticulum with endolysosomal vesicles (Lizée et al., 2003).

The adaptive immunity starts with the maturation of dendritic cells by direct and indirect activation by pathogens. Dendritic cells directly recognize conserved microbial molecules, known as pathogen-associated molecular patterns (PAMPs), with so called pattern-recognition receptors (PRRs). An important group of these PRRs are Toll-like receptors (TLRs), e.g. TLR4 recognizes lipopolysaccharide (LPS), which is contained in the cell walls of gram-negative bacteria. Indirectly, DCs are affected by the large series of inflammation associated tissue factors like cytokines, e.g. Tumor necrosis factor-α (TNF-α), Interferon-γ (IFN-γ) or Prostaglandin E_2 (PGE_2). Generally, the maturation process induces the up-regulation of MHC class I and MHC class II molecules and of co-stimulatory signals like CD80, CD86 and CD40. Furthermore their ability to take up antigen is lost. Such activation of DCs by microorganisms or tissue factors is known as polarization, because different types of dendritic cells are induced. This polarization results in either type 1, type 2 or regulatory type dendritic cells (illustrated in figure 2.7). These DCs differ in their activation of immune responses. While type 1 DCs induce a cell mediated immunity, accompanied with IgG1 and IgG3 isotype antibodies and an inflammatory response, type 2 DCs trigger a humoral immune response with IgG2, IgA and IgE antibodies.

The regulatory type DCs induce tolerance by a rather immature phenotype (Kapsenberg, 2003).

The maturation process is accompanied by up-regulation of chemokine receptors, namely CCR7, for homing to the lymph nodes and induction of T cells (figure 2.5 on page 11). The CCR7 ligands CCL19 and CCL21 play an important role in directing the migration of the antigen-loaded mature dendritic cells. Chemokines are also involved in attracting naive T and B cells into the lymph nodes, which results in the contact with the activated DCs. The chemokine CXCL10, a ligand for CXCR3 on activated T cells, mediates the immune response of T cells to the site of infection (Luster, 2002).

2.2.3 The T-helper Cell Pathway

Dendritic cells, as potent stimulators of the immune system, can process and present exogenous antigens in the context of MHC class I and MHC class II and therefore cross-prime $CD8^+$ cytotoxic T lymphocytes (CTL) and stimulate a T-helper cell response (Brossart et al., 1997, Guermonprez et al., 2002). Thereby DCs bias the development of specific effector $CD4^+$ T cell subsets, which induce different components of cellular and humoral immunity. The generation of these polarized T-helper type 1 (Th1) and type 2 (Th2) cells is promoted by the cytokine environment, the dose of the antigen, strength of antigenic stimulation, duration of the T cell receptor engagement and the nature and quantity of costimulatory molecules (Constant and Bottomly, 1997; Kapsenberg, 2003; Langenkamp et al., 2000; O'Garra and Arai, 2000). However, the T cell stimulation and Th1 and Th2 polarization require three dendritic cell derived signals. The first signal is the antigen-specific signal, the second is given by co-stimulatory molecules and the third, the polarizing signal, is mediated by soluble or membrane-bound factors (Kapsenberg, 2003). The differentiation into Th1 cells is encouraged by IL-12p70 and IFN-γ (figure 2.8). Interestingly, unlike IFN-γ, which is preventing the outgrowth of Th2 cells, IL-12p70 has no effect on the Th2 development (Constant and Bottomly, 1997). In contrast, IL-4 and IL-10 are affecting the priming of Th2 cells (figure 2.9). While IL-4 induces Th2 cell development directly, IL-10 has been reported to suppress IFN-γ producing Th1 cells (O'Garra and Arai, 2000). The enhancement of CD40 - CD40 ligand interaction is also important to induce either IL-12p70 or IL-4 production. Figure 2.10 on page 16 illustrates the interrelated control mechanism of the Th1 and Th2 pathway.

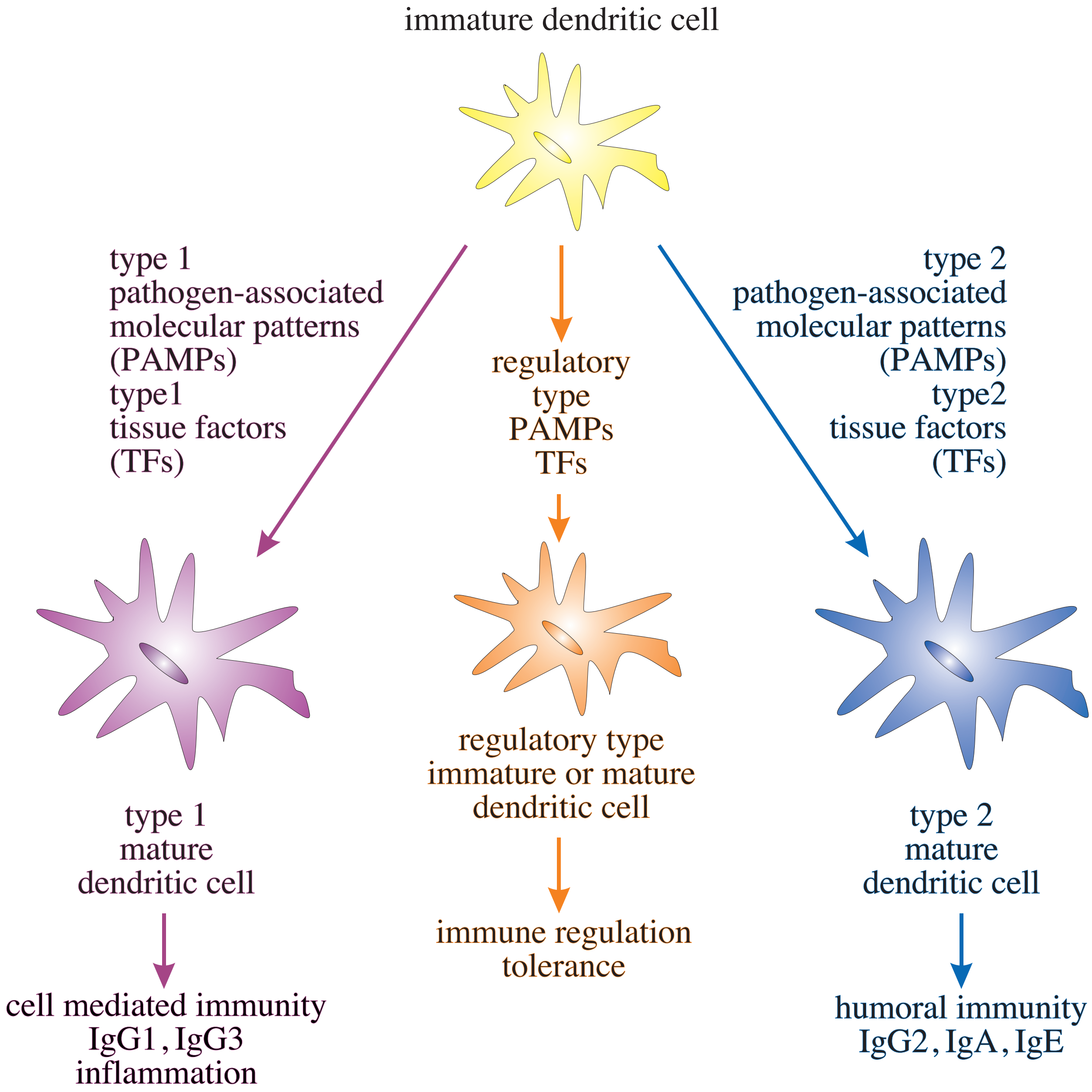


Figure 2.7: Immature dendritic cells (DCs) can be polarized by type 1, type 2 and regulatory type pathogen-associated molecular patterns (PAMPs) or tissue factors (TFs) to become different mature effector DCs

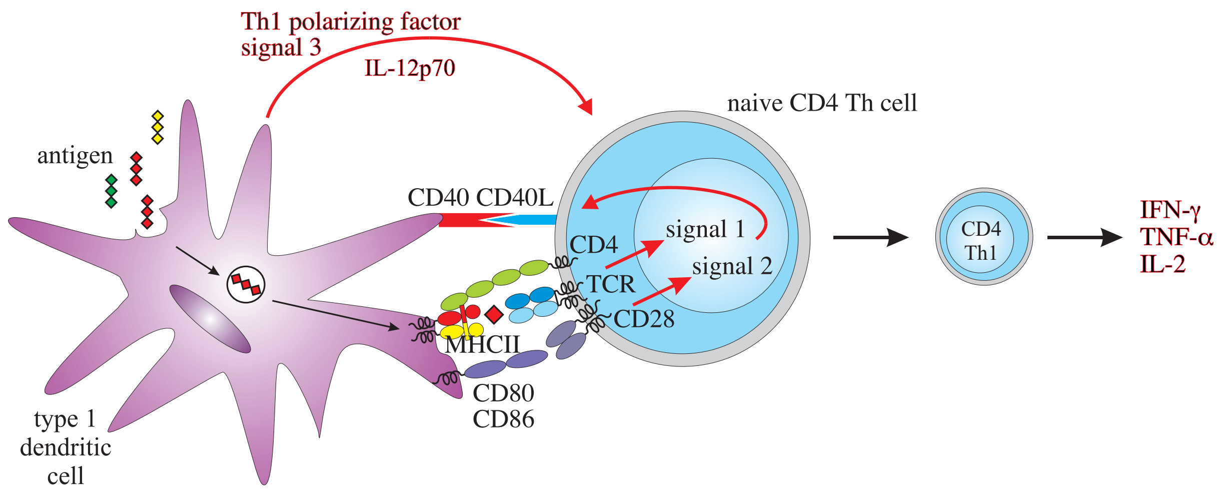


Figure 2.8: T cell stimulation and T-helper cell 1 (Th1) polarization require three dendritic cell signals: MHC class II:TCR interaction, the co-stimulatory signal and IL-12p70

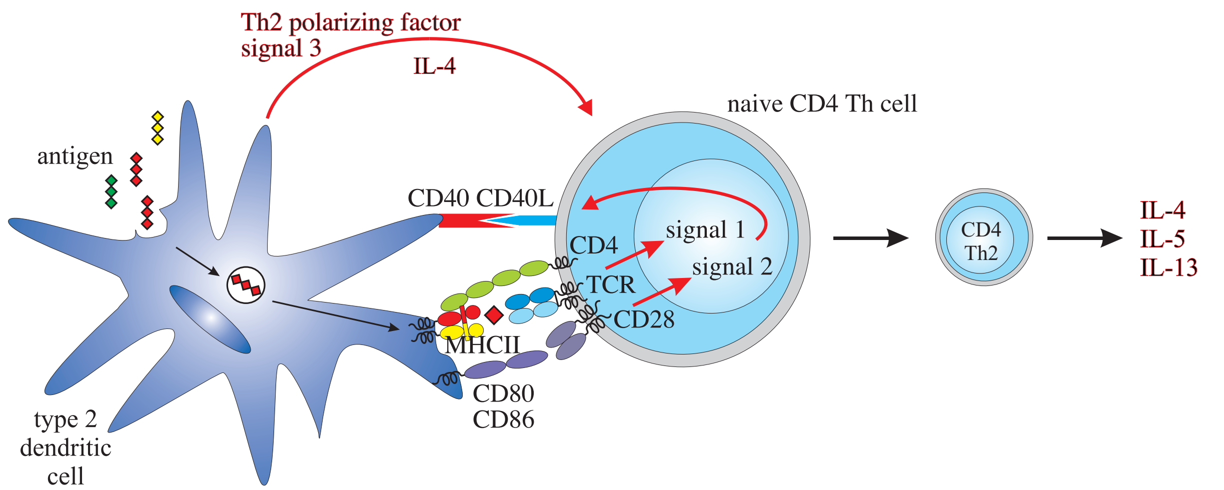


Figure 2.9: T cell stimulation and T-helper cell 2 (Th2) polarization require three dendritic cell signals: MHC class II:TCR interaction, the co-stimulatory signal and IL-4

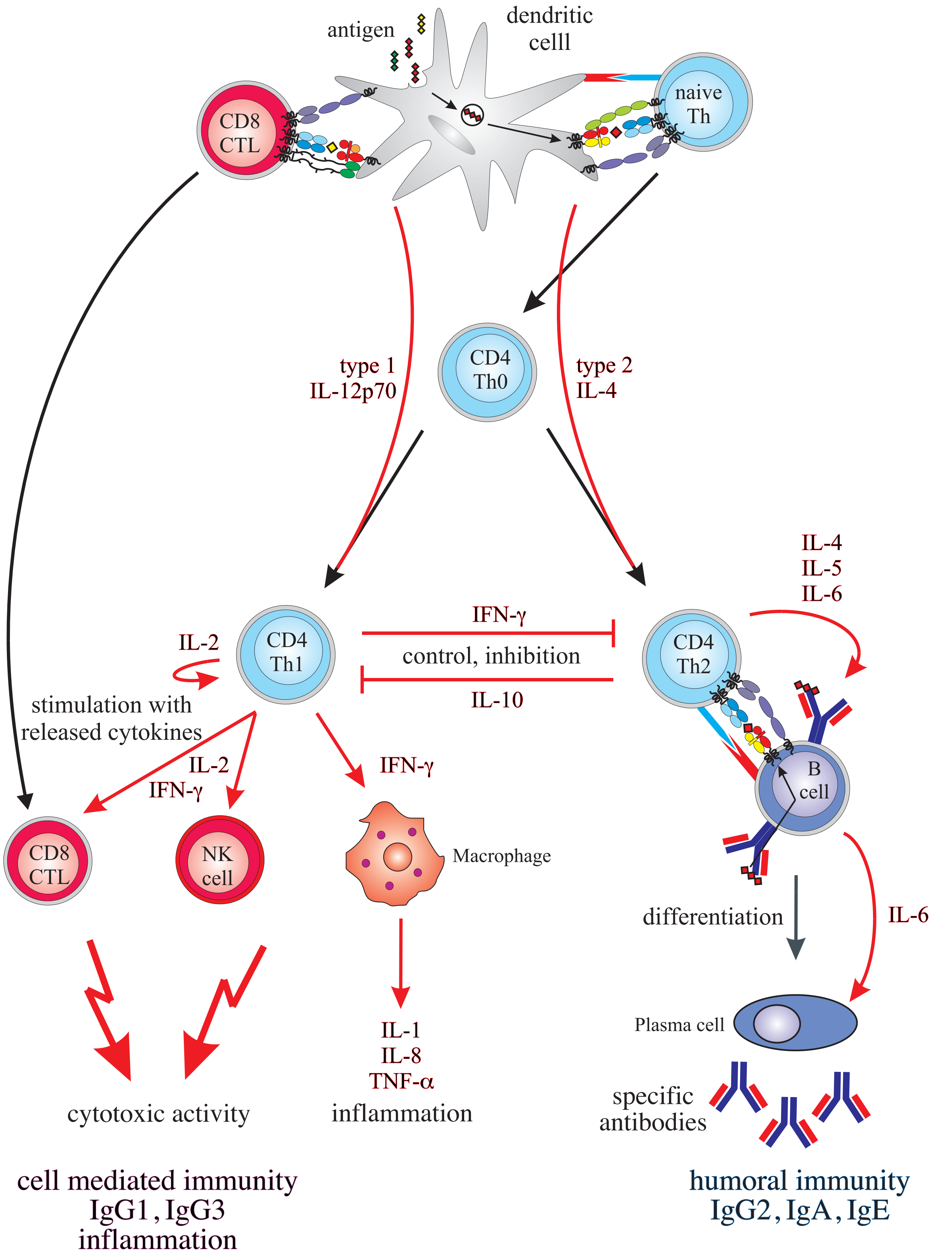


Figure 2.10: The T-helper cell type 1 and type 2 (Th1 and Th2) pathway is controlled by dendritic cells

2.2.4 Effector Functions of the Immune System

The effector function of the different components of the immune system depends on the kind of pathogen. There are three classes of effector T cells specialized in dealing with three classes of pathogen. Cytotoxic T cells (CTLs) induce apoptosis in their target cells that display peptides in the context of MHC class I on their surface. Th1 and Th2 cells recognize fragments of antigen bound to MHC class II molecules. Th1 cells activate macrophages to destroy intracellular microorganisms, activate B cells to produce IgG1 and IgG3 antibodies for opsonization and release IL-2 and IFN-γ for induction of NK cells and CTLs. On the other hand, Th2 cells direct an immune response to extracellular pathogens by initiating B cell responses to secrete IgG2, IgA and IgE isotypes of antibodies. All types of antibody-mediated responses contribute to the humoral immunity, whereas CTLs, NK cells and macrophages are members of the cell-mediated immunity (figure 2.10 on page 16).

2.3 Cancer Immunotherapy

2.3.1 Dendritic Cell Vaccines in Cancer Treatment

The previous sections described how adaptive immunity is induced and controlled by dendritic cells. These immune responses can be launched either to combat invading microorganisms or to eliminate aberant tissue of a body. The increased understanding of the complicated mechanisms in immunobiology resulted in the proposal of the immune surveillance hypothesis by Burnet in 1970: The body is kept under surveillance by the immune system to detect any aberrant cells, which may circumvent control mechanisms and lead to malignancies. The immune system depends on a number of subsystems, including cell-dependent and humoral immunity, which eliminate any aberrant tissue (Bremers et al., 2000a, b). However, the immune surveillance against tumors can fail because of immunological ignorance (Ochsenbein et al., 1999) or induction of tolerance (Pardoll, 2003). To overcome this tumor escape from immune surveillance (Costello et al., 1999), many new cancer vaccine strategies are based on the understanding that the nature of dendritic cells is central to the outcome of immune responses against tumors (Pardoll, 2003). The development of dendritic cell (DC) based vaccines, their in vitro generation and loading with tumor antigens, may circumvent tumor tolerance (McIlroy and Gregoire, 2003). Therefore, immature DCs need to efficiently take up exogenous tumor antigens, differentiate to a matured phenotype with up-regulation of MHC class I, MHC class II and co-stimulatory molecules, migrate towards lymphoid organs to elicit both a $CD4^{+}$ and $CD8^{+}$ T cell response (Schuler et al., 2003). Administering of immature dendritic cells need to be avoided, as suppressed CTL responses were observed (Dhodapkar et al., 2001). Furthermore, a cancer vaccine need to prime naive T lymphocytes, boost pre-existing tumor-specific immunity and overcome tumor-induced inactivation of tumor immunity (Gunzer and Grabbe, 2001). The principle of a dendritic cell based immunotherapy is shown in figure 2.11 on page 21. The success of an immunotherapeutical treatment depends on two major factors: the right tumor associated antigens and the delivery of these antigens to induce a tumor specific immune response (Gabrilovich, 2002). Therefore, many approaches have been developed for loading of dendritic cells with tumor associated antigens: Single antigens in form of peptides are loaded on dendritic cells or whole tumor cell information is delivered as apoptotic tumor cells, cell lysate, RNA, DNA or tumor cells are fused with dendritic cells.

Peptides

Known tumor associated antigens in form of molecular characterized peptides bound to certain MHC class I molecules are loaded on DCs that are able to generate a potent immune response. Thereby anti-tumor responses can be precisely analyzed and monitored by cytotoxic T cell, cytokine and tetramer analysis. However, this approach is only possible when the tumor epitopes are known and the peptides match the patients' MHC class I type (Parmiani et al., 2002).

Both, polymorphism and polygeny contribute to the extreme diversity of MHC molecules (see chapter 2.2.1). As a result, the peptide binding specificity varies for the different MHC surface proteins. However, it is noteworthy that a large fraction of the human population express a limited number of predominant alleles. In many of the performed clinical trials HLA-A*0201 positive patients are eligible for treatment, as HLA-A2 is the most common HLA class I specificity and is found at high frequency in most populations (Browning and Krausa, 1996). About 25% to 39% of the western European population are positive for HLA-A2 (Imanishi, 1991) and nearly 96% within this group are positive for the HLA-A*0201 allele (Browning and Krausa, 1996). For this reason a lot of the identified peptides are developed to bind to the HLA-A*0201 molecule. Nevertheless, utilizing just one TAA to target the heterogeneous population of tumor cells in vivo may result in tumor escape as not all cancerous cells are positive for the used TAA or the tumor might alter its expression. Therefore, other approaches have been developed to circumvent these disadvantages of HLA matching and usage of distinct TAAs.

Apoptotic Tumor Cells

Currently, the discussion of which method does result in the best cross-presentation of tumor-associated epitopes on the surface of dendritic cells led to plenty of approaches using apoptotic cells for the delivery of whole-tumor cell information. These apoptotic tumor cells are usually prepared by irradiation and endocytozed by receptor-mediated phagocytosis. This receptor-mediated uptake and the assumed access of the antigen to the cross-presentation pathway of dendritic cells were evaluated in different experiments (Jenne et al., 2000; Jenne and Sauter, 2002; Kotera et al., 2001; Lambert et al., 2001). Nonetheless, no differences were found to the method of whole-cell lysate (see next paragraph). Thus, the limitations of this method are the access to viable primary tumor cells that need to be subsequently irradiated.

Whole-cell Lysates

The method is based on DCs loaded with lysates prepared by repeated freezing-thawing cycles of tumor cells. This mixture of tumor cell associated proteins are processed by DCs and presented in the form of epitopes able to stimulate T cell responses (Gabrilovich, 2002). Loading of dendritic cells with whole tumor cell lysate preparations represents a promising method to utilize all potential known and unknown TAAs and circumvent the disadvantages like the requirement for known epitopes of the TAAs, HLA-matching of a patient and the necessity of exact tumor analysis (Schuler et al., 2003; Thumann et al., 2003). Furthermore, this approach seems to be one of the simplest and is easily to establish in a clinical environment as no viable tumor cells are needed for a total autologous system. In many in vitro studies it has been proven that whole tumor cell lysates are efficiently taken up by macropinocytosis and cross-presented on the surface of dendritic cells evidenced by cytotoxic assays (Bachleitner-Hofmann, 2002; Berger et al., 2001; Herr et al., 2000; Schnurr et al., 2001; Vegh et al., 2003; Wen et al., 2001). However, the precise monitoring in clinical settings is difficult, since the molecular characteristics are unknown.

RNA

This approach utilizes RNA isolated from tumor cells directly or after amplification via a RNA-cDNA (Polymerase Chain Reaction (PCR) amplification) -RNA route. Disadvantages are RNA instability and degradation during preparation, which does affect the expression level of the specific antigens (Gabrilovich, 2002). Furthermore, this method would be inefficient if TAAs are used, which are defined by a different, tumor-specific glycostructure. Produced proteins would be glycosilated by the unaltered machinery of the dendritic cell and therefore would not represent the malignant glycoform.

DNA Gene Transfer

Another method for loading of DCs is the gene transfer to the antigen presenting cells by transfection of defined tumor-associated genes or their parts (minigene) (Gabrilovich, 2002). This approach allows the expression of multiple and different epitopes that match patients' MHC class I molecules. The limitations of this method are similar to the RNA approach, especially regarding the glycosilation. Additionally, only known antigens can be used in this approach.

Fusion of Tumor Cells and DCs

With their article in 2000, Kugler and Stuhler published a startling report of regressions in renal cell carcinoma using dendritic cell - tumor cell fusion. After the discovery of several irregularities the paper was retracted in 2003. However, a number of studies showed that hybrids, fusion products of tumor cells with DCs, can elicit a T cell response to tumor cells (Gong et al., 1997; Gong et al., 2002). The concept of just fusing TAAs presented by tumor cells and the co-stimulatory signals expressed by DCs seems still quite interesting. Thus, it has still to be clarified if the generated hybrids are vital and can both process antigen and migrate towards lymph nodes to elicit an effective T cell response in vivo.

2.3.2 MUC1: A Tumor Associated Antigen in Breast Cancer

The human epithelial mucin MUC1 (CD227) is a large molecular weight type I transmembrane glycoprotein with a unique extracellular domain build of variable numbers of tandem repeats of 20 amino acids, each with 5 *O*-linked glycosilation sites, which is illustrated in figure 2.12 on

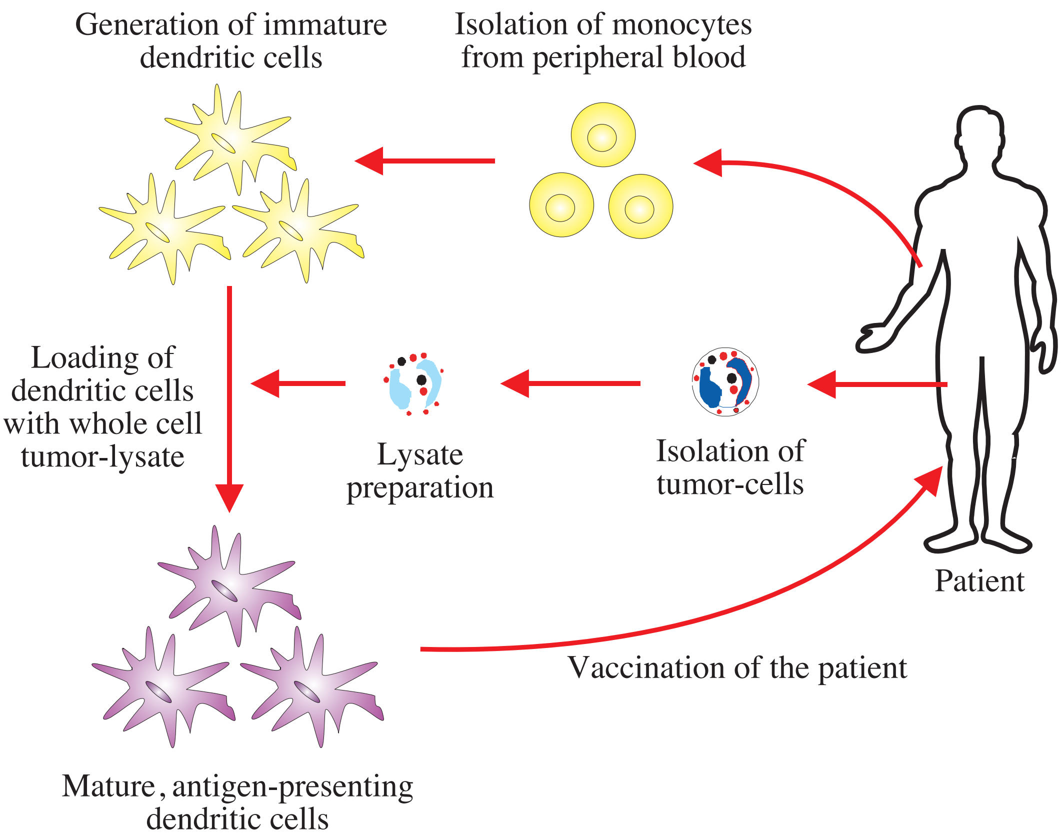


Figure 2.11: The principle of cancer immunotherapy using lysate-pulsed dendritic cells

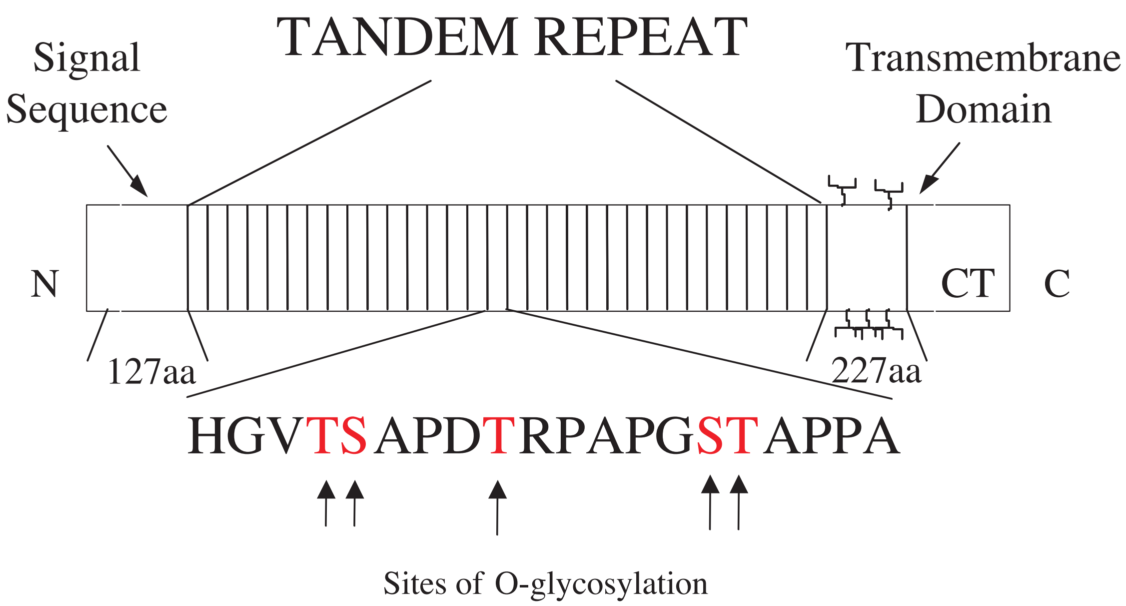


Figure 2.12: The structure of the MUC1 core protein with signal sequence, tandem repeat domain, transmembrane domain and cytoplasmic tail (CT)

page 21 (Gendler et al., 1988; Gendler et al., 1990; Lancaster et al., 1990). It was first identified in human milk (Shimiza et al., 1982), is widely expressed on glandular epithelial cells (Zotter et al., 1988) and was recently found on cells of the hematopoietic system like T-cells (Chang et al., 2000; Correa et al., 2003), monocytes and mature dendritic cells (personal observation). Aberrantly glycosilated MUC1 is overexpressed in the majority of breast, ovarian (Girling et al., 1989) and other cancer types (Noto et al., 1997; Zhang et al., 1998). The interest in using the cancer-associated form of MUC1 as potential target in cancer immunotherapy is reflected by both pre-clinical and clinical studies (Scholl et al., 2003; Taylor-Papadimitriou et al., 2002).

Epitopes of MUC1

In the cytoplasm proteins are degraded by proteasomes to fragments of 4 to 20 amino acids and transported by TAP into the endoplasmatic reticulum. These peptides are loaded on MHC class I molecules, usually 8-11 amino acids in length (see chapter 2.2.1). As MUC1 is aberrantly *O*-glycosilated, the pattern of the glycostructure on MUC1 is expected to influence the MHC class I restricted presentation of epitopes to T lymphocytes (Taylor-Papadimitriou et al., 2002). Galli-Stampino and colleagues (1997) reported that the glycan-structure influences the recognition of epitopes by T cells.

In searching for HLA-A*0201 matching epitopes of MUC1, peptides were derived from the area of the variable tandem repeats and regions flanking the tandem repeat, which are not affected by changes in the glycosilation. The epitopes F7 (MUC^{13-21}) LLLTVLTVV (Heukamp et al., 2001) and M1.2 (MUC^{12-20}) LLLLTVLTV (Brossart et al., 1999) are localized within the signal sequence of MUC1. Especially the M1.2 is of great interest as the frequency of T cells specific for this epitope were found increased in breast cancer patients compared to healthy individuals (Taylor-Papadimitriou et al., 2002).

2.4 Generation of Dendritic Cells

The usage of dendritic cells in cancer immunotherapy requires a reproducible generation method that can be performed under GMP (Good Manufacturing Practice) guidelines and be utilized in a clinical environment. For the generation of dendritic cells different sources can be used: proliferating $CD34^+$ precursors in blood (Caux et al., 1992) after granulocyte-colony stimulating

Table 2.1: The generation of dendritic cells as reported in several publications

Name	Year	Enrichment	Serum	Feeding	Yield*
Babatz et al.	2003	Magnetic beads	1%	YES	12.1%
Dietz et al.	2000	Magnetic beads	2%	YES	18.3 ± 6.3%
Felzmann et al.	2003	Magnetic beads	NO	YES	8.0 ± 3.0%
Meyer-Wentrup et al.	2003	Magnetic beads	1%	YES	41.0 ± 4.0%
Motta et al.	2003	Magnetic beads	1%	YES	20.7 ± 4.6%
Padley et al.	2001	Magnetic beads	1%	YES	30.3 ± 6.4%
Pullarkat et al.	2002	Magnetic beads	NO	NO	9.2 ± 5.2%
Ratta et al.	2000	Magnetic beads	10%	YES	4.8 ± 1.3%
Berger et al.	2002	Attachment	2%	YES	19.9 ± 9.6%
Goxe et al.	2000	Elutriation	2%	NO	48.3 %

*Yield: Yield of viable dendritic cells from inoculated monocytes

factor (G-CSF) mobilization (Mackensen et al., 2000), non-proliferating $CD14^+$ monocytes in peripheral blood after enrichment via magnetic beads (Babatz et al., 2003; Dietz et al., 2000; Felzmann et al., 2003; Meyer-Wentrup and Burdach, 2003; Motta et al., 2003; Padley et al., 2000, 2001; Pickl et al., 1996; Pullarkat et al., 2002; Ratta et al., 2000) or attachment (Araki et al., 2001; Bender et al., 1996; Berger et al., 2002; Moldenhauer et al., 2003; Morse et al., 1999; Pospisilova et al., 2002; Romani et al., 1996; Syme et al., 2001; Thurner et al., 1999b; Tuyaerts et al., 2002) and enrichment of DCs after cultivation of peripheral blood mononuclear cells (PBMCs) via elutriation (Bernard et al., 1998; Goxe et al., 1999, 2000; Guyre et al., 2002; Nguyen et al., 2002; Sorg et al., 2003; Spisek et al., 2001; Tazbirkova et al., 2003; Wong et al., 2001). Rare circulating DCs can also be isolated, but although patients can be pretreated with Flt3 ligand, the yield of DCs is comparably small (Fong et al., 2001). While DCs from $CD34^+$ cells require a prolonged culture and special cytokine setup in order to increase the small number of precursors, monocyte derived dendritic cells are easy to obtain after enrichment of monocytes by magnetic separation or adherence, followed by differentiation using granulocyte macrophage-colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4). This method developed by Sallusto and Lanzavecchia (1994) and Romani et al. (1994) is applied widely in experimental protocols (see figure 2.13 on page 24).

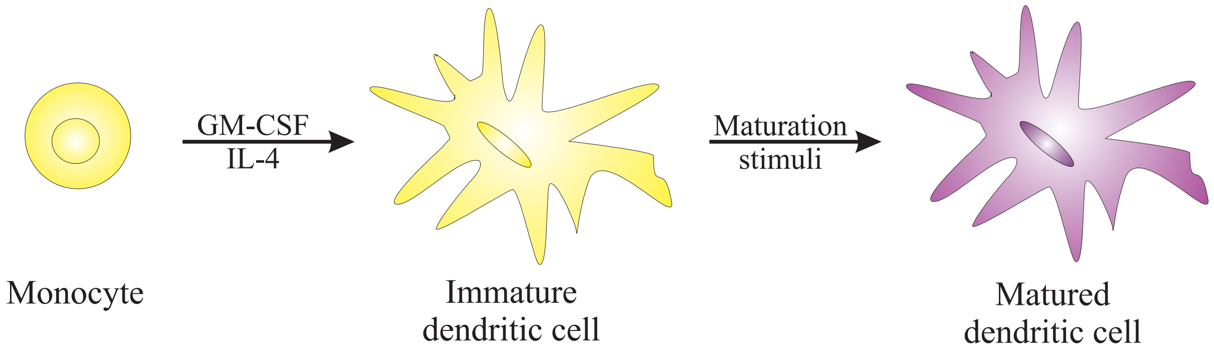


Figure 2.13: The differentiation of monocytes to dendritic cells in a two step process

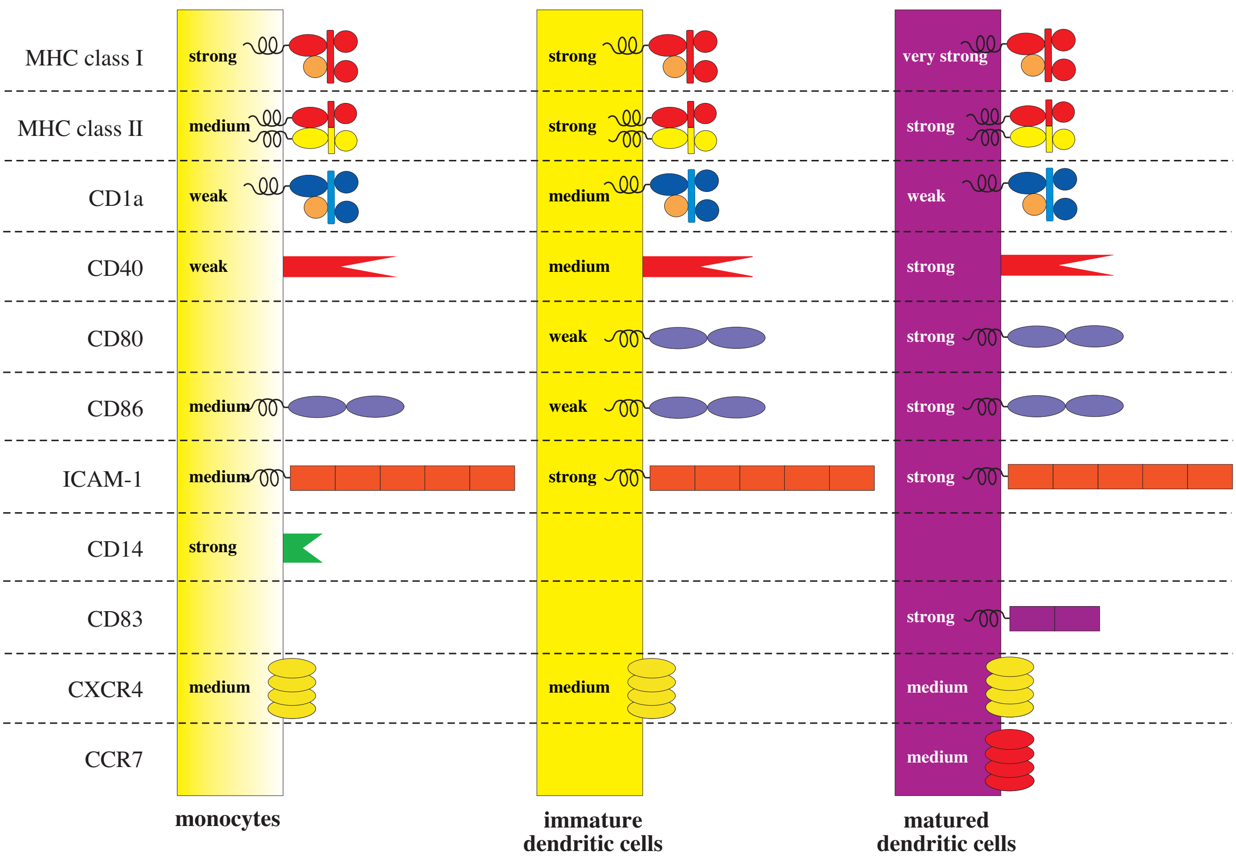


Figure 2.14: Surface marker expression on monocytes, immature dendritic cells and matured dendritic cells during differentiation

Due to the ease of availability of monocytes, rapidness of fully differentiation to unmature (5 to 6 days) or mature (6 to 8 days) DCs using special maturation stimuli (e.g. TNF-α, IL-1β, IL-6, PGE_2 (Jonuleit et al., 1997), TNF-α, PGE_2 (Kalinski et al., 1998) or lipopolysaccharide (LPS)) and the possibility of avoiding foreign antigens (fetal calf serum), this protocol was adapted for clinical applications (Thurner et al., 1999a, b). For studies where DCs are to be cultured ex vivo and then reintroduced to the patient, it is advisable to avoid fetal calf serum or any kind of foreign antigen due to possible infections and immunogenicity. However, most of the published protocols are using serum in their setup and usually feed the culture during the differentiation of monocytes to DCs, which does affect the reproduction, handling and simplicity. In table 2.1 different publications utilizing magnetic bead selection for monocyte-enrichment are listed and compared to selected reports using attachment or elutriation. Generally, it seems to be quite interesting to determine the cause of the low yield. In all published reports a common acceptance of the low yield of monocyte-derived dendritic cells was observed.

Maturation of Dendritic Cells

Monocytes can be differentiated in the presence of GM-CSF and IL-4 to immature dendritic cells. The second step is the addition of a defined stimulus to induce maturation that result in a phenotype for effective stimulation of both $CD4^+$ and $CD8^+$ T cells (see chapter 2.2.2). For the maturation of DCs various stimuli are described, which can induce a matured phenotype, like lipopolysaccharide (Langenkamp et al., 2000), double-stranded RNA (McGuirk and Mills, 2002), CpG oligonucleotides (Sparwasser et al., 1998; Weiner et al., 1997), CD40 ligand (Labeur et al., 1999) and different inflammatory cytokine combinations like TNF-α, IL-1β, IL-6, PGE_2 (Jonuleit et al., 1997) and TNF-α, PGE_2 (Kalinski et al., 1998). These stimuli can be categorized in type 1, type 2 and regulatory type inducing agents that affect the polarization of DCs (see also chapter 2.2.2). Table 2.2 shows different polarizing factors and pathogen-associated molecular patterns (PAMPs). However, for clinical applicability the stimulus should be in full accordance to GMP conditions, should result in reproducible induction of fully matured DCs and should be usable for large scale production (Thurner et al., 1999a, b).

The differentiation process of monocytes to fully matured dendritic cells goes along with changes in the expression of distinct surface molecules (see figure 2.14). The stimulatory

Table 2.2: Different stimuli that induce maturation and polarization in dendritic cells

Stimulus	Type 1	Type 2	Regulatory type
Cytokines	IFN-γ, IL-12p70, TNF-β	IL-4, IL-5, IL-13	IL-10, TGF-β
Chemokines	CCL21		
Eicosanoids		PGE_2	
Co-stimulatory factors	CD40 ligand		
PAMPs	LPS, dsRNA, CpG DNA		

PAMPs: pathogen-associated molecular patterns, dsRNA: double-stranded RNA, CpG DNA: oligodeoxynucleotides rich in unmethylated cytosine guanine dinucleotides, TGF: Transforming growth factor

molecules MHC class I and MHC class II are much stronger expressed on matured DCs. CD1a, a member of the Immunglobulin superfamily and structurally analog to MHC class I, serves as molecule for lipid presentation and is typically found on Langerhans cells (Calabi, 1991, 2000). Furthermore the co-stimulatory surface proteins CD40, CD80 and CD86, adhesion molecules like ICAM-1 (Intercellular adhesion molecule) and the maturation marker CD83 are expressed on matured DCs (Zhou and Tedder, 1995a, b, 1996). The receptor for the complex of LPS and LBP (LPS binding protein), CD14, is not found on both immature and matured DCs. The chemokine receptor expression, especially CCR7 for the chemotaxis to the lymph nodes, is up-regulated on fully matured dendritic cells (Luster, 2002).

The expression of these surface proteins are used for the phenotypical analysis of dendritic cells, whereby only CD83 is distinctively up-regulated on matured DCs.

Chapter 3

Material and Methods

3.1 Solutions

Phosphate Buffered Saline

Table 3.1: Formulation of phosphate buffered saline (PBS), which is an isotonic solution and not nutritionally complete. The pH was adjusted to 7.3. The solution was autoclaved for $30min$.

Distilled water	$1L$	
$NaCl$	$8.00\frac{g}{L}$ ($137mM$)	Sigma, Deisenhofen, Germany
KCl	$0.20\frac{g}{L}$ ($27mM$)	Sigma, Deisenhofen, Germany
Na_2HPO_4	$1.25\frac{g}{L}$ ($8mM$)	Sigma, Deisenhofen, Germany
KH_2PO_4	$0.20\frac{g}{L}$ ($15mM$)	Sigma, Deisenhofen, Germany

EDTA Buffer

Table 3.2: Formulation of EDTA buffer. The pH was adjusted to 7.3. The solution was autoclaved for $30min$.

PBS	$1L$	
$Na_2EDTA \cdot 2H_2O$	$0.74\frac{g}{L}$ ($2mM$)	Sigma, Deisenhofen, Germany

Analyzer Buffer

Table 3.3: Formulation of the buffer utilized for glucose, lactate, glutamine and glutamate analysis. The pH was adjusted to 7.3.

Distilled water	$1L$	
$NaCl$	$5.10\frac{g}{L}$ ($87mM$)	Sigma, Deisenhofen, Germany
Na_2HPO_4	$5.00\frac{g}{L}$ ($35mM$)	Sigma, Deisenhofen, Germany
NaH_2PO_4	$1.00\frac{g}{L}$ ($8mM$)	Sigma, Deisenhofen, Germany
Sodium benzoate	$0.30\frac{g}{L}$ ($2mM$)	Sigma, Deisenhofen, Germany
$Na_2EDTA \cdot 2H_2O$	$0.56\frac{g}{L}$ ($1.5mM$)	Sigma, Deisenhofen, Germany
Gentamicin	$50\frac{mg}{L}$	Sigma, Deisenhofen, Germany

3.2 Lysate from Human Breast Carcinoma Cell Lines

3.2.1 Cultivation of Human Tumor Cell Lines

Table 3.4: Materials for the cultivation of breast carcinoma cell lines

Cell lines	MCF-7	ATCC Number: HTB-22
	MDA-MB-231	ATCC Number: HTB-26
Medium	RPMI 1640	Biochrom, Berlin, Germany
Serum	10% fetal calf serum	Biochrom, Berlin, Germany
Versene	0.05% in EDTA / PBS	Biochrom, Berlin, Germany
Cultivation	Tissue culture flask, $75cm^2$	NUNC, Wiesbaden, Germany

The epithelial breast carcinoma cell lines used were MCF-7 (intraductal carcinoma, HLA-A*0201), (Soule et al., 1973) and MDA-MB-231 (adenocarcinoma, HLA-A*0201), (Cailleau et al., 1974). Both cell lines were derived from carcinomas that metastasized into the pleural fluid. Cells were cultivated in RPMI 1640 supplemented with 10% heat-inactivated ($56°C$, $30min$) fetal calf serum in standard tissue culture flasks ($75cm^2$) at $37°C$ and 5% CO_2.

Cells were detached by addition of versene, subcultivated at a ratio of 1:6 and maintained at low passage number (5 to 20).

3.2.2 Preparation of Tumor Cell Lysate

Table 3.5: Materials for the preparation of tumor cell lysate

Ultrasonic bath	U50	Ultrawave, Cardiff, UK
BCA assay		Pierce, Perbio Science, Tattenhill, UK

Confluent cells were detached by addition of $3mL$ of 0.05% versene in 0.02% EDTA solution and incubated for 10 min at $37°C$. After washing twice with PBS cells were resuspended at $1 \cdot 10^7\ \frac{1}{mL}$ in PBS and frozen at $-80°C$. Disruption of cells was carried out by four freeze-thaw cycles. Cells were thawed by $10min$ sonication at $4°C$ in an ultrasonic bath and subsequently frozen at $-80°C$. The supernatant was collected after centrifugation ($1000g$ / $15min$ / $20°C$) and passed through a $0.2\mu m$ filter. The protein concentration was determined by a BCA assay according to the manufacturer's instructions (Wiechelman et al., 1988).

3.3 Immunoblotting of Prepared Lysate

3.3.1 Lysate Preparation

Table 3.6: Solutions used for lysate preparation

RIPA buffer	PBS	
	0.5% Deoxycholic acid sodiumsalt	Sigma, Deisenhofen, Germany
	1.0% Nonidate P40	Sigma, Deisenhofen, Germany
	0.1% Sodium dodecyl sulfate (SDS)	Sigma, Deisenhofen, Germany
Lysate solution	$10mL$ RIPA buffer	
	1 tablet of complete MIM	Roche, Mannheim, Germany
	$0.1mL$ Sodium orthovanadate ($100mM$)	Sigma, Deisenhofen, Germany

Tumor cell lysates were prepared either as described above or otherwise confluent MDA-MB-231 and MCF-7 cells were harvested, washed twice in PBS and resuspended at $1 \cdot 10^7 \frac{1}{mL}$ in lysis buffer (PBS, 0.5% sodium deoxycholate, 1.0% nonidate P40, 0.1% SDS, one tablet of protease inhibitor (complete MIM) and $0.1mL$ sodium orthovanadate ($100mM$) per $10mL$ of lysis buffer). The homogenate was passed several times through a fine-gauge needle, incubated for $30min$ at $4°C$, passed again through a fine-gauge needle, then centrifuged and frozen at $-80°C$. The protein concentration was determined by a BCA assay according to the manufacturer's instructions (Wiechelman et al., 1988).

3.3.2 Denaturing Electrophoresis

Table 3.7: Materials and solutions used for SDS-PAGE

Novex Tris-Glycine gel	4-20%	Invitrogen, Paisley, UK
Rainbow marker	RPN800	Invitrogen, Paisley, UK
Sample buffer ($5x$)	$250mM$ Tris-HCl, pH 6.8	Sigma, Deisenhofen, Germany
	50% Glycerol	Sigma, Deisenhofen, Germany
	10% SDS	Sigma, Deisenhofen, Germany
	per $285mL$ $5x$ sample buffer:	
	$20mL$ Mercaptoethanol	Sigma, Deisenhofen, Germany
	$20mL$ Bromophenolblue	
	(1% in Trizma Base pH 6.8)	Sigma, Deisenhofen, Germany
Running buffer ($10x$)	$30g$ Tris-HCl	Sigma, Deisenhofen, Germany
	$144g$ Glycine	Sigma, Deisenhofen, Germany
	1% SDS	Sigma, Deisenhofen, Germany
	$1L$ distilled water	

To identify the presence of MUC1 protein in the prepared cell lysates, the lysate was run on SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) to separate the proteins. Protein samples were boiled for $5min$ in equal volume of $5x$ SDS-PAGE sample buffer ($250mM$ Tris-HCL, pH 6.8, 50% glycerol, 10% SDS, 7% mercaptoethanol and 7% bromophenolblue/Tris-HCL (1% bromophenolblue in Tris-HCL, pH 6.8)). A volume corresponding to $20\mu g$ protein was separated on a 4-20% Tris-Glycine gradient gel with the in table 3.8 listed running conditions.

Table 3.8: Running conditions for SDS-PAGE

Voltage	$100V$ constant
Time	$2h$
Expected current	$30-40mA$ (start); $8-12mA$ (end)
Temperature	$20°C$

3.3.3 Blotting and Detection

Table 3.9: Materials and solutions used for blotting and detection

Hybond-C membrane	Nitrocellulose (RPN203E)	Amersham, Little Chaltford, UK
Wet blotter	Mini Trans-Blot Cell	BioRad, Hertfordshire, UK
Blotting buffer	$5.3g$ Trizma Base	Sigma, Deisenhofen, Germany
	$29.0g$ glycine	Sigma, Deisenhofen, Germany
	$1.0g$ SDS	Sigma, Deisenhofen, Germany
	$200mL$ methanol	Sigma, Deisenhofen, Germany
	$800mL$ distilled water	
Detection	SuperSignal	
	Chemoluminescence Kit	Pierce, Perbio Science, Tattenhill, UK
X-ray film		Fuji, London, UK

The protein was transferred to a Hybond-C membrane using a wet-blotter with the in table 3.10 listed blotting conditions. Blots were blocked for $1h$ in 5% BSA and 0.1% Tween 20 in PBS and incubated with HMFG-2 monoclonal antibody supernatant (neat) (Burchell et al., 1983) overnight at $4°C$. After thorough washing, blots were probed with rabbit anti-mouse (1:1000) secondary antibody conjugated to HRP for $1h$ and washed 3 times. The protein signal was detected using a chemoluminescence kit and exposed to X-ray film.

Table 3.10: Blotting conditions

Voltage	$30V$ constant
Time	$14h$
Expected current	$150mA$ (start)
Temperature	$4°C$

3.4 Generation of Dendritic Cells

3.4.1 Isolation of CD14$^+$ cells

Table 3.11: Materials and solutions used for the isolation of CD14$^+$ cells

Ficoll-Paque	Density: $1.077\frac{g}{mL}$	Amersham, Uppsala, Sweden
MACS-buffer	PBS	
	0.5% Bovine serum albumine	Life Technologies, Karlsruhe, Germany
MACS columns	small: MS (up to $2 \cdot 10^8$ cells)	Miltenyi, Bergisch Gladbach, Germany
	big: LS (up to $2 \cdot 10^9$ cells)	Miltenyi, Bergisch Gladbach, Germany
Filter	3M	Miltenyi, Bergisch Gladbach, Germany
Micro-beads	CD14$^+$	Miltenyi, Bergisch Gladbach, Germany
Magnet	MIDI cell sorting kit	Miltenyi, Bergisch Gladbach, Germany
Leucosep tubes	$50mL$	Greiner, Solingen, Germany
Tubes	$15mL$	Greiner, Solingen, Germany
Tubes	$50mL$	Greiner, Solingen, Germany

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coat preparations or blood samples from healthy donors or breast cancer patients by density-gradient centrifugation on Ficoll-Paque. $15mL$ of Ficoll-Paque was filled in Leucosep tubes and centrifuged ($1000g$ / $30sec$ / $20°C$). After that, 15 to $20mL$ of buffy coat or $30mL$ of blood samples were overlaid and centrifuged ($1000g$ / $15min$ / $20°C$). The plasma (supernatant) was collected for further processing, the white cell band was transferred into a new $50mL$ tube and washed twice with PBS (centrifugation at $400g$ and $20°C$ for $10min$). Finally, cells were resuspended in $50mL$

PBS, counted and flowcytometrically analyzed.

$CD14^+$ cells were affinity-purified utilizing the MIDI magnetic cell sorting kit (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions (Manz et al., 1995; Thiel et al., 1998). Therefore, PBMCs were incubated with CD14 Micro-beads ($50\mu L$ per 10^8 PBMC) in MACS-buffer ($500\mu L$ per 10^8 PBMC) for $15min$ at $4°C$ and washed once. Labeled cells were passed through a positive selection column and eluted after removal of the column from the magnetic device. For higher purity a second column was used and the phenotype of cells was assessed by flow cytometry. The step by step protocol is described below:

- Centrifuge cells ($200g$ / $10min$ / $20°C$)
- Remove all supernatant
- Labeling: MS (small) Columns: $50\mu L$ buffer and $5\mu L$ Micro-beads per 10^7 total cells
- Labeling: LS (big) Columns: $500\mu L$ buffer and $50\mu L$ Micro-beads per 10^8 total cells
- Incubate $30min$ at $4°C$
- Fill up to $2mL$ (small) or $10mL$ (big)
- Centrifuge cells ($200g$ / $10min$ / $20°C$)
- Resuspend in $500\mu L$ (small) or $3mL$ (big) buffer
- Place separation column in the separation unit (with filter)
- Apply $500\mu L$ (small) or $3mL$ (big) buffer
- Pipette labeled cells onto the column
- Wash 3x with $500\mu L$ (small) or $3mL$ (big) buffer
- Apply $2mL$ (small) or $5mL$ (big) buffer onto the column and flush the column
- Repeat the separation by using a second column but without filter
- Centrifuge cells ($200g$ / $10min$ / $20°C$)
- Resuspent cells in $10mL$ X-VIVO 15
- Counting of cells using CASY 1 and flowcytometric analysis (CD14 / CD3 / HLA-A,B,C)

3.4.2 Processing of Plasma

Plasma obtained after Ficoll-Paque centrifugation was heat-inactivated at $56°C$ for $30min$. This was followed by centrifugation at $1000g$ and $20°C$ for $10min$ and passing through a $2\mu m$ filter. Finally, the plasma was either utilized directly for freezing of PBMCs or itself frozen at $-20°C$ and thawed for usage.

3.4.3 Differentiation of Monocytes to Dendritic Cells

Table 3.12: Materials and solutions used for differentiation of monocytes to immature dendritic cells

Medium	X-VIVO 15	Life Technologies, Karlsruhe, Germany
48 well plates	$500\mu L$	NUNC, Wiesbaden, Germany
Tissue culture flasks	$75cm^2$	Greiner, Solingen, Germany
Teflon bags	$30mL$	CellGenix, Freiburg, Germany
rhuGM-CSF	$400\frac{U}{mL}$	Leucomax, Norvartis, Nuernberg, Germany
rhuIL-4	$2000\frac{U}{mL}$	R&D, Wiesbaden, Germany

Isolated $CD14^+$ cells were cultured in X-VIVO 15, $400\frac{U}{mL}$ rhuGM-CSF and $2000\frac{U}{mL}$ rhuIL-4 at a cell concentration described in the results section (standard concentration: $1.3 \cdot 10^6\ \frac{1}{mL}$) in 48 well plates ($500\mu L$), tissue culture flasks ($75cm^2$, $30mL$) or hydrophobic cell bags ($30mL$) at $37°C$ and 5% CO_2 for 6 days (Bohnenkamp and Noll, 2003).

3.4.4 Maturation of Dendritic Cells

For the maturation of dendritic cells different cytokine stimuli were used: The cytokine cocktails I or II listed in table 3.13 were added on day 6. For lysate-pulsing of dendritic cells the lysate + adjuvant combination was supplemented on day 6. The resultant suspension cells were harvested on day 8 (Bohnenkamp and Noll, 2003).

Table 3.13: Cytokines and compounds used for the maturation of dendritic cells

Cocktail I	TNF-α	$1000\frac{U}{mL}$	R&D, Wiesbaden, Germany
	IL-1β	$1000\frac{U}{mL}$	R&D, Wiesbaden, Germany
	IL-6	$1000\frac{U}{mL}$	R&D, Wiesbaden, Germany
	PGE_2	$1\frac{\mu g}{mL}$ $(0.003\mu M)$	Sigma, Deisenhofen, Germany
Cocktail II	TNF-α	$1000\frac{U}{mL}$	R&D, Wiesbaden, Germany
	PGE_2	$18\frac{\mu g}{mL}$ $(0.051\mu M)$	Sigma, Deisenhofen, Germany
Lysate + adjuvant	Tumor cell lysate	$100\frac{\mu g}{mL}$	
	TNF-α	$1000\frac{U}{mL}$	R&D, Wiesbaden, Germany
	PGE_2	$1\frac{\mu g}{mL}$ $(0.003\mu M)$	Sigma, Deisenhofen, Germany

3.4.5 Cryopreservation

For the cryopreservation of PBMCs and dendritic cells heat-inactivated ($56°C$ for $30min$) autologous plasma was used (see section 3.4.2 on page 34). Cells were centrifuged ($200g$ / $10min$ / $20°C$) and resuspended at a cell density of $1 \cdot 10^7\ \frac{1}{mL}$ (PBMCs) and between $1.1 \cdot 10^6$ and $3.5 \cdot 10^6\ \frac{1}{mL}$ (mature dendritic cells) in autologous plasma + 10% DMSO and frozen at $-80°C$ in a polysterene box that allowed cooling at nearly $1°C$ per minute. For thawing, cells were transferred into a $50mL$ tube and suspended in $10mL$ of icecold X-VIVO 15 (dendritic cells) or icecold AIM V (PBMCs) medium. After centrifugation ($200g$ / $10min$ / $20°C$) cells were resuspended in the corresponding medium at $37°C$.

Table 3.14: Materials and solutions used for the cryopreservation of primary cells

Medium	X-VIVO 15	Life Technologies, Karlsruhe, Germany
Medium	AIM V	Gibco, Carlsbad, CA, USA
Cryovials	$1.8mL$	NUNC, Wiesbaden, Germany
Dimethyl sulfoxide	DMSO	Sigma, Deisenhofen, Germany
Tubes	$50mL$	Greiner, Solingen, Germany

3.5 Analytics of Cell Parameter

3.5.1 Cell Counting and Viability

Routinely, for the determination of the cell number and the viability a haemocytometer with standard trypan blue dye exclusion and a CASY 1 particle counting system (model TT, Schaerfe System, Reutlingen, Germany) was used.

The cell concentration with a haemocytometer is determined with the term:

$$\frac{\text{number of cells}}{mL} = \frac{\text{number of cells in 4 quadrants}}{4} \cdot 10^4 \cdot \text{dilution factor} \tag{3.1}$$

The viability (trypan blue dye exlusion method) is calcualted by utilizing following formula:

$$\text{viability} = \frac{\text{viable cells}}{\text{total number of cells}} \cdot 100 \tag{3.2}$$

3.5.2 Flow Cytometric Analysis

Flow cytometry is a useful tool for analyzing certain physical and chemical characteristics of single cells or particles as they are moving in suspension past a sensing point. The modern flow cytometer consists of a light source, collection optics, electronics and a computer to translate generated signals to data. In the used cytometers (FACSCalibur, Becton Dickinson and EPICS XL Flow Cytometer, Beckman-Coulter) the light source is an argon-laser, which emits coherent light at a wavelength of $488nm$. Scattered and emitted fluorescent light is collected in front of the light source and at a right angle (90°) to detect the forward scatter (front) or the side scattering and fluorescence (right angle) respectively. With flow cytometry different physical characteristics such as cell size, shape and internal complexity as well as surface molecules, that can be detected by a fluorescent compound, can be examined. Additionally, many flow cytometers have the ability to sort, or physically separate, particles from a sample.

The data generated can be displayed using either a linear or a logarithmic scale. The use of a logarithmic scale is indicated in most biological situations where distributions are skewed to the right. In this case the effect is to normalize the distribution. Linear scaling is used when there is not such a broad range of signals, e.g. in DNA analysis. For analysis of surface molecules antibodies bound to fluorescent dyes were used. The utilized fluorochromes and their characteristic excitation and emission wavelength is illustrated in table 3.15.

Table 3.15: Table of fluorochromes used in flow cytometry and their characteristic excitation and emission wavelength. The channel corresponds to the flow cytometer, in which distinct emissions are detected.

Channel	Sensor* [nm]	Fluorochrome	Excitation [nm]	Emission [nm]
FL-1	505-545	Fluorescein		
		isothiocyanate (FITC)	495	519
FL-2	560-590	R-Phycoerythrin (PE)	480 / 565	578
FL-3	605-635	PE-Cy5 (CyChrome)*	480 / 565 / 650	670
		PE-Texas Red (ECD)	480 / 565	613
FL-4	≥660	PE-cyanin 5.1 (PC5)	488 / 565 / 652	670

*Filters and sensors used in the EPICS XL Flow Cytometer, Beckman-Coulter; Cy-Chrome was utilized with the FACSCalibur, Becton Dickinson (different sensor and filter array)

Cells were stained with directly conjugated antibodies, which are listed in table 3.16 on page 38. Samples of $40\mu L$ of cell suspension ($1 \cdot 10^5$ cells) were incubated for $15min$ on ice in PBS containing 5% human serum albumin with corresponding antibodies. Cells were then fixed in 0.5% paraformaldehyde (Sigma, Deisenhofen, Germany). Appropriate isotype controls were used. Cell lines were analyzed for MUC1 expression with FITC-conjugated HMFG-1 (Burchell et al., 1983), SM-3 (Burchell et al., 1987) and 12c10 antibodies. Samples were analyzed using an EPICS XL Flow Cytometer (Beckman-Coulter, High Wycombe, UK) and WinMDA 2.8 software (Scripps Research Institute, La Jolla, CA) or with a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and CellQuest 3.1 software (Beckton Dickinson).

3.5.3 Apoptosis Analysis

For quantification of monocytes and dendritic cells undergoing early programmed cell death an annexin V – propidiumiodide apoptosis detection kit (Becton Dickinson, Heidelberg, Germany) was utilized. Analyzed cells were washed once in PBS and resuspended in the provided binding buffer. Annexin V (FITC-conjugated) and propidiumiodide were added and incubated for $15min$ at room temperature in the dark. Immediate flow cytometric analysis was performed using an EPICS XL Flow Cytometer (Beckman-Coulter, High Wycombe, UK) and WinMDA 2.8 software (Scripps Research Institute, La Jolla, CA). The cell population was selected by

Table 3.16: Antibodies used for surface protein analysis

Antibody reacts with	Clone	Isotype	Fluorochrome	Company
Isotype control	MOPC-21	Mouse IgG_1, κ	all	Becton Dickinson
HLA-A,B,C	ω6/32	Mouse IgG_1, κ	FITC	Cancer Research UK
HLA-A*0201	BB7.2	Mouse IgG_{2b}, κ	FITC	Cancer Research UK
HLA-DR	G46-6	Mouse IgG_{2a}, κ	CyChrome	Becton Dickinson
CD1a	HI149	Mouse IgG_1, κ	CyChrome	Becton Dickinson
CD3	UCHT1	Mouse IgG_1, κ	CyChrome	Becton Dickinson
CD4	RPA-T4	Mouse IgG_1, κ	FITC	Becton Dickinson
CD8	RPA-T8	Mouse IgG_1, κ	PE	Becton Dickinson
CD14	M5E2	Mouse IgG_{2a}, κ	FITC	Becton Dickinson
CD16	3G8	Mouse IgG_1, κ	FITC	Becton Dickinson
CD19	HIB19	Mouse IgG_1, κ	PE	Becton Dickinson
CD25	M-A251	Mouse IgG_1, κ	PE	Becton Dickinson
CD40	5C3	Mouse IgG_1, κ	FITC	Becton Dickinson
CD54 (ICAM-1)	HA58	Mouse IgG_1, κ	PE	Becton Dickinson
CD56	B159	Mouse IgG_1, κ	PE	Becton Dickinson
CD69	FN50	Mouse IgG_1, κ	PE	Becton Dickinson
CD71	M-A712	Mouse IgG_{2a}, κ	PE	Becton Dickinson
CD80	BB1	Mouse IgM, κ	FITC, PE	Becton Dickinson
CD83	HB15e	Mouse IgG_1, κ	PE	Becton Dickinson
CD86	FUN-1	Mouse IgG_1, κ	FITC, PE	Becton Dickinson
CXCR4	12G5	Mouse IgG_{2a}, κ	PE	Becton Dickinson
CCR7	3D12	Rat IgG_{2a}, κ	PE	Becton Dickinson
12c10		Mouse IgG_1, κ	FITC	Cancer Research UK
HMFG-1		Mouse IgG_1, κ	FITC	Cancer Research UK
HMFG-2		Mouse IgG_1, κ	FITC	Cancer Research UK
SM-3		Mouse IgG_1, κ	FITC	Cancer Research UK

forward (FSC) and side scatter (SSC).

3.5.4 Cytospin

With the cytospin method cells were brought up on a slide and subsequently stained with haematoxylin and eosin (HE), a combination of a basophil (blueish-purple) nuclear stain and an acidophil (pink) cytoplasmic stain. The detailed protocol is described below:

- Collect cells and adjust to a concentration of $3 \cdot 10^5 \ \frac{1}{mL}$
- Assemble slide filter card (Cytofunnel, Shandon, Cheshire, UK) and sample chamber in the slide clip and place it in the cytospin head (Cytospin 3, Shandon)
- Pipette 1 drop (by Pasteuer pipette) into sample chamber
- Centrifuge ($1500rpm$ / $2min$ / $20°C$)
- Leave slides to air dry for $10min$
- Fix cells in 100% ethanol (Sigma)
- Leave slides to air dry for $10min$
- Put slides into a slide holder and wash once in water
- Stain in haematoxylin (Sigma) for $30sec$
- Wash slides for $2min$ with water
- Stain in eosin (Sigma) for $30sec$
- Wash slides for $2min$ with water
- Dehydrate cells twice in ethanol (Sigma) and twice in xylene (Sigma) for $30sec$
- Protection: Coverslips were mounted with Mowiol-DABCO mounting medium

3.5.5 Scanning Electron Microscopy

Particularly, the Scanning Electron Microscopy (SEM) serves for the representation of surfaces. Therefore, the object of interest have to fulfill the following conditions: The object needs to be clean, free of dust and water-free, must not be gassing out in the vacuum and the surface needs to be electroconductive. For this reason, biological surfaces are sputtered with gold.

The protocol for the preparation of biological specimen is listed below:

- Overlay specimen with a 2.5% glutaraldehyde solution for 1 day
- Wash twice with PBS
- Dehydrate specimen with acetone (increasing percentage from 20% up to 100% for $1h$). Finally, incubate object for 1 day in 100% acetone.
- Critical point drying
- Sputtering with gold
- SEM analysis

3.6 Immunobiological Analysis

3.6.1 Analysis of Endocytosis

The endocytic activity of immature dendritic cells was assessed with FITC-conjugated dextran (MW=10,000, Molecular Probes, MoBiTec, Göttingen, Germany), Alexa Fluor 488 conjugated LPS (Molecular Probes) and collagen-I labeled microspheres ($2\mu m$, FluoSpheres, Molecular Probes). For the analysis of dextran (Duperrier et al., 2000) and LPS (Vasselon et al., 1999) uptake, $2 \cdot 10^5$ dendritic cells were harvested, incubated with $100\mu L$ fetal calf serum for $15min$, centrifuged ($200g$ / $10min$ / $20°C$) and incubated for $1h$ with $100\mu L$ of $1\frac{mg}{mL}$ dextran or $20\frac{\mu g}{mL}$ LPS at $37°C$ (positive) and $0°C$ (negative) respectively. Afterwards, cells were washed three times in ice cold PBS and resuspended in $300\mu L$ PBS containing 0.1% trypan blue (Sigma) to quench fluorescence from the cell surface. The flow cytometrical analysis was carried out immediately.

For FluoSpere uptake, $5 \cdot 10^5$ dendritic cells were incubated in PBS supplemented with 5% FCS with $5 \cdot 10^6$ collagen-I labeled microspheres at $37°C$ for $2h$ and $20h$ respectively. After addition of $200\mu L$ PBS cells were analyzed directly on a flow cytometer.

3.6.2 Migration of Dendritic Cells

Matured dendritic cells were harvested, washed twice and adjusted to a cell density of $1 \cdot 10^6$ $\frac{1}{mL}$ in X-VIVO 15 medium. The chemotaxis toward chemokines was tested using a transwell assay ($6\mu m$ pore size, NUNC, Wiesbaden, Germany). The lower chambers were filled with $500\mu L$ medium containing the chemokines CCL19 (MIP-3β, $250\frac{ng}{mL}$, R&D), CXCL12 (SDF-1α, $100\frac{ng}{mL}$, R&D) or no chemokines (Luft et al., 2002). In the upper chamber $100\mu L$ of cell suspension was inoculated and the assay was incubated at $37°C$ and 5% CO_2. After $2h$ cells in the lower chamber were harvested and counted by a CASY1 particle counter. All migratory studies were done in triplicate.

3.6.3 Allogeneic T Cell Response

T Cell Proliferation: Flow Cytometric Analysis

Allogeneic responder PBMCs ($1 \cdot 10^6$ $\frac{1}{mL}$) with known MHC mismatch were cultured with matured dendritic cells ($1 \cdot 10^5$ $\frac{1}{mL}$ (1:10), $5 \cdot 10^4$ $\frac{1}{mL}$ (1:20), $2.5 \cdot 10^4$ $\frac{1}{mL}$ (1:40) and $1.25 \cdot 10^4$ $\frac{1}{mL}$ (1:80) respectively) in AIM-V medium (Gibco, Carlsbad, CA) for 4 days in 24-well plates (NUNC, Wiesbaden, Germany) at $37°C$ in a humidified 5% CO_2 containing atmosphere. On day 4 proliferation was analyzed by flow cytometer.

Characterization of surface markers involved in activation and proliferation gives insight into specific cell subsets and T cell responses in the presence of dendritic cells (Nguyen et al., 2003). Therefore, the expression on T cells of the activation markers CD25 (Interleukin-2 α-chain) and CD71 (transferrin receptor) was investigated on day 4 of the mixed leukocyte reaction. Cell samples were stained for CD3 (ECD), CD25 (FITC) and CD71 (PE) respectively. Corresponding isotype controls were used.

T Cell Proliferation: Counting

Dendritic cells were added to $5 \cdot 10^5$ allogeneic PBMCs at a ratio of 1:10 in 6 well plates and co-incubated for 4 days in AIM V. After 4 and 7 days 50% of the culture medium were replaced and 100$\frac{U}{mL}$ rhuIL-2 (R&D) were added. After 8 days the proliferating T cells were counted, phenotyped and an Interferon-γ secretion assay was performed (see section 3.7.3 on page 47).

3.6.4 Synthetic Peptides

HLA-A*0201-binding peptides Melan-A / Mart-1_{26-35} A27L ELAGIGILTV (Kawakami et al., 1994; Romero et al., 1997; Valmori et al., 1998) and influenza A virus matrix$_{58-66}$ peptide (FLU M1) with the sequence GILGFVFTL (Gotch et al., 1987; Pogue et al., 1995) were used for in vitro stimulation of T cells. The Pan-HLA-DR binding peptide PADRE AGVAAWTLKAAA (Alexander et al. (1994), Brossart et al. (1999)) was used to enhance T-helper cell responses. All peptides were made by Fmoc chemistry with a Syro II peptide synthesizer (Multisyntech, Witten, Germany). The peptide was analyzed by reversed-phase liquid chromatography that showed over 90% purity.

3.6.5 MHC Class I Restricted T Cell Response

Table 3.17: Materials used for the MHC class I restricted stimulation of T cells

Medium	AIM V	Gibco, Carlsbad, CA, USA
rhuIL-7	2400$\frac{U}{mL}$	R&D, Wiesbaden, Germany
rhuIL-2	20$\frac{U}{mL}$	R&D, Wiesbaden, Germany
FLU M1 peptide	40$\frac{\mu g}{mL}$ / 20$\frac{\mu g}{mL}$	Cancer Research UK
Melan-A / Mart-1 peptide	40$\frac{\mu g}{mL}$ / 20$\frac{\mu g}{mL}$	Cancer Research UK
PADRE helper peptide	50$\frac{\mu g}{mL}$	Cancer Research UK
β_2-microglobulin	5$\frac{\mu g}{mL}$	Sigma, Deisenhofen, Germany
6 well plates	$4mL$	NUNC, Wiesbaden, Germany
rhuIL-4		R&D, Wiesbaden, Germany
Tissue culture dishes		NUNC, Wiesbaden, Germany

PBMCs from buffy coats from healthy donors were obtained and enriched for $CD14^+$ cells as described (see section 3.4.1 on page 32). The negative fraction was frozen in autologous, heat-inactivated plasma containing 10% dimethyl sulfoxide (DMSO). The TNF-α and PGE_2 matured monocyte-derived dendritic cells were pulsed for $2h$ at $37°C$ with the appropriate peptide ($40\frac{\mu g}{mL}$), PADRE helper peptide ($50\frac{\mu g}{mL}$) and human β_2-microglobulin ($5\frac{\mu g}{mL}$) in serum-free AIM V medium. The $CD14^+$ depleted fraction of PBMCs was thawed and co-cultured at a concentration of $1 \cdot 10^6\ \frac{1}{mL}$ with $1 \cdot 10^5\ \frac{1}{mL}$ peptide-pulsed autologous DCs in a volume of $4mL$ (triplicates) AIM-V medium and $2400\frac{U}{mL}$ IL-7 in 6 well plates. After 7 days a second stimulation was performed. $CD4^+$ cells were depleted by CD4 mAb coated tissue culture dishes. Enriched cells were centrifuged, resuspended in fresh AIM-V medium containing $20\frac{U}{mL}$ IL-2, adjusted to a cell concentration of $1 \cdot 10^6\ \frac{1}{mL}$ and incubated with $5 \cdot 10^4\ \frac{1}{mL}$ DCs, which had previously been pulsed with appropriate peptide ($20\frac{\mu g}{mL}$) and human β_2-microglobulin ($5\frac{\mu g}{mL}$). Feeding of cells was performed every 2^{nd} day by addition of medium supplemented with $20\frac{U}{mL}$ IL-2 according to proliferation. Seven days after stimulation T-cells were phenotypically and functionally analyzed.

3.6.6 Stimulation of Autologous T Cells with Tumor Lysate-pulsed Dendritic Cells

Table 3.18: Materials utilized for the autologous stimulation of T cells with lysate-pulsed dendritic cells

Medium	AIM V	Gibco, Carlsbad, CA, USA
rhuIL-7	$2400\frac{U}{mL}$	R&D, Wiesbaden, Germany
rhuIL-2	$20\frac{U}{mL}$	R&D, Wiesbaden, Germany
6 well plates	$4mL$	NUNC, Wiesbaden, Germany

Carcinoma cell lysate-pulsed dendritic cells were harvested on day 8 and co-cultured at a cell density of $1 \cdot 10^5\ \frac{1}{mL}$ with $1 \cdot 10^6\ \frac{1}{mL}$ autologous PBMCs in a volume of $4mL$ (triplicates) AIM-V medium and $2400\frac{U}{mL}$ IL-7 in 6 well plates. Restimulation of PBMCs was performed weekly with thawed lysate-pulsed dendritic cells at a ratio of 1:20 with fresh medium and $20\frac{\mu g}{mL}$ IL-2. Cells were fed according to proliferation. The cell density of PBMCs was kept between $5 \cdot 10^5\ \frac{1}{mL}$ and $1 \cdot 10^6\ \frac{1}{mL}$ to avoid substrate limitations such as glucose and glutamine (see also Bohnenkamp et al., 2002). PBMCs were harvested $48h$ after the 3^{rd} stimulation and analyzed as described.

3.6.7 Tetramer Staining

Antigen-specific T cells were identified by using Phycoerythrin-labeled HLA-A*0201 tetramer complexes (ProImmune, Oxford, UK) folded around the decapeptide analogue Melan-A / Mart-1_{26-35} A27L ELAGIGILTV, the synthetic analogue of influenza A virus matrix_{58-66} peptide GILGFVFTL or the MUC1 specific epitopes F7 (MUC_{13-21}) LLLTVLTVV (Heukamp et al., 2001) and M1.2 (MUC_{12-20}) LLLLTVLTV (Brossart et al., 1999). To minimize non-specific staining, each tetramer was titered and used at the lowest concentration that revealed a positive population for specific cytotoxic T cells. PBMCs or specifically stimulated T cells were resuspended in PBS containing 5% human serum albumin and incubated with indicated tetramer for $20min$ at $20°C$ followed by staining with CD8 (PC5) for $15min$ at $20°C$ in the dark. Cells were washed twice and fixed with 0.5% paraformaldehyde before analyzing by flow cytometry. A minimum of 50,000 CD8^+ events were collected.

3.7 Cytokine Analysis

3.7.1 The Cytometric Bead Array

To analyze the profile of cytokines produced by matured dendritic cells and during autologous T cell stimulation, supernatants were collected and frozen at $-80°C$. The concentrations of the typical Th1 / Th2 cytokines IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-10 respectively were measured using a cytometric bead array (CBA) kit (Becton Dickinson) for detection of all six cytokines simultaneously (detection limit: $5\frac{pg}{mL}$). In this test, six populations of beads with distinct fluorescence intensities are coated with corresponding antibodies specific for the proteins to be investigated and analyzed by flow cytometer (see figure 3.1 on page 45). The human cytokine capture bead suspension ($50\mu L$ per test) and detection reagent ($50\mu L$ per test) were transferred to assay tubes and incubated with samples and provided standards ($50\mu L$ per test) for $3h$ at $20°C$. After washing with $300\mu L$ of provided wash buffer, $300\mu L$ of wash buffer was added and FACS analysis was performed using a FACSCalibur (Becton Dickinson), CellQuest 3.1, CBA software (Becton Dickinson) and Excel (Office 2001, Microsoft, Redmond, USA) according to the manufacturer's instructions. The standard curves for the analyzed cytokines are shown in figure 3.2 on page 46.

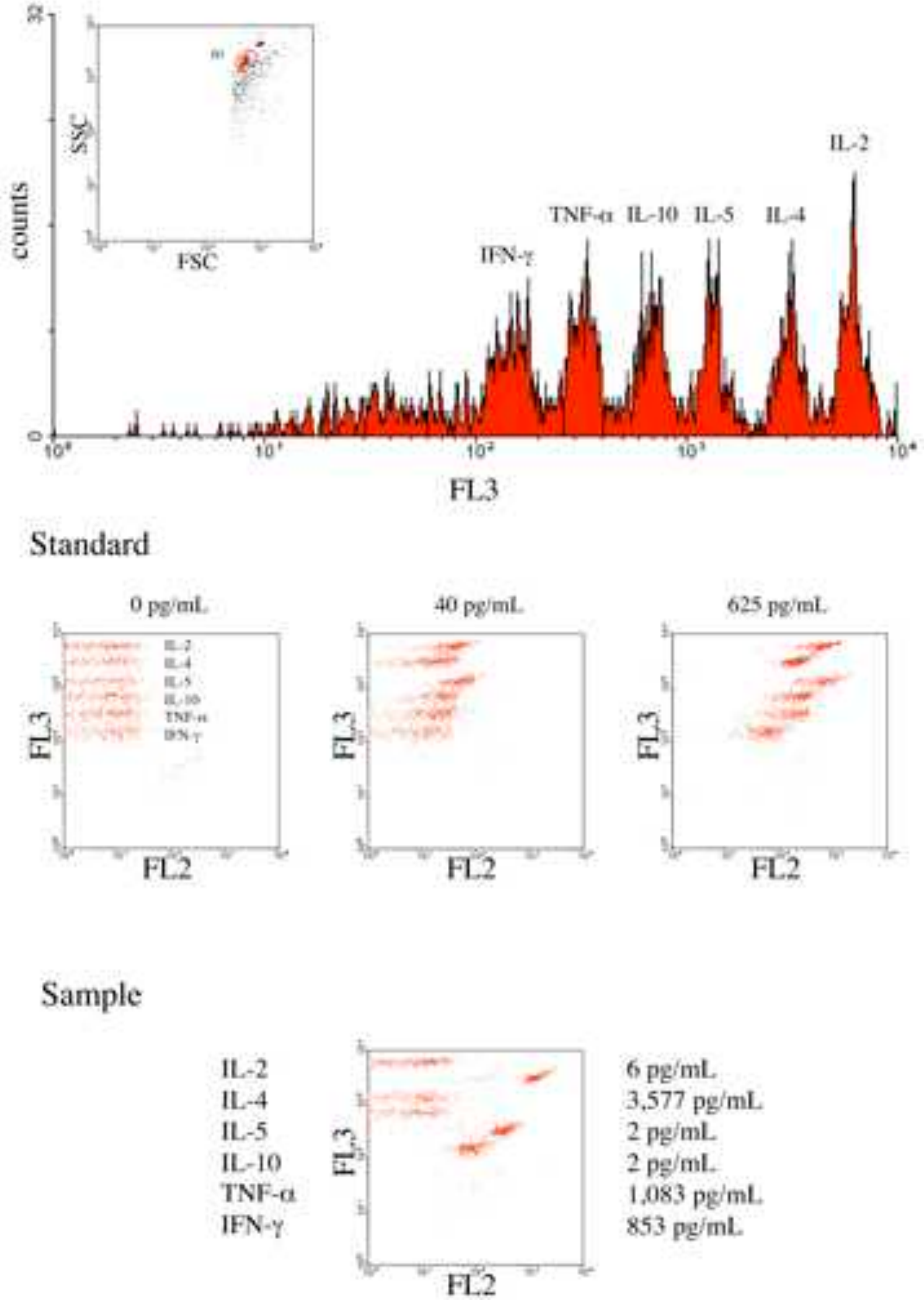


Figure 3.1: Flowcytometric analysis of different cytokines with the cytometric bead array. Beads specific for different cytokines (IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2 respectively) can be distinguished by their fluorescence intensity in the channel FL-3. After incubation with supernatants, cytokines bind to specific beads and are identified by a specific fluorescent labeled antibody. The concentration is detected in the channel FL-2.

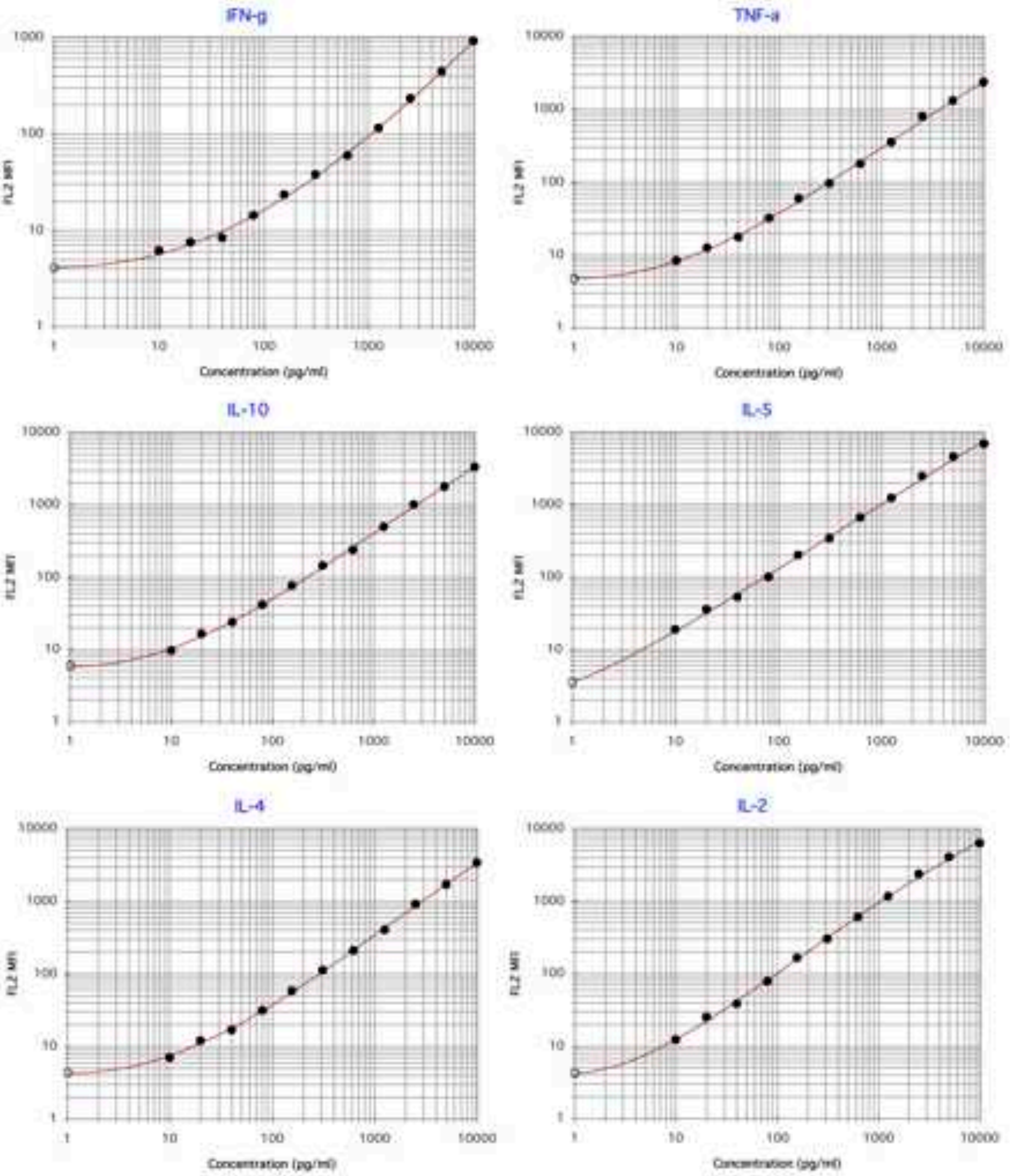


Figure 3.2: The standard curves are analyzed with the software Excel (Office 2001, Microsoft) and the CBA plugin (Becton Dickinson). Measured samples are compared with the standard curve for calculation of the concentration. The standard curve range for the cytokine concentrations is between $5\frac{pg}{mL}$ and $10{,}000\frac{pg}{mL}$.

3.7.2 Enzyme Linked Immunosorbent Assay

Cytokine ELISA kits for rhuGM-CSF (detection limit: $4.7\frac{pg}{mL}$), rhuIL-4 (detection limit: $7.8\frac{pg}{mL}$) and rhuIL-12p70 (detection limit: $7.8\frac{pg}{mL}$) were purchased from Becton Dickinson (OptEIA human ELISA Set, BD PharMingen, Heidelberg, Germany) and were used following the manufacturer's instructions. The readout of the ELISA-plates was performed using a photometer (Wallac Victor^2, PerkinElmer Life Science, Bad Wildbad, Germany) reader at $450nm$ with a correction at $570nm$.

3.7.3 Interferon-γ Secretion Assay

For determination of the portion of IFN-γ producing T cells an IFN-γ Cytokine Secretion Assay (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instructions (Scheffold et al., 1995). PBMCs ($1 \cdot 10^6$) were washed once, resuspended in $90\mu L$ of cold AIM V and incubated with $10\mu L$ of the provided cytokine catch reagent for $5min$ on ice. After adding of $10mL$ of AIM V ($37°C$), cells were placed in an incubator for $45min$ and moved every $5min$. Subsequently, the PBMCs were centrifuged ($300g$ / $10min$ / $4°C$), resuspended in $90\mu L$ of PBS containing 2% bovine serum albumine and incubated with $10\mu L$ of detection antibody for $10min$ on ice. Cells were washed, stained with antibodies for flow cytometry (see section 3.5.2 on page 36) and analyzed. In figure 3.3 on page 48 the principle of the secretion assay is illustrated.

3.8 Analysis of Medium Components

Osmolality was measured using a freezing-point osmometer Osmomat 030 (Gonotec, Berlin, Germany). Glucose (Ebio compact, Eppendorf, Hamburg, Germany), lactate (YSI 1500L, Yellow Springs Instruments, Yellow Springs, USA), glutamine and glutamate (YSI 2700 select, Yellow Springs Instruments, Yellow Springs, USA) were quantified enzymatically using the indicated automatic analyzer according to the manufacturer's instructions. Amino acid analysis was realized using HPLC (Amino Quant 1090 AX, Hewlett Packard, Waldbronn, Germany).

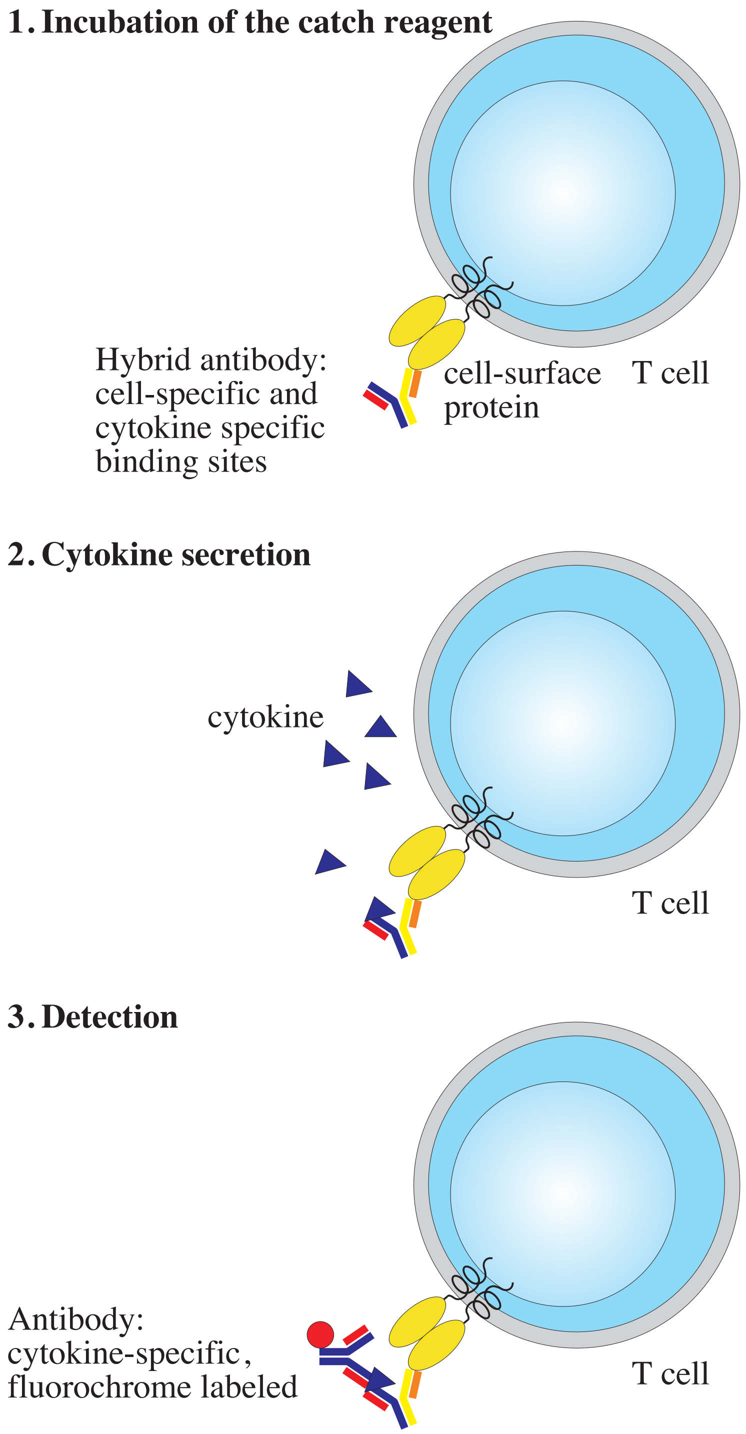


Figure 3.3: The principle of the IFN-γ Cytokine Secretion Assay. Hybrid antibodies are used, which contain a cell-specific and a cytokine-specific binding site. After binding of the antibody to a common surface molecule, the cells are incubated for $45min$ to secrete IFN-γ, which binds to the cytokine-specific binding site. A second fluorochrome labeled antibody detects the bound cytokine and cells are analyzed by a flow cytometer.

Chapter 4

Results

4.1 The Generation of Dendritic Cells

The aim of the development of a simple and standardized protocol was to obtain a homogenous population of fully matured dendritic cells in a high yield. For that reason, the disadvantages of non-uniform culture conditions caused by supplemented serum, non-defined cell densities and adherence steps need to be circumvented. Furthermore, the need for feeding of cells is to be avoided to decrease the risk of contamination and labour intensive steps. In this study the influence of the main important cultivation parameters were investigated to optimize and setup the serum-free generation of monocyte-derived dendritic cells in the medium X-VIVO 15. Cell density, GM-CSF and IL-4 concentration, medium components like glucose, lactate and amino acids were assessed. Moreover, the influence of different maturation stimuli (TNF-α, IL-1β, IL-6, PGE_2 and TNF-α, PGE_2) on the maturation status of DCs was examined. During all optimization steps the requirements for full accordance to GMP (good manufacturing practice) conditions were considered.

4.1.1 Cell Enrichment and Starting Population of Monocytes

As source of monocytes fresh blood as well as buffy coat preparations from healthy donors were used. After blood donation ($500mL$) the blood bag is centrifuged for 16 to $23min$ at room temperature at up to $4000g$ (Deutsches Rotes Kreuz, 2004). Afterwards, three distinct layers can be distinguished: the plasma layer on the top, the red cell fraction on the bottom and the intermediate layer, the buffy coat, which contains the white leukocytes of the blood.

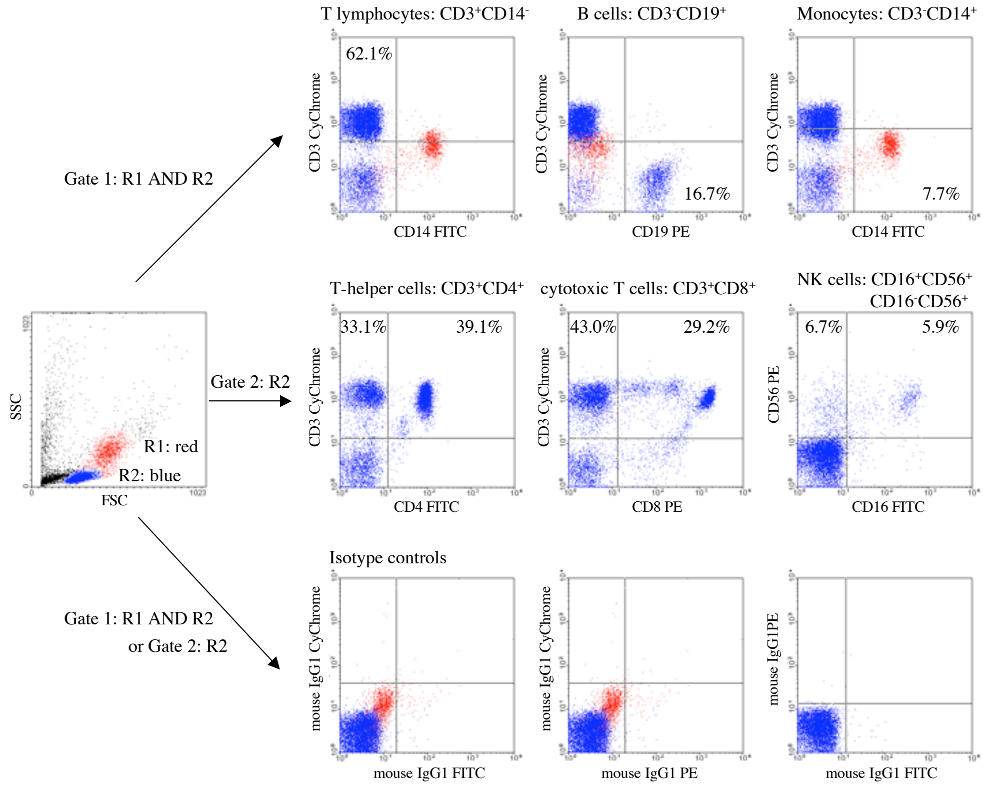


Figure 4.1: Flowcytometric analysis of peripheral blood mononuclear cells after ficoll density gradient centrifugation. Shown are dot plots of one representative donor. The quadrants of the dot plot analyzing monocytes (top right) is adjusted due to the higher auto fluorescence scattering. The isotype control for the center dot plots refers to the blue population.

This intermediate layer was utilized as source of monocytes from peripheral blood mononuclear cells.

Buffy coat preparations are usually containing not only lymphocytes and monocytes but also erythrocytes, granulocytes and plasma. For further separation of the lymphocyte and monocyte fraction a ficoll density gradient centrifugation was done. In this step the cells were divided by their density and again the intermediate fraction was collected, containing the leukocytes without granulocytes. In figure 4.1 the flowcytometrical analysis of PBMCs after ficoll density gradient centrifugation is illustrated. In the forward / sideward scatter (FSC / SSC) dot plot two different cell populations can be observed, a blue population (R2 = region 2) and a red population (R1 = region 1). The upper three dot plots represent both regions, which is termed

Table 4.1: The distribution of cell subpopulations in peripheral blood from one representative donor (see also figure 4.1)

Subpopulation	T cell subset	Phenotype	Whole population Gate 1 [%]	Lymphocytes Gate 2 [%]
T cells		$CD3^+$	62.1	
	T-helper cells	$CD3^+CD4^+$	33.6*	39.1
	Cytotoxic T cells	$CD3^+CD8^+$	25.1*	29.2
	Not defined	$CD3^+CD4^-CD8^-$	3.4*	
B cells		$CD19^+$	16.7	
NK cells		$CD56^+CD16^+$	5.2	5.9
NK cells		$CD56^+CD16^-$	5.9	6.7
Monocytes		$CD14^+$	7.7	
Total*			94.6	
Not defined			5.4	

*Subpopulation of T cells do not contribute to the calculation of the total cell number.

gate 1 (in this case gate 1 = R1 and R2). By analysis with monoclonal antibodies against surface molecules specific for T cells (CD3, binds to the T cell receptor), B cells (CD19, an immunglobulin specific to B cells) and monocytes (CD14, receptor for LPS and LBP complex), the distribution of the three main components of the mononuclear cells can be demonstrated. Thereby, the red population, the monocytes ($CD14^+$), can be distinguished by the higher level of auto fluorescence and the larger size in the forward and sideward scatter. The center dot plots represent gate 2 (gate 2 = R2). Only the blue region of cells is analyzed in these dot plots to circumvent interference with the higher level of auto fluorescence of monocytes. In these dot plots the T cell subpopulations, namely T-helper cells ($CD4^+$, binds to MHC class II molecules) and cytotoxic T cells ($CD8^+$, binds to MHC class I molecules), and the different natural killer (NK) cell populations ($CD16^+CD56^+$ and $CD16^-CD56^+$, CD16: FcγRIII, component of Fc receptor that binds to different isotypes of antibodies (IgG1, IgG3); CD56: immunglobulin, cell adhesion molecule) can be distinguished. The three dot plots on the bottom of figure 4.1 show the isotype controls. In table 4.1 the different cell components of peripheral blood mononuclear cells are shown.

Different methods of monocyte enrichment are currently discussed in literature (see also

Table 4.2: Yield of monocytes of 8 different donors after magnetic-bead enrichment

	Number of PBMCs after ficoll [-]	Number of monocytes after enrichment [-]	Yield of monocytes after enrichment [%] of PBMCs
Donor 1	$6.7 \cdot 10^8$	$4.6 \cdot 10^7$	6.9
Donor 2	$4.9 \cdot 10^8$	$4.2 \cdot 10^7$	8.6
Donor 3	$5.6 \cdot 10^8$	$1.0 \cdot 10^8$	17.9
Donor 4	$8.8 \cdot 10^8$	$8.8 \cdot 10^7$	10.0
Donor 5	$4.7 \cdot 10^8$	$8.8 \cdot 10^7$	18.7
Donor 6	$8.9 \cdot 10^8$	$1.2 \cdot 10^8$	13.5
Donor 7	$7.5 \cdot 10^8$	$1.1 \cdot 10^8$	14.7
Donor 8	$9.0 \cdot 10^8$	$1.1 \cdot 10^8$	12.2
Mean	$7.0 \cdot 10^8$	$8.8 \cdot 10^7$	12.8
Standard deviation	$1.7 \cdot 10^8$	$2.7 \cdot 10^7$	4.0

chapter 2.4). For the process development of a standardized protocol a homogenous starting population of monocytes is inevitable for investigation of cytokine influences, medium limitations and metabolite influences. Due to the availability of GMP quality immunomagnetic beads from Miltenyi (CliniMACS) to isolate $CD14^+$ monocytes (Dzionek et al., 2002), this method was used in a laboratory setup with Mini- and Midi-MACS magnets and appropriate columns. The cells were purified using two columns to increase the enrichment efficiency. This magnetic cell sorting approach was highly effective in isolating $CD14^+$ monocytes from PBMCs with a yield of $12.8 \pm 4.0\%$ ($n = 8$) (illustrated in table 4.2) and a viability of $\geq 98.0\%$ (Trypan blue dye exclusion).

In figure 4.2 on page 54 the analysis of magnetic-bead enriched monocytes by forward / sideward scatter and expression of several surface proteins is shown. As determined by forward side scatter (FSC / SSC) the purity of enriched monocytes was 97.0% of this representative donor. Furthermore, the monocytes expressed high levels of MHC class I molecules (HLA-A,B,C^+), medium levels of MHC class II (HLA-DR^+) and low levels of CD1a. The co-stimulatory molecules CD86 (medium expression) and CD40 (weak) were found on their surface. The adhesion molecule ICAM-1(high), the receptor CD14 (high) and the chemokine receptor

CXCR4 (medium) were also expressed.

This highly pure population of monocytes were used as source for the development of a protocol for the generation of dendritic cells.

4.1.2 Cytokine Kinetics

During 6 days monocytes differentiate in the presence of GM-CSF and IL-4 to immature DCs. Additional two days are required for the maturation of DCs, whereby both cytokines need to be present to assist the maturation process. Therefore, both GM-CSF and IL-4 must not be exhausted after 8 days of cultivation, especially if feeding of the culture should be avoided. Thus, for the cultivation in the presence of these cytokines the concentration should be maintained or the concentration accordingly adjusted.

Several experiments were done to test the stability and half-life of the mentioned cytokines. The stability and half-life test was established utilizing triplicates of tissue culture flasks, which were also used for the generation of DCs. $40\frac{U}{mL}$ ($3{,}600\frac{pg}{mL}$) GM-CSF and $40\frac{U}{mL}$ ($227\frac{pg}{mL}$) IL-4 respectively were inoculated in X-VIVO 15 and incubated at 37°C and 5% CO_2 for 30 days. Samples were taken every 2^{nd} day, frozen and after collection thawed for quantification of GM-CSF and IL-4. The GM-CSF (Leucomax, Sandoz) concentration remained constant after 30 days. In contrast, the utilized IL-4 was not as stable as the GM-CSF. After 2 days the IL-4 (R&D) decreased to 60% of the initial inoculated cytokine concentration (see figure 4.3 on page 55). Afterwards the remaining IL-4 concentration showed further decay following a first order reaction, which is demonstrated in figure 4.4 on page 55. The rate constant was determined to $k = 0.03\frac{1}{d}$ and the half-life to $t_{\frac{1}{2}} = \frac{\ln 2}{k} = 23d$. A possible mechanism of the strong decrease in IL-4 concentration after 2 days may be the attachment of the cytokine to the positively charged surface of the tissue culture flask. Consequently, for the calculation of the rate constant only values between the 2^{nd} and the 30^{th} day were used.

4.1.3 Influence of Different Cell Densities on Yield and Maturation of DCs

To setup and optimize the protocol for the generation of dendritic cells, the influence of different cell densities on the consumption and accumulation of medium components such as glucose, lactate, amino acids and supplemented cytokines was investigated. The goal of these experiments

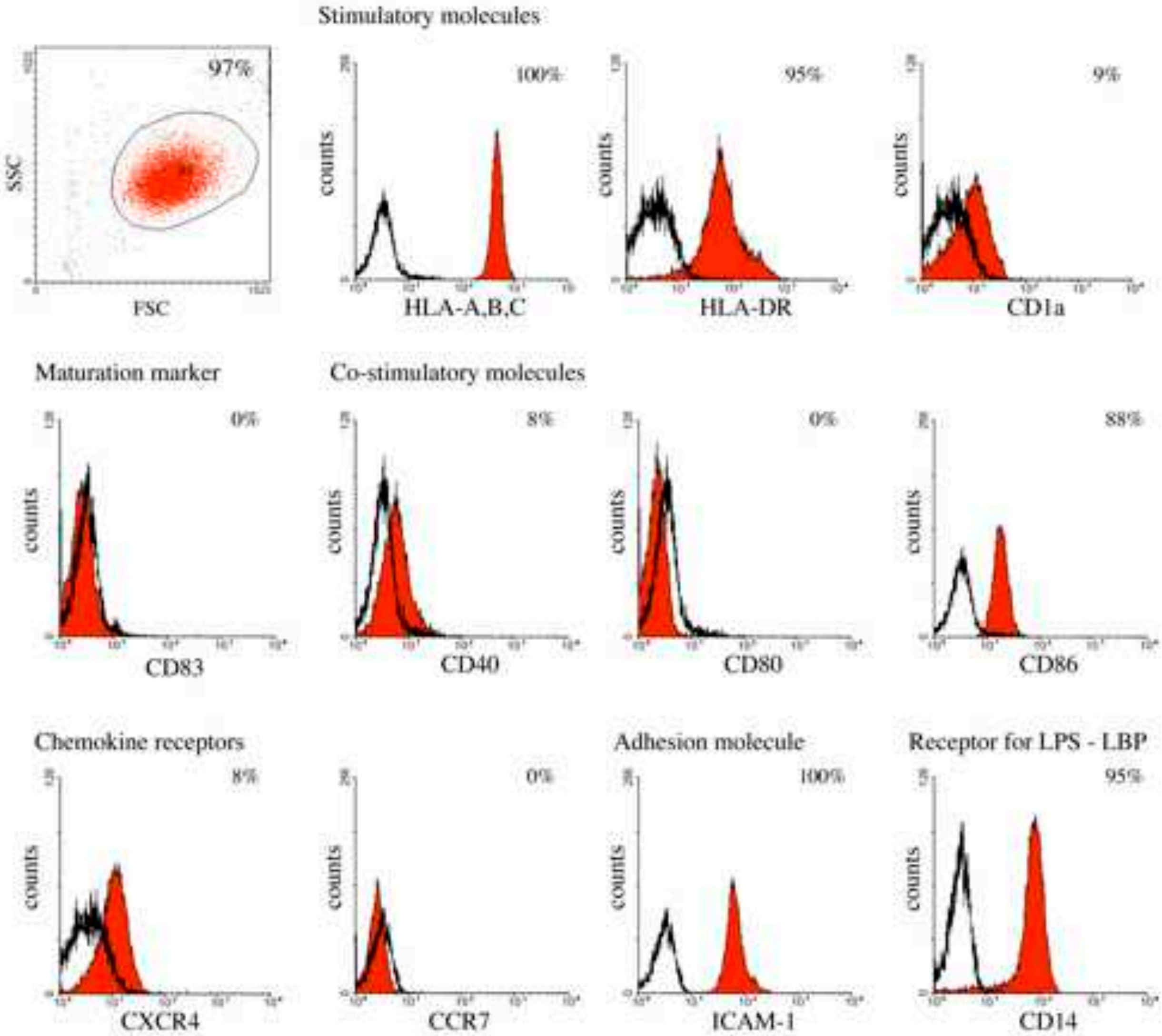


Figure 4.2: Flowcytometric analysis of monocytes after magnetic-bead enrichment (MACS). Shown is one representative donor. Outlined histograms indicate isotype controls, histograms correspond to gated cells

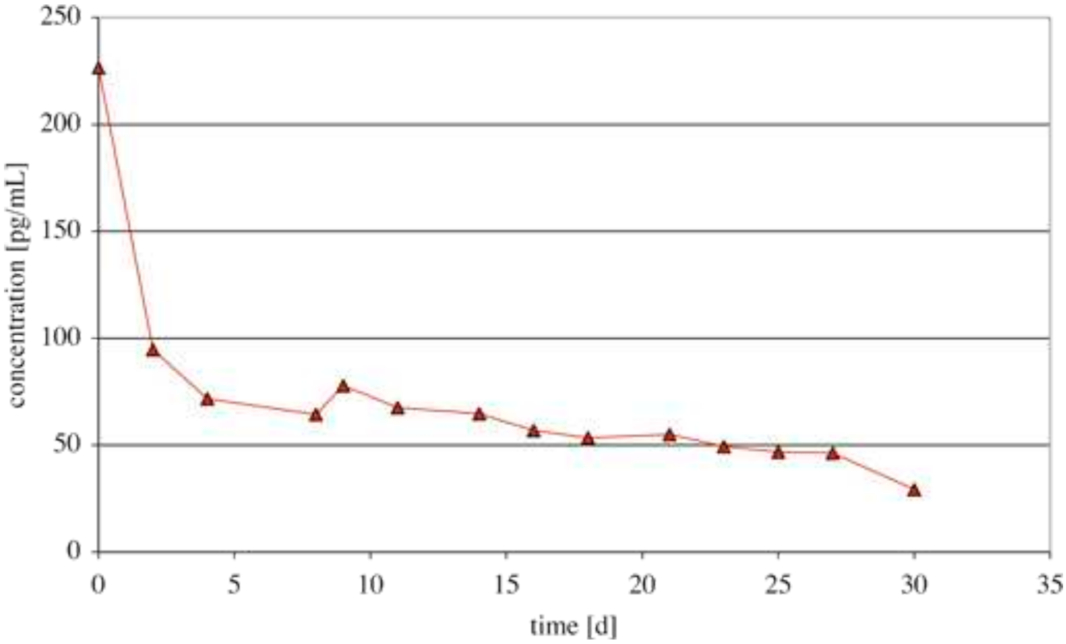


Figure 4.3: The concentration of IL-4 during the incubation for 30 days. The cytokine concentration was analyzed flowcytometrically by an cytometric bead array.

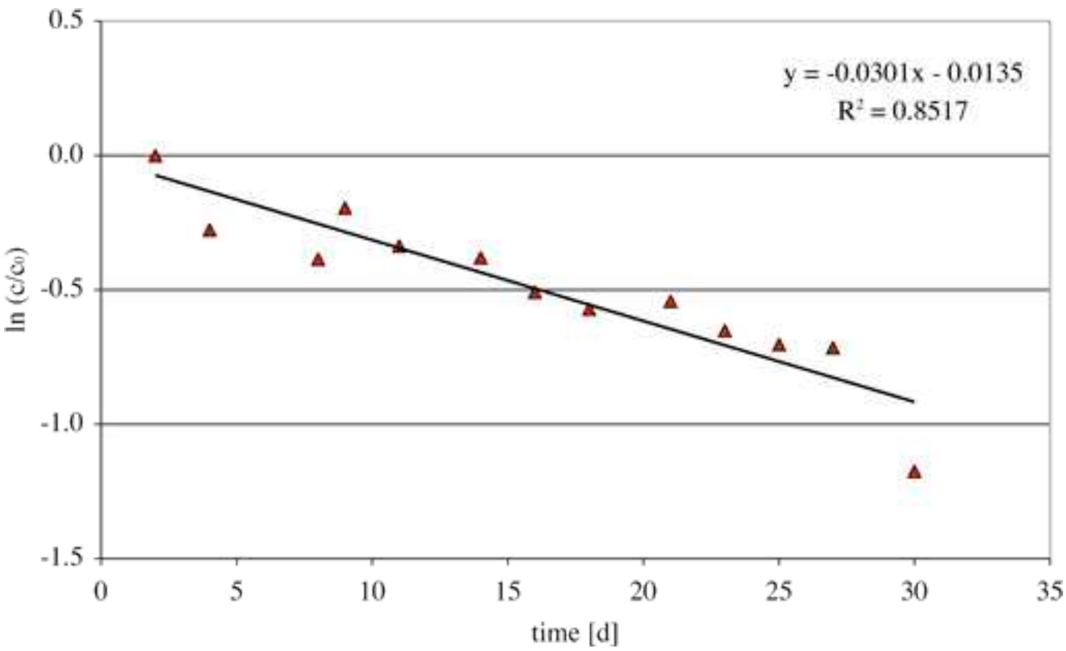


Figure 4.4: The determination of the rate constant k of the first order reaction; ln (c/c_0) is plotted against time; the slope gives k.

was to show that a batch cultivation strategy (without feeding) can be used for the differentiation of monocytes to dendritic cells. However, it has to be proven that the metabolism of these non proliferating cells does not enrich metabolites toxic for the differentiation. The parameters of performed experiments are shown in table 4.3.

Table 4.3: The parameters of the experiments to test the influence of different cell densities

Cell densities	$3.3 \cdot 10^5$, $6.6 \cdot 10^5$, $1.3 \cdot 10^6$, $2.6 \cdot 10^6$ $\frac{1}{mL}$
Donors	three
Cultivation system	48 well plates, $500\mu L$ volume
Medium	X-VIVO 15
GM-CSF	$800\frac{U}{mL}$
IL-4	$500\frac{U}{mL}$
Maturation stimulus	TNF-α ($1000\frac{U}{mL}$), IL-1β ($1000\frac{U}{mL}$), IL-6 ($1000\frac{U}{mL}$), PGE_2 ($1\frac{\mu g}{mL}$)
Time (differentiation / maturation)	6 days / 2 days

In different experiments $3.3 \cdot 10^5$, $6.6 \cdot 10^5$, $1.3 \cdot 10^6$ and $2.6 \cdot 10^6$ $\frac{1}{mL}$ monocytes were inoculated in X-VIVO 15 supplemented with $800\frac{U}{mL}$ GM-CSF and $500\frac{U}{mL}$ IL-4 (Feuerstein et al., 2000) and incubated for 6 days without feeding. The maturation cytokines TNF-α ($1000\frac{U}{mL}$), IL-1β ($1000\frac{U}{mL}$), IL-6 ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$) (Jonuleit et al., 1997) were added on day 6 and incubated for further two days.

However, as described in the previous section 4.1.2, the cytokine IL-4 might attach to the positive charged surface of the tissue culture flasks. For that reason both GM-CSF and IL-4 were added two hours after the inoculation of the cells. Monocytes are strongly plastic adherent, which results in the occupation of the bottom of the flask leaving less space for the binding of the IL-4. This finding was further assured by the fact that inoculation of GM-CSF and IL-4 directly with cells resulted in lower yield of dendritic cells compared to the latter method.

After 6 days of cultivation partly non-adherent dendritic cells could be observed, which did not express CD83, a marker of mature dendritic cells (Zhou and Tedder, 1995). The maturation stimulus added for two additional days induced the expression of CD83 and increased the surface proteins CD80 and CD86 as well as HLA-DR (MHC class II). These non-adherent dendritic cells with many motile veils showed the typical pattern of mature DCs, which is il-

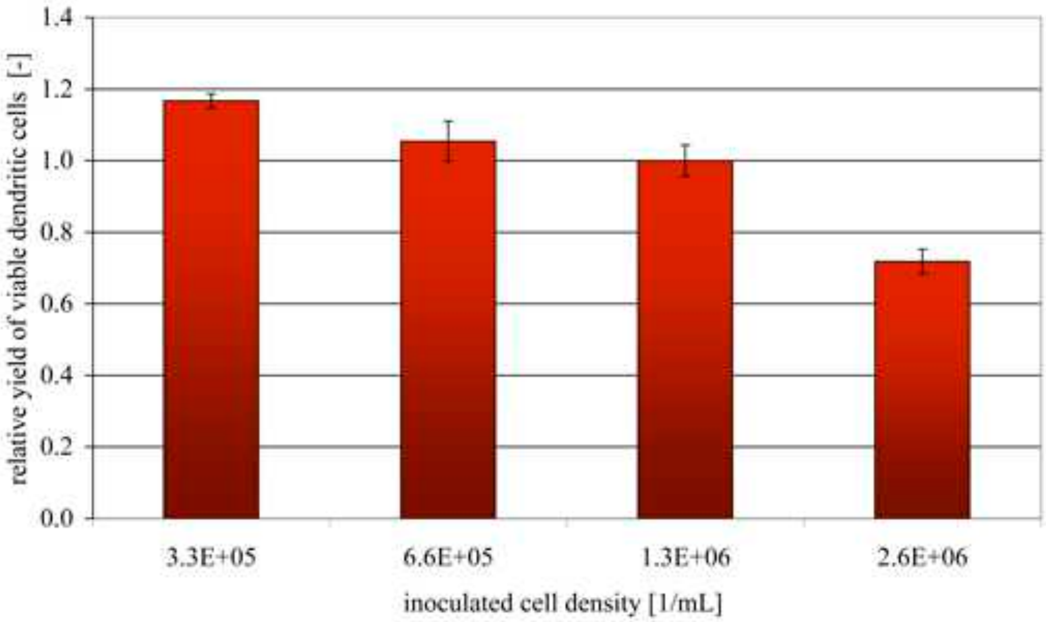


Figure 4.5: Influence of different cell densities on yield after generation of DCs. Monocytes were enriched via immunomagnetic beads, inoculated at specified cell densities and differentiated with $800\frac{U}{mL}$ GM-CSF and $500\frac{U}{mL}$ IL-4. For maturation a cytokine cocktail consisting of TNF-α ($1000\frac{U}{mL}$), IL-1β ($1000\frac{U}{mL}$), IL-6 ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$) was used. The data represent the mean ± SD (standard deviation) of triplicates from a single donor. The relative yield was calculated in relation to $1.3 \cdot 10^6\ \frac{1}{mL}$. From (Bohnenkamp and Noll, 2003)

lustrated in figure 4.6 on page 58. The size of the cells increased from an average of $10\mu m$ (monocytes) to $16\mu m$ (DCs), which was determined by the CASY 1 particle counter.

The yield (as defined by size of cells, morphology and surface antigen expression) of matured DCs was similar for the cultures initially inoculated with $3.3 \cdot 10^5$, $6.6 \cdot 10^5$ and $1.3 \cdot 10^6\ \frac{1}{mL}$ monocytes and about 25% lower for the highest inoculated cell density (see figure 4.5). All cell densities except for the $2.6 \cdot 10^6\ \frac{1}{mL}$ showed the typical mature DC phenotype with high expression of HLA-DR (MHC class II), CD80, CD83 and CD86, which is illustrated in figure 4.7. The DCs differentiated and matured from $2.6 \cdot 10^6\ \frac{1}{mL}$ monocytes expressed reduced HLA-DR, CD80, CD83 and CD86 antigens. The HLA-DR / CD80 and HLA-DR / CD83 dot plots for $6.6 \cdot 10^5$ and $1.3 \cdot 10^6\ \frac{1}{mL}$ showed two distinct populations of dendritic cells: a population with higher HLA-DR / CD80 and HLA-DR / CD83 expression and a lower one, which may be caused by a different maturation status. Especially for the HLA-DR / CD80 dot plot for the cell density of $1.3 \cdot 10^6\ \frac{1}{mL}$ this observation was obvious. Lower HLA-DR expression seems to

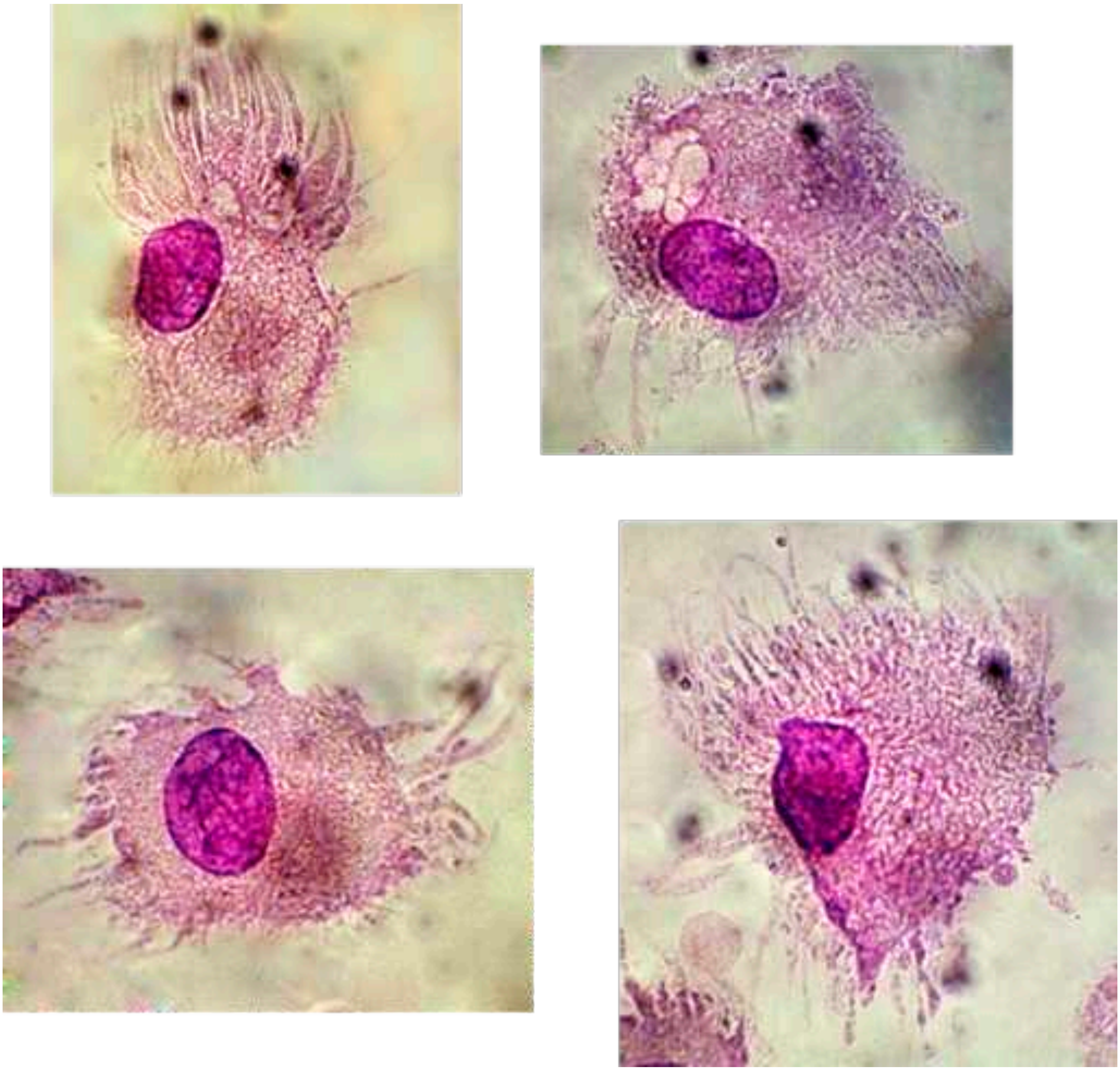


Figure 4.6: Cytospins of dendritic cells after the cultivation of 8 days. After centrifugation on a cytofunnel, cells were stained with haematoxylin and eosin. Photographs were taken at a 100x magnification with a Fuji Finepix S2 and subsequently edited in contrast and color for original reproduction.

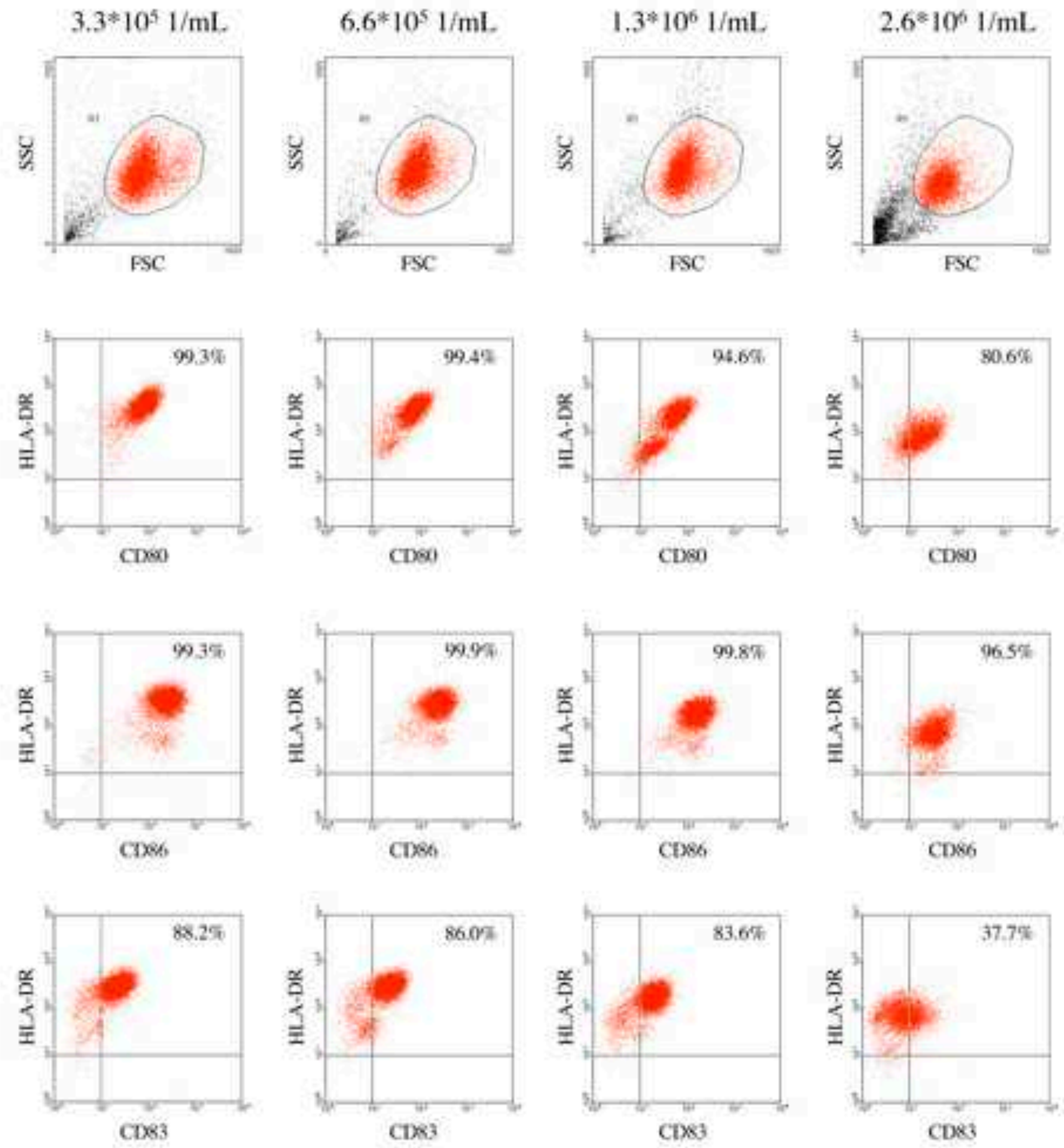


Figure 4.7: Phenotype of DCs generated with in table 4.3 specified cultivation parameters. HLA-DR / CD80, HLA-DR / CD86 and HLA-DR / CD83 dot plots are shown for different cell densities ($3.3 \cdot 10^5$, $6.6 \cdot 10^5$, $1.3 \cdot 10^6$ and $2.6 \cdot 10^6$ $\frac{1}{mL}$ respectively). Decreased levels of CD80, CD83 and CD86 were expressed for the highest cell density, HLA-DR / CD80 and HLA-DR /CD83 dot plots for $6.6 \cdot 10^5$ and $1.3 \cdot 10^6$ $\frac{1}{mL}$ featured a higher and a lower expressing population. The data shown are from one representative experiment out of 3 performed. From (Bohnenkamp and Noll, 2003)

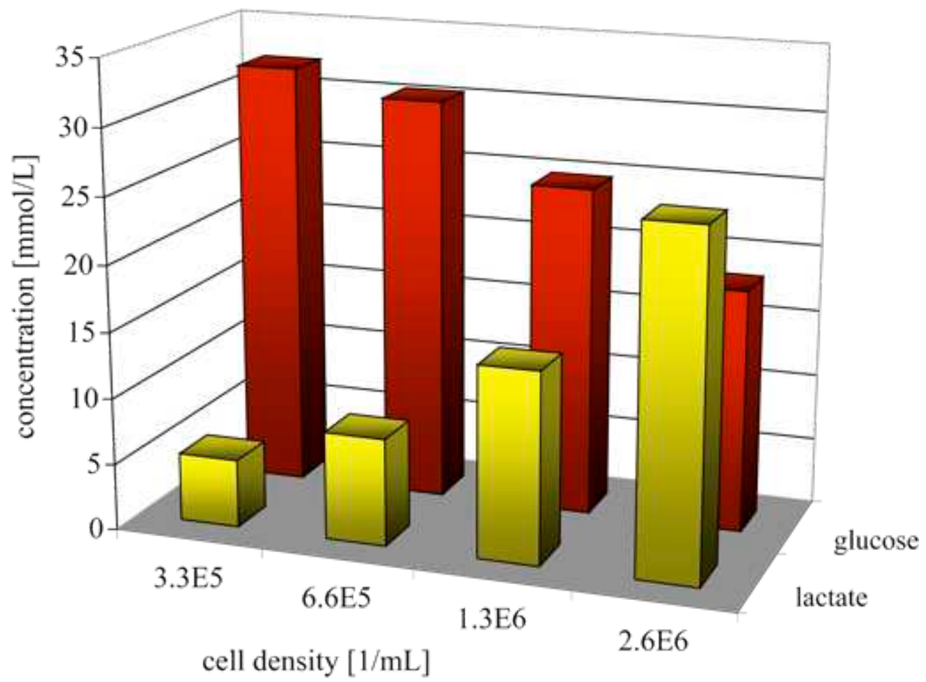


Figure 4.8: Glucose and lactate analysis on day 8 after generation of DCs. Shown is one representative experiment out of three. From (Bohnenkamp and Noll, 2003)

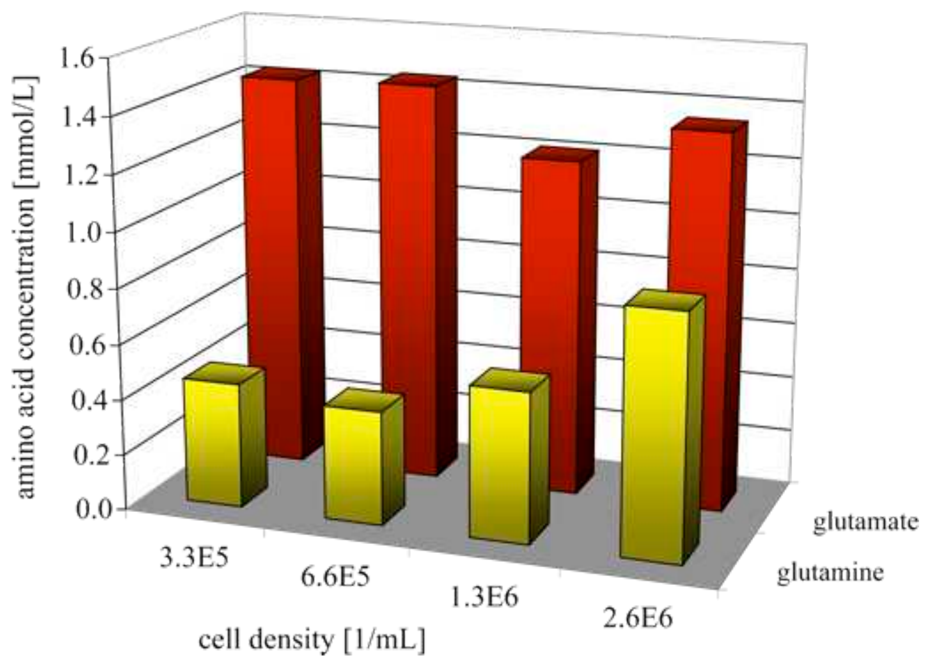


Figure 4.9: Glutamine and glutamate analysis on day 8 after generation of DCs. Shown is one representative experiment out of three.

correlate with a decrease in CD80 surface antigen expression. The mean fluorescent intensity as an indicator for the level of expression of CD40 decreased about 50% with the highest cell density (data not shown). No differences in expression of CD1a were observed (data not shown).

The medium components were also analyzed, whereby an increase in lactate concentration at the highest cell density at $\geq 25\frac{mmol}{L}$ (see figure 4.8 on page 60) was found, which caused a decreased culture pH and might be responsible for the lower yield. Accumulation of metabolic products like lactate produces an acidic environment and therefore inhibits proliferation (Bohnenkamp et al., 2002, Patel et al., 2000). The glucose concentration in all experiments remains above limiting levels. Amino acid analysis demonstrated no limitation for glutamine and serine. The highest glutamate concentration was $1.4\frac{mmol}{L}$, which is illustrated in figure 4.9 on page 60.

An ELISA for GM-CSF and IL-4 showed no limitation for GM-CSF (GM-CSF residual content: $3.3 \cdot 10^5$ $\frac{1}{mL}$: 780 $\frac{U}{mL}$, $6.6 \cdot 10^5$ $\frac{1}{mL}$: $720\frac{U}{mL}$, $1.3 \cdot 10^6$ $\frac{1}{mL}$: $450\frac{U}{mL}$ and $2.6 \cdot 10^6$ $\frac{1}{mL}$: $500\frac{U}{mL}$ respectively) but IL-4 was limiting (detection limit: $7.8\frac{pg}{mL}$) (data not shown), which might explain the non-homogenous DC population especially for the $6.6 \cdot 10^5$ and $1.3 \cdot 10^6$ $\frac{1}{mL}$ inoculated monocytes.

Due to a cost-effective utilization of the cultivation system in terms of highest yield of matured dendritic cells per volume, all further experiments were performed at a cell density of $1.3 \cdot 10^6$ $\frac{1}{mL}$ inoculated monocyte.

4.1.4 Optimization of GM-CSF and IL-4 Concentrations

Based on the finding that no substrate feeding is necessary for the generation of matured dendritic cells at a cell density of $1.3 \cdot 10^6$ $\frac{1}{mL}$ inoculated monocytes, the influence of different concentrations of GM-CSF and IL-4 on yield and phenotype of the cells was investigated in order to get a homogenous DC population.

The next step was to investigate the consumption of GM-CSF and IL-4. Therefore 200, 400 and $800\frac{U}{mL}$ GM-CSF were inoculated, while IL-4 remained at $500\frac{U}{mL}$. The $200\frac{U}{mL}$ were exhausted completely while in the other two experiments about $300\frac{U}{mL}$ were consumed ($296\frac{U}{mL}$ and $320\frac{U}{mL}$ respectively), which is shown in figure 4.10 on page 62. The yield and phenotype was comparable to previous experiments (see figure 4.7, $1.3 \cdot 10^6$ $\frac{1}{mL}$ inoculated monocytes). The $200\frac{U}{mL}$ GM-CSF concentration resulted in the same number of DCs but in a lower expression of CD80, CD83 and CD86 (data not shown).

Table 4.4: The parameters of the optimization of the GM-CSF and IL-4 concentration

Cell densitiy	$1.3 \cdot 10^6\ \frac{1}{mL}$
Donors	three
Cultivation system	48 well plates, $500\mu L$ volume
Medium	X-VIVO 15
GM-CSF	200, 400, 800$\frac{U}{mL}$
IL-4	500, 1000, 2000$\frac{U}{mL}$
Maturation stimulus	TNF-α (1000$\frac{U}{mL}$), IL-1β (1000$\frac{U}{mL}$), IL-6 (1000$\frac{U}{mL}$), PGE_2 (1$\frac{\mu g}{mL}$)
Time (differentiation / maturation)	6 days / 2 days

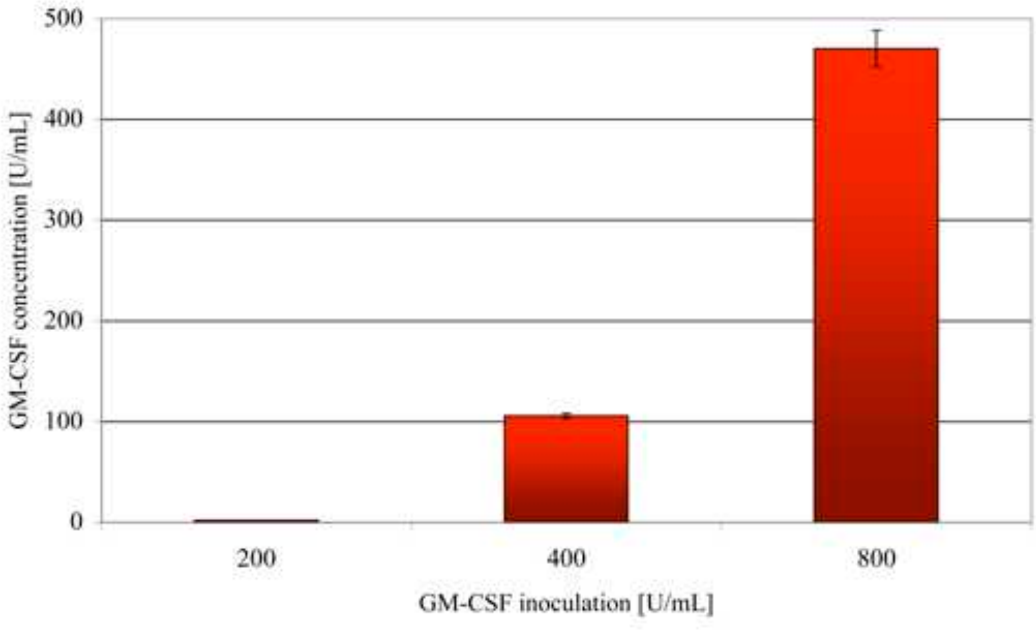


Figure 4.10: GM-CSF consumption of cultivated cells after 8 days. Data shown are the mean ± SD of triplicate cultures from one representative experiment of three performed. From (Bohnenkamp and Noll, 2003)

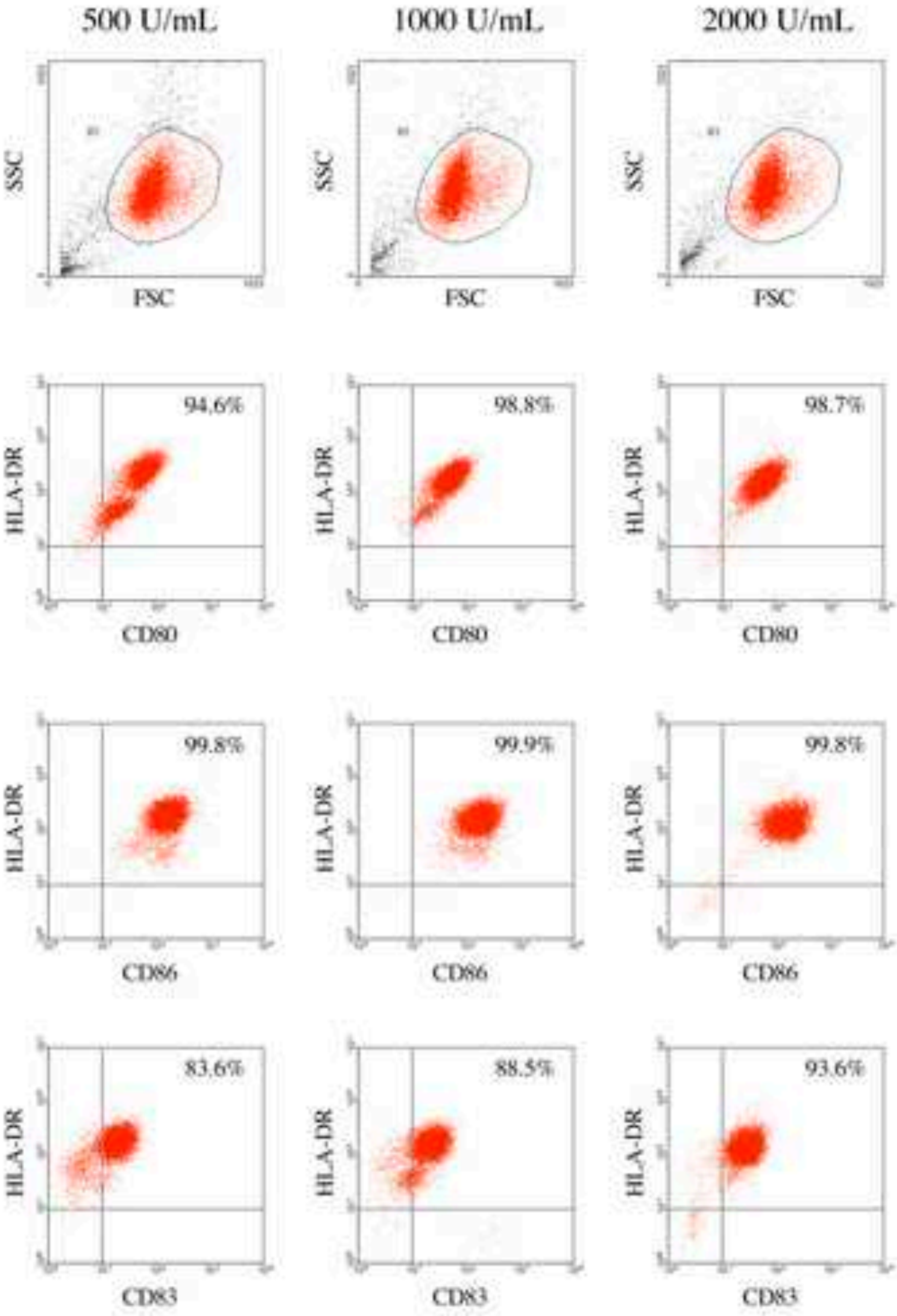


Figure 4.11: Phenotype of DCs generated with different IL-4 concentrations ($500\frac{U}{mL}$, $1000\frac{U}{mL}$ and $2000\frac{U}{mL}$). Only DCs generated with $2000\frac{U}{mL}$ resulted in a homogenous population of fully matured dendritic cells. The data shown are from one representative experiment of 3 performed. From (Bohnenkamp and Noll, 2003)

Afterwards the determination of the required IL-4 concentration, which results in a homogenous populations of matured dendritic cells, was performed. Different IL-4 concentrations (500, 1000 and 2000$\frac{U}{mL}$ respectively) with a constant concentration of 800$\frac{U}{mL}$ GM-CSF, to alter only one parameter, were inoculated. The IL-4 ELISA indicated that only at an initial concentration of 2000$\frac{U}{mL}$ the IL-4 was not limiting, as still 25$\frac{U}{mL}$ were measured after 8 days of cultivation (data not shown) and phenotypical analysis showed a homogenous population of matured DCs only for the highest IL-4 concentration (see figure 4.11 on page 63). However, the number of DCs was similar for all cytokine concentrations.

These results clearly demonstrated that 400$\frac{U}{mL}$ GM-CSF and 2000$\frac{U}{mL}$ IL-4 are required but also sufficient for the generation of matured dendritic cells without feeding.

4.1.5 The Influence of Different Maturation Stimuli

In previous experiments for the maturation of dendritic cells a cocktail consisting of TNF-α (1000$\frac{U}{mL}$), IL-1β (1000$\frac{U}{mL}$), IL-6 (1000$\frac{U}{mL}$) and PGE_2 (1$\frac{\mu g}{mL}$) was used. In the following experiments the feasibility to simplify this maturation cocktail while obtaining the same yield, viability, phenotype and distinct functional capacity of DCs were examined. 400$\frac{U}{mL}$ GM-CSF and 2000$\frac{U}{mL}$ IL-4 were inoculated to differentiate monocytes to dendritic cells. The parameters of this experiment are listed in table 4.5.

Table 4.5: Experimental parameters to test different maturation stimuli

Cell densitiy	$1.3 \cdot 10^6\ \frac{1}{mL}$
Donors	four
Cultivation system	48 well plates, 500μL volume
Medium	X-VIVO 15
GM-CSF	400$\frac{U}{mL}$
IL-4	2000$\frac{U}{mL}$
Maturation stimulus I	TNF-α (1000$\frac{U}{mL}$), IL-1β (1000$\frac{U}{mL}$), IL-6 (1000$\frac{U}{mL}$), PGE_2 (1$\frac{\mu g}{mL}$)
Maturation stimulus II	TNF-α (1000$\frac{U}{mL}$), PGE_2 (18$\frac{\mu g}{mL}$)
Time (differentiation / maturation)	6 days / 2 days

Therefore two maturation cocktails were compared: Cocktail I composed of TNF-α

($1000\frac{U}{mL}$), IL-1β ($1000\frac{U}{mL}$), IL-6 ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$) (Jonuleit et al., 1997) and Cocktail II consisted of TNF-α ($1000\frac{U}{mL}$) and PGE_2 ($18\frac{\mu g}{mL}$) (Kalinsky et al., 1998). Using monocytes from 4 different donors, similar results in yield, viability and phenotype for both cocktails were obtained (data not shown).

A consequence of maturation can be the secretion of cytokines by dendritic cells (Sallusto and Lanzavecchia, 1999). In particular, the cytokine IL-12p70 is of major interest because it polarizes the T-helper cell pathway to a Th1 response (see also figure 2.10 on page 16) However, it has been demonstrated that PGE_2 induces the final maturation of IL-12p70 deficient dendritic cells (Kalinski et al., 1998). To investigate the amount of bioreactive IL-12p70, the cell culture supernatant of DCs after 2 days of stimulation with the respective cytokine cocktail was analyzed by ELISA . With both stimuli no detectable level of IL-12p70 was produced at any time point (data not shown).

4.1.6 Mixed Leukocyte Reaction

In section 4.1.5 it has been described that dendritic cells matured with either stimulus I or stimulus II were obtained with the same yield, viability and phenotype. An important readout of the dendritic cell functionality is the allostimulatory capacity in a mixed leukocyte reaction (MLR). Thereby, the capacity of dendritic cells of activation of T lymphocytes is determined by measurement of proliferation (see also Background - MLR on page 67).

The allostimulatory capacity of matured DCs was tested using a ratio of dendritic cells to T cells of 1:10. After an induction phase cells were fed twice at $92h$ and $163h$ by replacement of 50% of the medium and adding of $100\frac{U}{mL}$ IL-2. Figure 4.12 on page 66 illustrates the potent stimulatory capacity of dendritic cells matured with either cytokine stimulus I: TNF-α ($1000\frac{U}{mL}$), IL-1β ($1000\frac{U}{mL}$), IL-6 ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$) or stimulus II: TNF-α ($1000\frac{U}{mL}$) and PGE_2 ($18\frac{\mu g}{mL}$). In contrast, immature dendritic cells failed to induce a potent allostimulatory response of T lymphocytes.

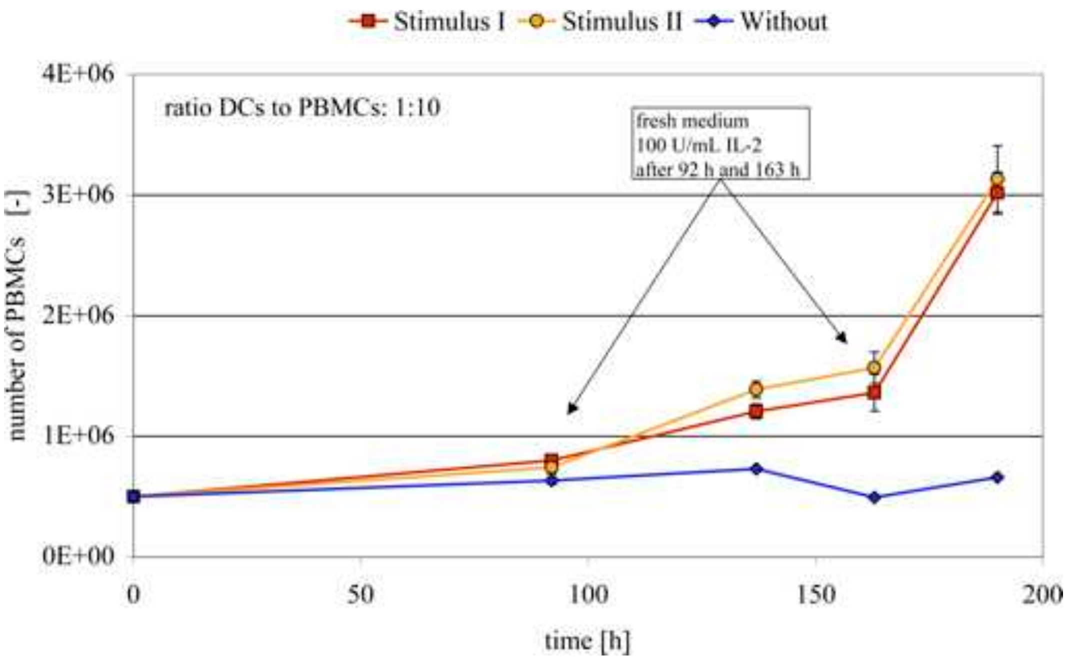


Figure 4.12: Allostimulatory capacity for PBMC from healthy donors. DCs matured with different maturation stimuli (stimulus I: TNF-α ($1000\frac{U}{mL}$), IL-1β ($1000\frac{U}{mL}$), IL-6 ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$); stimulus II: TNF-α ($1000\frac{U}{mL}$) and PGE_2 ($18\frac{\mu g}{mL}$)) induced a similar stimulatory capacity in the allogeneic MLR. Shown are mean values ± SD from three different experiments in triplicates. From (Bohnenkamp and Noll, 2003)

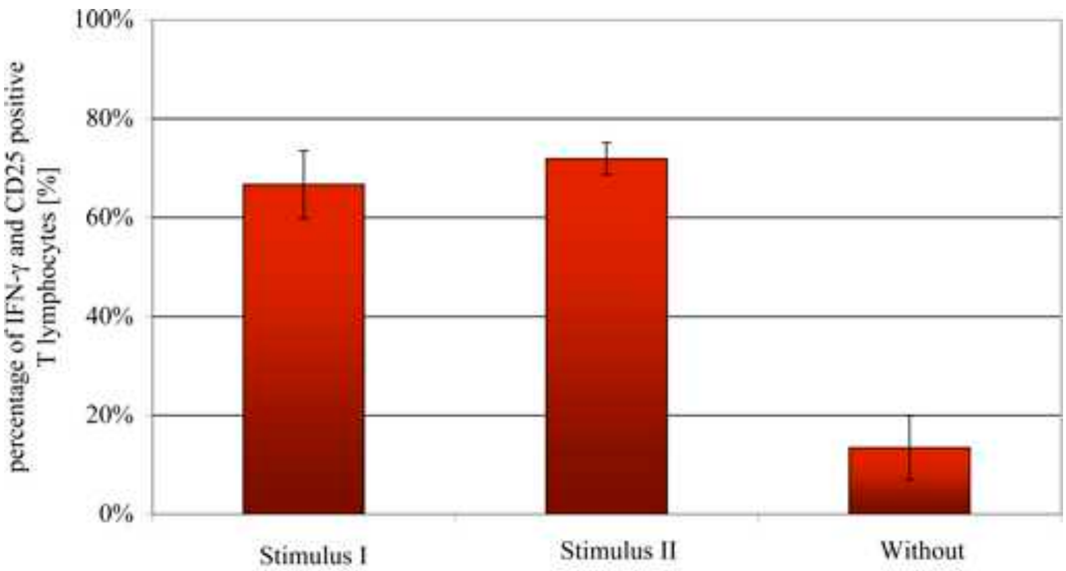


Figure 4.13: Phenotype of PBMC after MLR. Either maturation stimulus I and II induced IFN-γ producing T cells which were also CD25 (IL-2 α-chain) positive. Results are expressed as mean ± SD from three experiments in triplicates. From (Bohnenkamp and Noll, 2003)

> BACKGROUND - MLR: The mixed leukocyte reaction is a test for the stimulatory capacity that is induced by dendritic cells. In the presence of allogeneic (two individuals or cell populations that differ in their MHC complexes) T cells, dendritic cells induce a strong activation based on the different MHC types. The more MHC complexes and co-stimulatory molecules dendritic cells express the better T cells are stimulated. T cell activation is determined by their proliferation, e.g. DNA synthesis (BrdU incorporation), expression of 'proliferation markers' like CD25, the high affinity IL-2 receptor, and CD71, the transferrin receptor, or counting of proliferating cells. Usually, dendritic cells and T cells are co-cultivated at different ratios, to analyze up to which dilution T cells are stimulated (NGUYEN ET AL., 2003).
> There are two modes of cross-reactive recognition that may explain the alloreactivity of T cells. Allogeneic T cells can be activated in a peptide-dominant way, recognizing the peptide expressed by the non-self MHC complexes. On the other hand, the allogeneic MHC molecule may fit to the T cell receptor and may give a tight binding that is less dependent on the peptide bound to the MHC molecule (JANEWAY ET AL., 2001).

Testing of the supernatant of the MLR after 4 days for levels of IL-12p70 showed no detectable amount of cytokine. Since PGE_2 suppressed IL-12p70 production in matured DCs, it was investigated whether a typical Th1 or Th2 cytokine pattern was being induced. After $190h$ of cultivation and proliferation of T lymphocytes, cells were tested for IFN-γ by a cytokine secretion assay. Either T cells stimulated by DCs matured with cocktail I or II were IFN-γ and CD25 positive (see figure 4.13 on page 66). Performed ELISA for IL-4 showed no level of cytokine (data not shown). These data suggest that T lymphocytes stimulated by DCs matured by either cytokine stimulus were polarized towards the Th1 type dendritic cell.

4.1.7 Generation of Dendritic Cells with Optimized Parameters

Based on the optimized parameters, the generation of dendritic cells was carried out in $75cm^2$ tissue culture flasks with a volume of $30mL$. Monocytes were inoculated at a cell density of $1.3 \cdot 10^6\ \frac{1}{mL}$ and differentiated with $400\frac{U}{mL}$ GM-CSF and $2000\frac{U}{mL}$ IL-4 (see table 4.7). On day 6 $1000\frac{U}{mL}$ TNF-α and $18\frac{\mu g}{mL}$ PGE_2 were added for additional 2 days for maturation of DCs. On day 8 the cells were harvested (see also table 4.7 on page 70).

Table 4.6: The results of the generation of dendritic cells using the optimized protocol

($n = 8$)	Mean	Standard deviation
Inoculated cells [$\frac{1}{mL}$]	$10.9 \cdot 10^5$	$1.0 \cdot 10^5$
Number of immature DCs on day 6 [$\frac{1}{mL}$]	$7.0 \cdot 10^5$	$2.4 \cdot 10^5$
Yield of immature DCs [%]	64.2	22.3
Number of matured DCs on day 8 [$\frac{1}{mL}$]	$7.2 \cdot 10^5$	$1.5 \cdot 10^5$
Yield of matured DCs [%]	66.3	13.6
Purity (FSC / SSC) [%]	95.4	2.1
Viability (Trypan blue dye exclusion) [%]	93.4	5.9
Number of matured DCs per buffy coat [-]	$5.8 \cdot 10^7$	$1.2 \cdot 10^7$

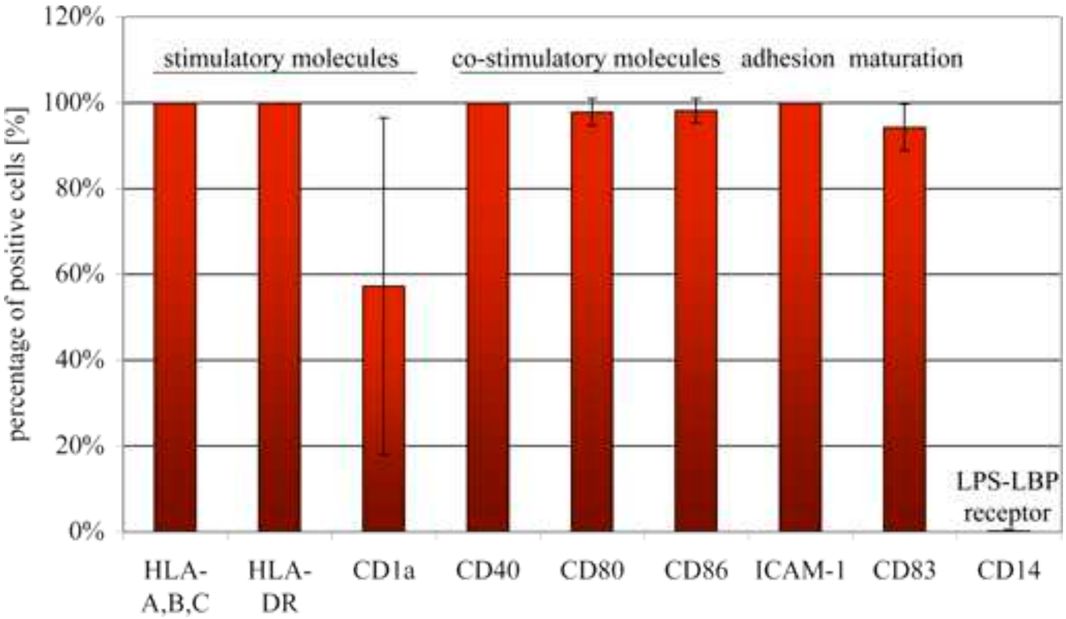


Figure 4.14: Phenotypical analysis of mature DCs generated with the standardized protocol. Shown are results of surface antigen expression as indicated as mean ± SD from 8 independent experiments. From (Bohnenkamp and Noll, 2003)

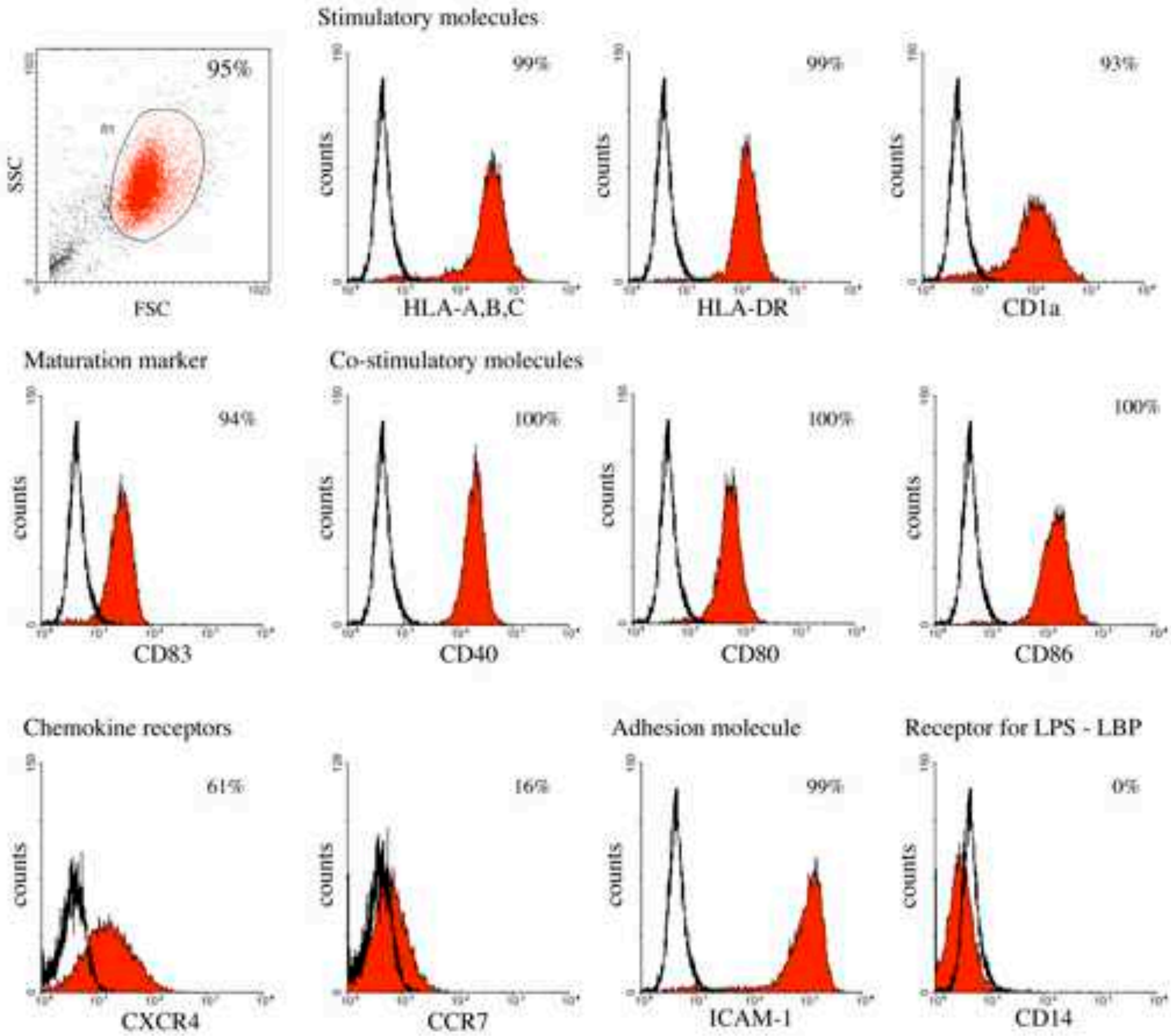


Figure 4.15: Expression of surface antigens on matured DCs from one typical donor out of 8. Outlined histograms represent isotype staining. Histograms indicate gated cells.

Table 4.7: Optimized parameters for the generation of dendritic cells

Cell densitiy	$1.3 \cdot 10^6 \frac{1}{mL}$
Cultivation system	$75cm^2$ tissue culture flasks, $30mL$ volume
Medium	X-VIVO 15
GM-CSF	$400\frac{U}{mL}$
IL-4	$2000\frac{U}{mL}$
Maturation stimulus	TNF-α ($1000\frac{U}{mL}$), PGE_2 ($18\frac{\mu g}{mL}$)
Time (differentiation / maturation)	6 days / 2 days

During the differentiation of monocytes to fully mature dendritic cells from 8 different donors no difference was observed regardless of the cultivation system (48 well plates compared with tissue culture flasks). The mean yield as calculated from inoculated $CD14^+$ monocytes was $66.3 \pm 13.6\%$ with a mean viability of $93.4 \pm 5.9\%$ ($n = 8$), which is shown in table 4.6 on page 68. Figure 4.14 illustrates the mean percentage of positive dendritic cells for important surface antigens like HLA-DR, HLA-A,B,C, co-stimulatory molecules like CD40, CD80 and CD86, the intercellular adhesion molecule ICAM-1 and the maturation marker CD83 of dendritic cells. The expression of surface proteins from one representative donor is demonstrated in figure 4.15 on page 69.

4.2 Apoptosis of Monocytes

The optimized protocol for the standardized generation of monocyte-derived dendritic cells resulted in a yield of $66.3 \pm 13.6\%$ with a mean viability of $93.4 \pm 5.9\%$ ($n = 8$). In this context, an important question is whether the yield of DCs is determined by the cultivation method or by the properties of the monocytes being used.

The aim of the following experiments was to investigate whether the degree of apoptosis of the starting population of $CD14^+$ enriched monocytes is the crucial parameter determining the yield of matured monocyte-derived dendritic cells. As programmed cell death regulates the monocytes population in vivo, the analysis of $CD14^+$ enriched monocytes by flow cytometry for apoptosis using an assay for phospatidylserine expression on early apoptotic cells with fluorescein labeled annexin V combined with propidiumiodide staining, originally developed by Vermes et al. (1995), was carried out.

Background - apoptosis of monocytes: Monocytes play a major role as circulating precursors of the different types of macrophages and dendritic cells and participate in inflammatory responses by releasing cell-signaling molecules (Fahy et al., 1999, Lund et al., 2001). The number of monocytes, which migrate into tissue, generally exceeds the number needed to replace dying macrophages or dendritic cells. Excess monocytes, entering non-inflamed tissue, are therefore removed by resident macrophages (Mangan et al., 1991). On the other hand, up-regulation of the number of monocytes during inflammatory responses may be required. In general, the balance of cell proliferation and removal is crucial for the regulation of cell populations (Kerr et al., 1972). Additionally, as a part of normal physiological cell processes, apoptosis regulates equilibrium between cell proliferation and cell death (Vermes et al., 2000). In considering the differentiation of monocytes from freshly isolated peripheral blood, it is likely that some of the monocytes are apoptotic and cannot be differentiated to DCs. Moreover, the culture environment and the isolation procedure itself (Lund et al., 2002) may affect the level of apoptosis. Thus, when cultured in medium in the absence of an appropriate stimulus, monocytes rapidly undergo programmed cell death (Mangan et al., 1991).

Background - annexin V: The phenomenon of highly asymmetric biological membranes is not only restricted to membrane proteins but also found in the distribution of lipids (Bevers et al., 1989). An extreme distribution is found for phospatidylserine (PS), which is almost entirely located in the inner side of the plasma membrane. An alteration of plasma membrane asymmetry occurs early after the onset of the execution phase of apoptosis and results in the translocation of PS from the inner to the outer layer of the plasma membrane (Cornelissen et al., 2002). When PS is initially exposed on the outer surface, the membrane integrity has not been compromised and no nuclear disintegration and alterations in the cytoplasm are detectable (van Engeland et al., 1998). By combining the annexin V staining, which was shown to have a high affinity for PS, with propidiumiodide (PI), discrimination between early apoptotic cells (annexin V^+, PI^-) and late apoptotic or necrotic cells (annexin V^+, PI^+) is possible.

Table 4.8: Analysis for annexin V (AV) and propidiumiodide (PI) of monocytes after CD14 enrichment by immunomagnetic bead selection. From (Bohnenkamp et al., submitted)

	Yield of monocytes after enrichment [%] of PBMCs	$AV^{+}PI^{-}$ Apoptotic cells [%] of monocytes	$AV^{+}PI^{+}$ Necrotic cells [%] of monocytes
Donor 1	6.9	29.9	0.8
Donor 2	8.6	37.7	0.2
Donor 3	17.9	62.6	1.2
Donor 4	10.0	34.5	1.6
Donor 5	18.7	23.7	0.3
Donor 6	13.5	33.2	0.3
Donor 7	14.7	45.9	0.2
Donor 8	12.2	34.5	0.1
Mean	12.8	37.8	0.6
Standard deviation	4.0	11.1	0.5

Monocyte apoptosis and the fate during differentiation to matured dendritic cells

As the yield from monocyte-derived dendritic cells was $66.3 \pm 13.6\%$, the initial studies focused on analysis of monocyte apoptosis and viability. After enrichment of monocytes by immunomagnetic beads (see also chapter 4.1.1), the $CD14^{+}$ cell population showed evidence of apoptosis determined by annexin V staining. As shown in table 4.8, the mean of 8 different donors was 37.8%, varying between 23.7% and 62.6%.

After this finding it was aimed to follow the fate of apoptotic monocytes during differentiation to matured dendritic cells. $10.9 \cdot 10^5 \pm 1.0 \cdot 10^5 \frac{1}{mL}$ $CD14^{+}$ monocytes ($n = 8$) in X-VIVO 15 containing GM-CSF and IL-4 were inoculated into tissue culture flasks and the non-adherent population was analyzed by flow-cytometry for apoptosis and by staining for viable cells. Figure 4.16 on page 73 shows the flowcytometrical analysis from one donor and Figure 4.17 illustrates the changes in the viable cells and apoptotic cells in the non-adherent population over time. The viable cell number decreased rapidly on day 1 when viable monocytes attached to the surface of the tissue culture flask while most apoptotic cells remained non-attached (this represents 53% of initial apoptotic cell number for the case illustrated in Figure 4.17).

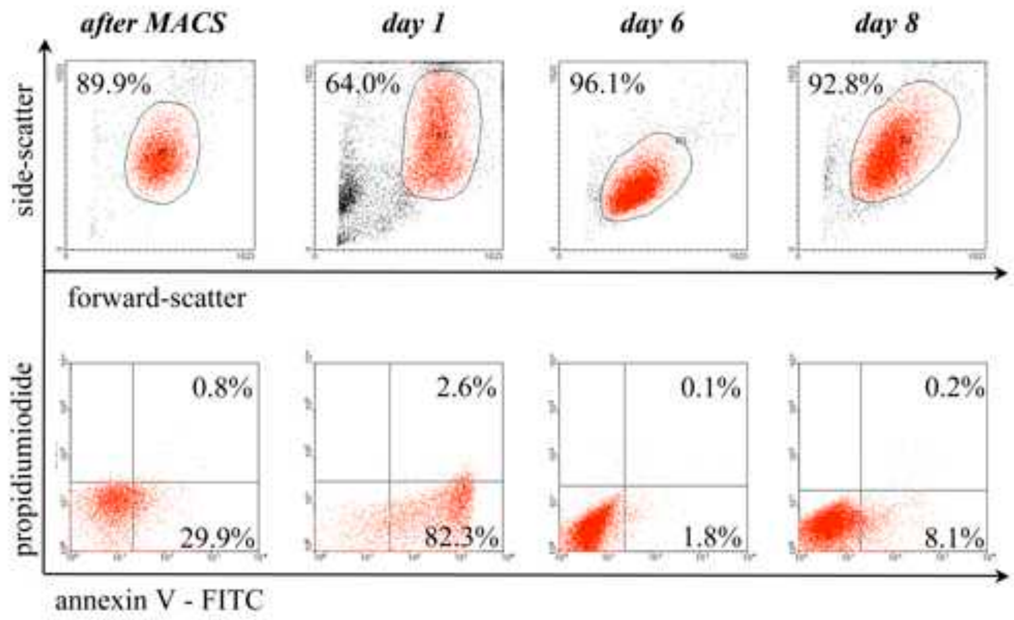


Figure 4.16: Analysis of one representative donor out of 8 for annexin V and propidiumiodide during differentiation of monocytes to dendritic cells. Only suspension cells were stained, attached cells were not analysed. From (Bohnenkamp et al., submitted)

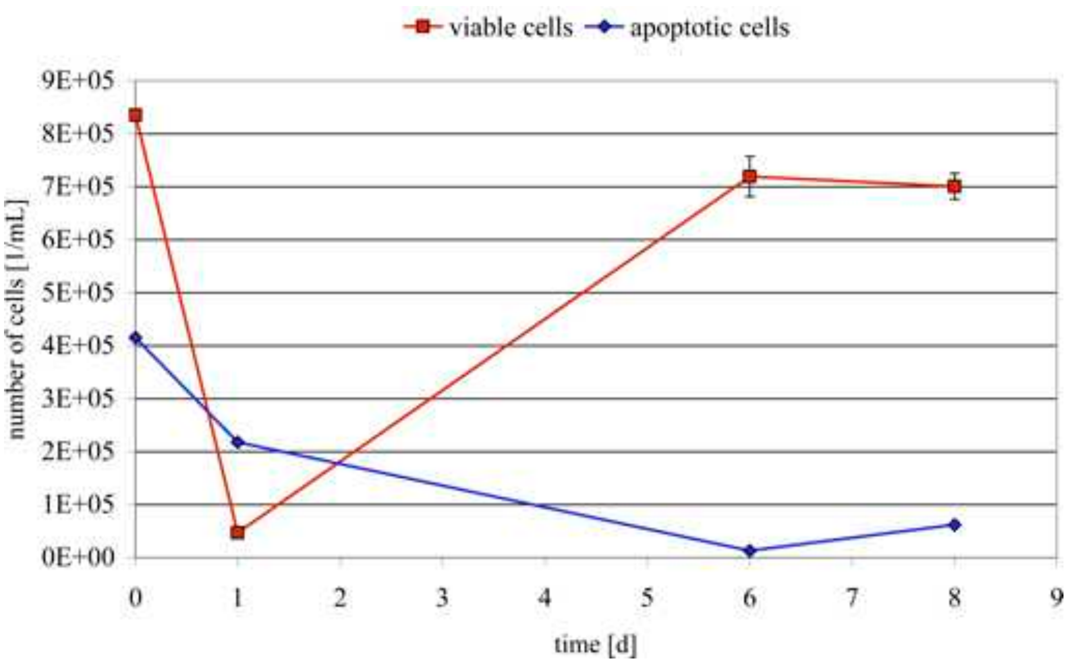


Figure 4.17: Number of cells in suspension for one representative donor out of 8. Results are from triplicates and expressed as mean ± standard deviation. From (Bohnenkamp et al., submitted)

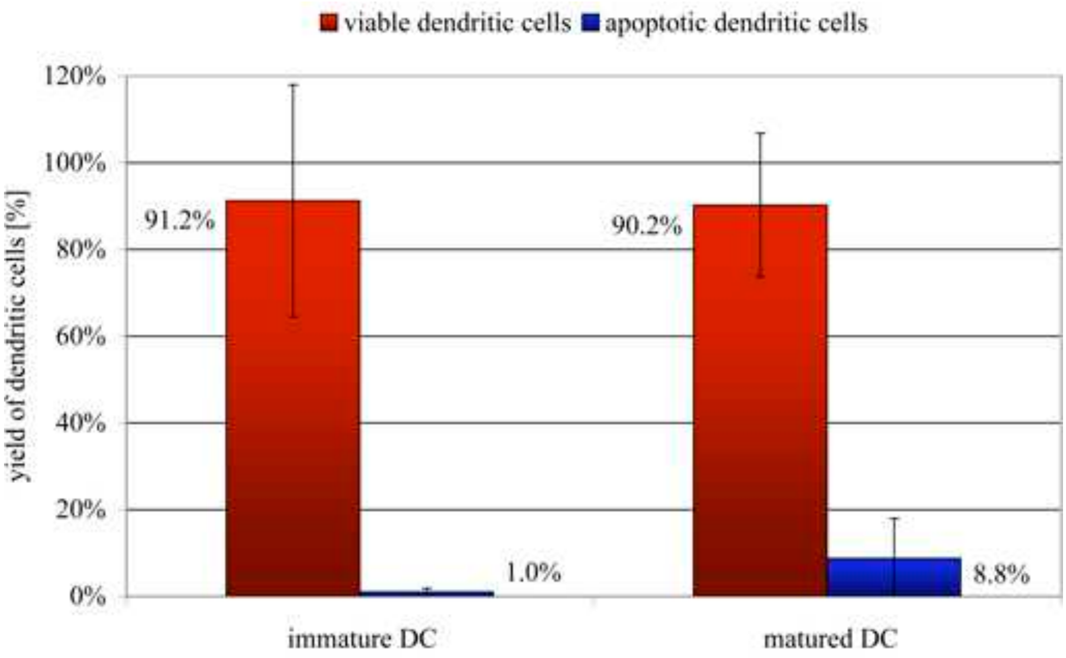


Figure 4.18: Yield of immature and TNF-α and PGE_2 matured dendritic cells. Results are from 8 different donors and presented as mean ± standard deviation. Shown are viable (AV^- / PI^-) and apoptotic (AV^+ / PI^-) cells. From (Bohnenkamp et al., submitted)

After 6 days of differentiation most of the apoptotic cells have disappeared and the cells found in suspension represent immature dendritic cells (increased to $7.2 \pm 0.4 \cdot 10^5 \frac{1}{mL}$ for one representative donor) as determined by the expression of high levels of HLA-A,B,C and HLA-DR, weak levels of CD1a, CD80, CD83 and CD86 and no expression of CD14 (data not shown). An analysis of the number of annexin V positive cells present after differentiation for 6 days showed that this value decreased on average to just $1.0 \pm 0.9\%$ (Figure 4.18). The addition of TNF-α and PGE_2 induced maturation of the DCs, as demonstrated by the high expression of HLA-A,B,C, HLA-DR, CD80, CD83 and CD86, a weak level of CD1a and no expression of CD14 (see figure 4.15 on page 69). However, the percentage of apoptosis was increased to $8.8 \pm 9.3\%$ ($n = 8$) of all suspension cells on day 8 (illustrated in figure 4.18). Thus, in the presence of the maturation cytokines, the number of viable cells decreased slightly, while an increased number of apoptotic cells was observed.

These results suggest that GM-CSF and IL-4 prevent monocytes from undergoing further spontaneous apoptosis. Moreover, phospatidylserine presenting monocytes were apparently removed by phagocytosis by viable monocytes or immature dendritic cells resulting in a homogenous, viable cell population on day 6. Using the protocol described, the yield of dendritic cells was calculated considering only viable, non-apoptotic monocytes and DCs. Combining the data from the 8 donors, the yield (as defined by size of cells, morphology, surface antigen expression and apoptosis) of immature DCs and of matured DCs was $91.2 \pm 26.8\%$ and 90.2 ± 16.6 ($n = 8$) respectively (figure 4.18).

4.3 Induction of MUC1 Specific T Lymphocytes

In the chapters 4.1 and 4.2 the development of a reproducible and standardized protocol for the generation of monocyte-derived dendritic cells was described. With GM-CSF and IL-4 DCs differentiate in the medium X-VIVO 15 under serum-free conditions in 6 days to immature dendritic cells. Additionally, it was shown that the cytokine cocktail TNF-α and PGE_2 induced maturation, which resulted in a homogenous population of fully matured dendritic cells. Furthermore, the percentage of apoptotic monocytes in the starting population was proved to be crucial for the determination of the yield of dendritic cells.

In the following chapter different in vitro assays were established to investigate the functionality of DCs. Above all, a dendritic cell system based on lysate-pulsed DCs was to be developed that efficiently present tumor-associated antigens for induction of a strong T cell response. The following questions regarding the immunobiological functionality of dendritic cells were addressed:

- A reproducible method for lysate preparation
- Antigen uptake of DCs
- Lysate-pulsing and maturation
- Migration towards important chemokines
- Cryopreservation of dendritic cells
- MHC class I restricted T cell responses
- Induction of autologous T cells with lysate-pulsed DCs

The goal of the described experiments was to investigate whether lysate-pulsed dendritic cells induce tumor-associated antigen specific T lymphocytes in an MHC class I restricted

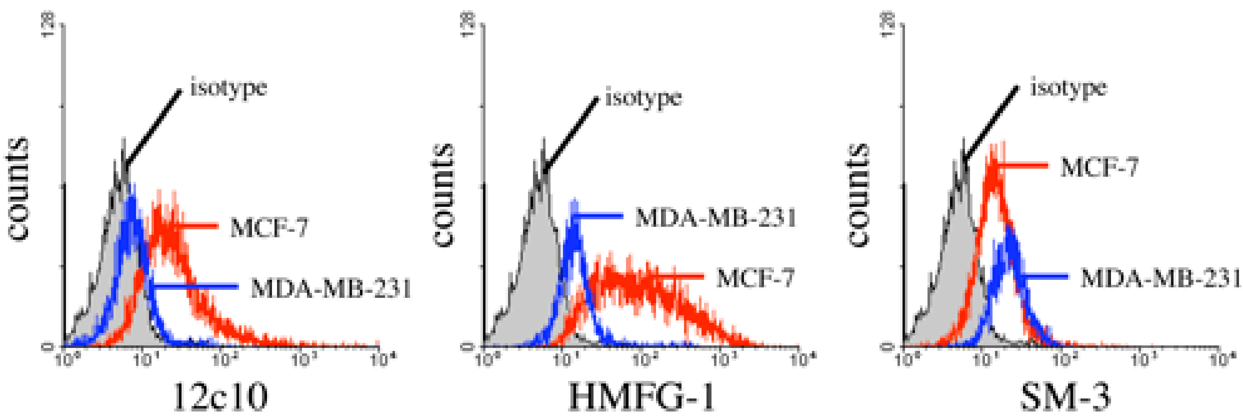


Figure 4.19: Analysis of MUC1 expression on the breast carcinoma cell lines MCF-7 and MDA-MB-231 with the antibodies 12c10, which binds outside the tandem repeat, HMFG-1 (binds to a epitope defined as PDTR) and SM-3 (binds to a epitope defined as PDTRP). From (Bohnenkamp et al., in preparation)

fashion. MUC1, which is expressed by the breast carcinoma cell lines MCF-7 and MDA-MB-231, was used as a model antigen. For detection of MUC1 specific T cells, tetramers with the M1.2 (Brossart et al., 1999) and F7 (Heukamp et al., 2001) peptides, both localized within the signal sequence of MUC1, were chosen. Furthermore, the induction of a strong Th1 polarized response by lysate-pulsed DCs matured with the adjuvants TNF-α and PGE_2 were investigated. Because the dose as well as the type and structure of the antigen influences the differentiation and polarization of an induced immune response, the cytokine profile induced by lysate-pulsed DCs and the secreted cytokines induced by DCs pulsed with the MHC class I restricted peptides FLU M1 and Melan-A / Mart-1 was analyzed.

4.3.1 Evaluation of Breast Carcinoma Cell Lysates

The delivery of tumor-associated antigens to dendritic cells is critical for the induction of a strong T cell response. Consequently, the preparation method for lysates from cancer cells need to be a simple procedure under sterile conditions and result in the solubilization of unaltered proteins. Lysates from breast carcinoma cell lines were evaluated utilizing MUC1 as model tumor-associated antigen, on which the experiments were based.

The expression of MUC1 on the breast carcinoma cell lines MCF-7 and MDA-MB-231 was

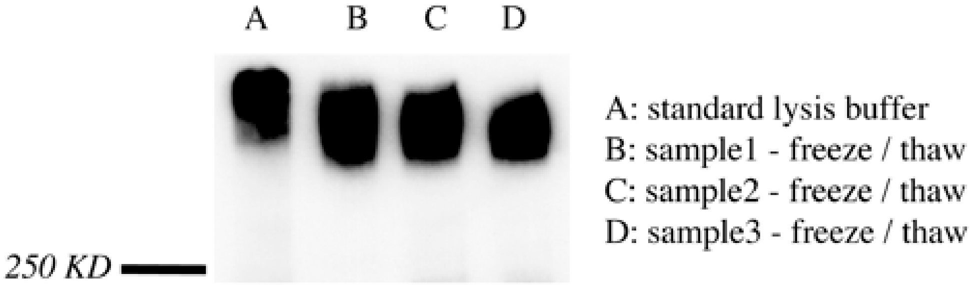


Figure 4.20: Detection of MUC1 with HMFG-2 (binds to an epitope defined as DTR) antibody. Western blot analysis of MCF-7 lysate from three independent experiments. Lysate was prepared by four repetitive freeze and thaw cycles (thawing at $4°C$ by sonication). As a control a standard lysate buffer was used. From (Bohnenkamp et al., in preparation)

determined by flow cytometry using the 12c10 monoclonal antibody, which binds to epitopes outside the tandem repeat region, and the HMFG-1 and SM-3 monoclonal antibodies, which react with a core protein epitope defined as PDTR and PDTRP respectively (figure 4.19) (Blockzjil et al., 1998). The difference in MUC1 expression by both cell lines was evident. The MCF-7 cell line showed higher expression compared with the MDA-MB-231 cell line as determined by 12c10 and HMFG-1 antibody (also reported by Walsh et al. (2000)). However, MDA-MB-231 proved to show a more homogeneous expression level of MUC1 (the relative variation from cell to cell).

Usually, for the generation of lysate-pulsed dendritic cells, the lysates are prepared by repetitive freez-thaw cycles. However, the method of choice need to avoid proteolytic reactions by active proteases or phosphatases and need to solubilize all cell proteins without degradation. For this reason, lysates were prepared at low temperatures ($\leq 4°C$) and lysis of tumor cells was performed by four freeze-thaw cycles: thawing was done by sonication at $4°C$ in an ultrasonic bath. Lysates from carcinoma cell lines were tested for MUC1 by western blot analysis and compared with a homogenate prepared with standard lysis buffer. As demonstrated in figure 4.20, freeze-thaw lysates contained MUC1 protein at nearly the same concentration as standard lysates. Furthermore, it was shown in three independent experiments that the preparation method was highly reproducible.

4.3.2 Immature DCs Efficiently Take Up Antigen

Because of antigens have to be taken up efficiently by dendritic cells, the endocytosis of model antigens by DCs prepared using the method described in chapter 4.1 on page 49 was analyzed. So, model antigens labeled with a fluorescent dye are loaded on immature DCs, incubated for a certain time and analyzed by a flowcytometer. As antigens labeled dextran, lipopolysaccharide and collagen-I labeled microspheres with a size of $2\mu m$ were used. In table 4.9 the parameters such as incubation time and concentration of the corresponding antigen are listed.

Table 4.9: Parameters for the evaluation of the antigen uptake by dendritic cells

	Dextran	LPS	Microspheres
Concentration	$1\frac{mg}{mL}$	$20\frac{\mu g}{mL}$	$1.0 \cdot 10^6\ \frac{1}{mL}$
Incubation time	$2h$	$2h$	$2h$ and $20h$
Number of DCs	$1.0 \cdot 10^5\ \frac{1}{mL}$	$1.0 \cdot 10^5\ \frac{1}{mL}$	$1.0 \cdot 10^5\ \frac{1}{mL}$
Day of differentiation	d6	d6	d6 / d8

Monocyte-derived dendritic cells were generated with the described protocol. After 6 days of differentiation in the presence of GM-CSF and IL-4 immature dendritic cells were incubated with the antigens FITC conjugated dextran and Alexa-Fluor-488 conjugated LPS, as well as collagen-I labeled microspheres (size of $2\mu m$) at $37°C$. As negative control cells were incubated with the same antigen and concentration at $0°C$. The uptake of the different compounds is shown in figure 4.21. Immature dendritic cells took up dextran and LPS compared with the negative control.

The histograms that plots the uptake of the incubated microspheres demonstrates the efficient macropinocytosis of incubated collagen-I labeled beads. In these histograms every peak corresponds to the number of endocytozed microspheres: The higher the fluorescence intensity the more beads are taken up. Furthermore, a prolonged incubation time increased the number of pinocytozed beads. Mature dendritic cell showed a reduced endocytic capacity compared with immature DCs.

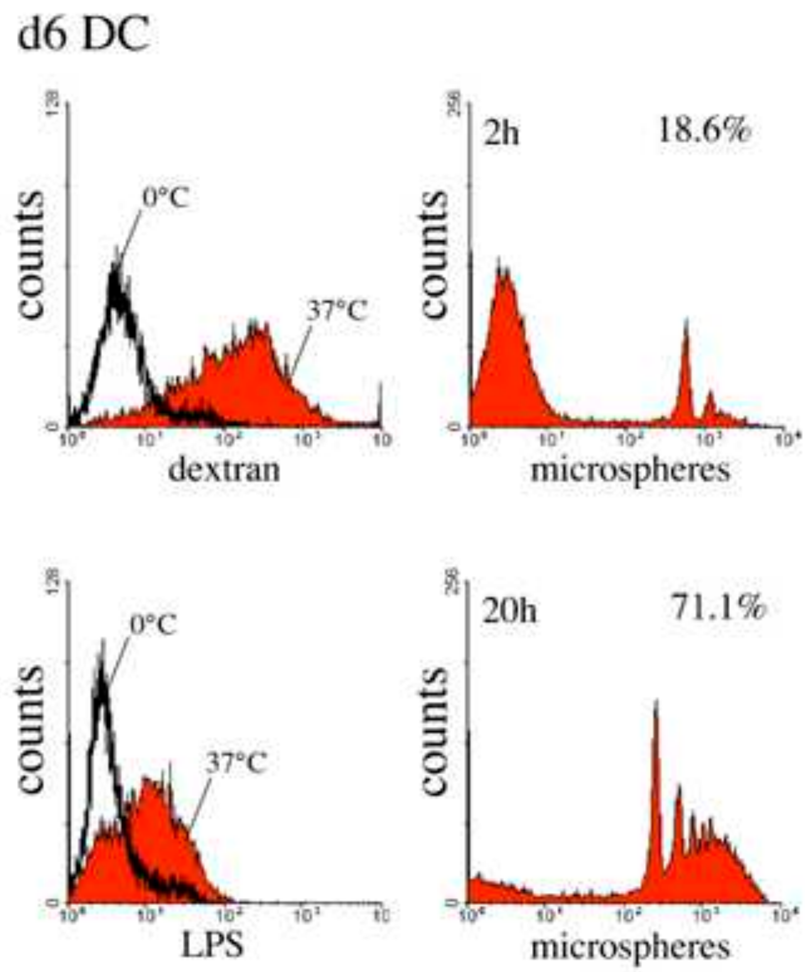


Figure 4.21: Analysis of the capacity of immature (d6) and matured (d8) DCs to endocytose different antigens. FITC labeled dextran ($1\frac{mg}{mL}$), Alexa-Fluor-488 labeled LPS ($20\frac{\mu g}{mL}$) and collagen-I microspheres ($1.0 \cdot 10^6\ \frac{1}{mL}$) were compared. Shown is one representative experiment out of four. From (Bohnenkamp et al., in preparation)

4.3.3 Adjuvant Induced Maturation of Lysate-pulsed Dendritic Cells

In section 4.3.2 it has been confirmed that immature dendritic cells efficiently take up antigens, which were loaded in a solubilized form on the cells. Thereby, different concentrations varying between $20\frac{\mu g}{mL}$ (LPS) and $1\frac{mg}{mL}$ (dextran) were used. In this section the influence of different breast carcinoma cell lysates and lysate concentrations was investigated. Moreover, the contribution of the lysate to the maturation of DCs and the necessity for adaption of the maturation cytokine cocktail consisting of TNF-α and PGE_2 was investigated.

To investigate the effect of tumor cell lysates on dendritic cells, lysates containing total protein levels of $50\frac{\mu g}{mL}$ up to $300\frac{\mu g}{mL}$ of both cell lines were incubated with DCs on day 6 for $48h$. Lysate concentrations of $150\frac{\mu g}{mL}$ and higher were found to decrease the yield and viability of harvested DCs (data not shown). None of the concentrations of protein induced maturation as determined by the expression of low levels of HLA-A,B,C, weak levels of CD80, CD83 and CD86 (data not shown). As indicated by these results, protein lysates from MCF-7 and MDA-MB-231 breast carcinoma cell lines were not immunogenic and therefore did not mature loaded dendritic cells making the addition of a maturation stimulus necessary.

To induce maturation $100\frac{\mu g}{mL}$ protein (equivalent to approximately one tumor cell per DC) were used, the highest concentration which did not decrease yield and viability, and additionally, as adjuvants, TNF-α ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$). Surface receptor analysis demonstrated that DCs pulsed with $100\frac{\mu g}{mL}$ lysate (MCF-7 or MDA-MB-231) and matured with TNF-α ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$) were comparable to DCs matured with TNF-α ($1000\frac{U}{mL}$) and PGE_2 ($18\frac{\mu g}{mL}$) (figure 4.22). Both maturation stimuli resulted in high levels of MHC class I and MHC class II molecules, the costimulatory proteins CD80 and CD86 and the maturation marker CD83.

Assessment of the allostimulatory capacity in a mixed leukocyte reaction comparing MCF-7 and MDA-MB-231 lysate-pulsed DCs showed slight differences in T cell activation analyzed by CD25 (α-chain of the IL-2 receptor) and CD71 (transferrin receptor), which is illustrated in the figures 4.23 and 4.24 on page 82. Analysis of IL-12p70, IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-10 in the supernatant of lysate-pulsed matured DCs evidenced a DC phenotype, which did not secrete any of the determined cytokines (data not shown).

As described in chapter 4.2, monocytes from PBMCs contain a high percentage of apoptotic cells (37.8% $\pm$ 11.1%, as shown in table 4.8 on page 72), which need to be considered in the

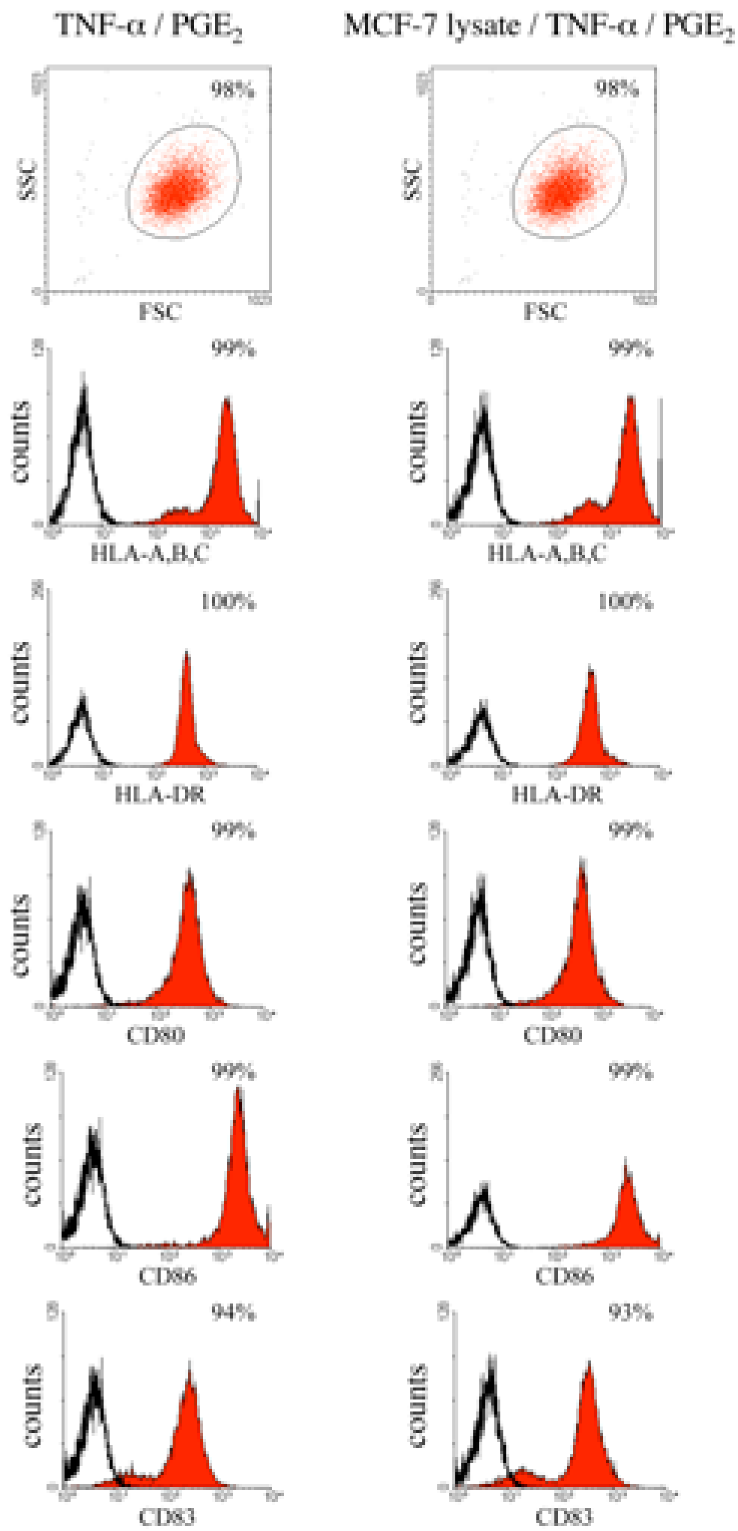


Figure 4.22: After a 48 h incubation with TNF-α and PGE_2 or with lysate in the presence of TNF-α and PGE_2 DCs highly express the surface molecules MHC class I (HLA-A,B,C) and MHC class II (HLA-DR), CD80, CD86 and the maturation marker CD83. Outlined histograms represent isotype staining. Histograms correspond to gated cells. From (Bohnenkamp et al., in preparation)

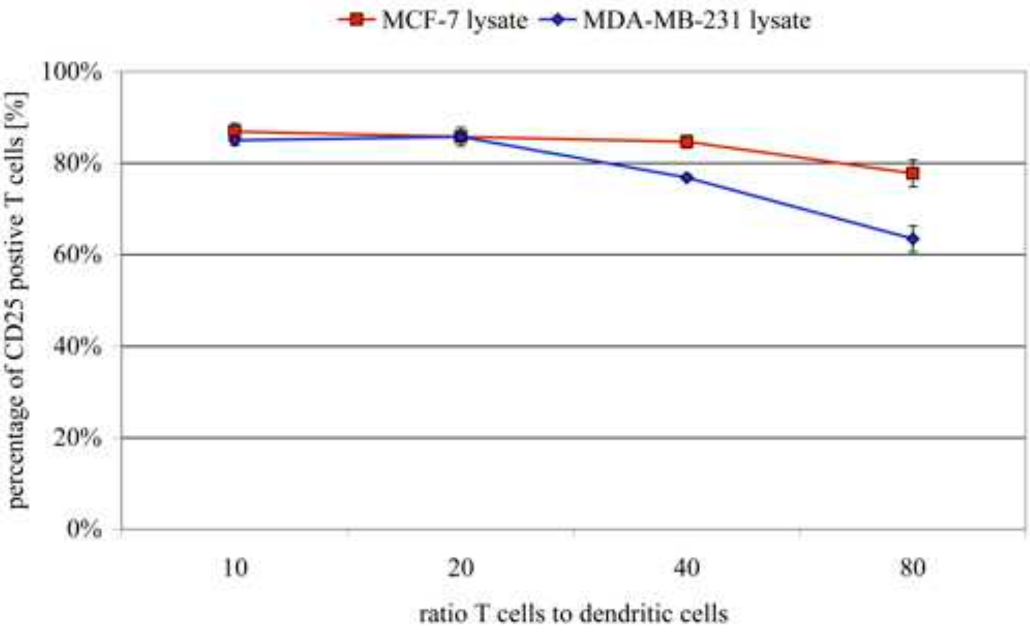


Figure 4.23: Activation of positive T cells (CD3) was assessed by CD25 (α-chain of the IL-2 receptor) staining after 4 days of co-cultivation. Results are from three different donors (triplicate) and are presented as mean ± SD. From (Bohnenkamp et al., in preparation)

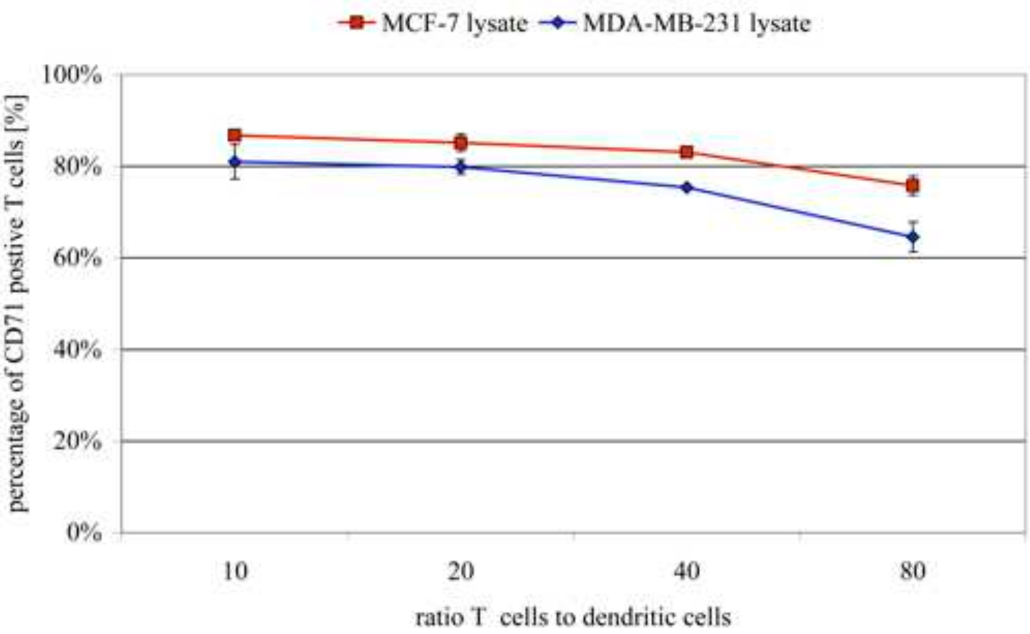


Figure 4.24: Activation of positive T cells (CD3) was assessed by CD71 (transferrin receptor) staining after 4 days of co-cultivation. Results are from three different donors (triplicate) and are presented as mean ± SD. From (Bohnenkamp et al., in preparation)

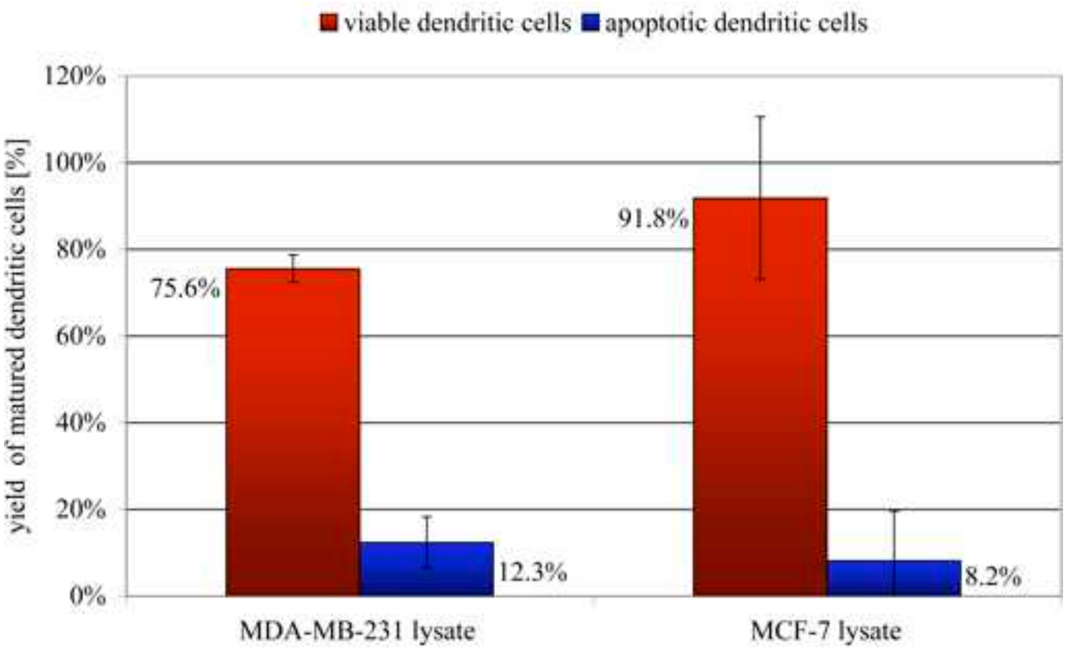


Figure 4.25: Yield of matured DCs pulsed with $100\frac{\mu g}{mL}$ lysate of breast carcinoma cell lines MCF-7 and MDA-MB-231 in the presence of TNF-α and PGE_2. Results are from 6 (MCF-7) and 3 (MDA-MB-231) different donors and presented as mean ± SD. Viable (AV^- / PI^-) and apoptotic (AV^+ / PI^-) cells are shown. From (Bohnenkamp et al., in preparation)

determination of the yield. By using an annexin V propidiumiodide assay, only viable, non-apoptotic monocytes and DCs were applied to calculation. The yield of MDA-MB-231 and MCF-7 lysate-pulsed, matured dendritic cells (as defined by size of cells, morphology, surface antigen expression and apoptosis) was 75.6±3.1% ($n = 3$) and 91.8±18.8% ($n = 6$) respectively, which is illustrated in figure 4.25. Furthermore, the percentage of apoptotic DCs was 12.3±6.0% and 8.2 ± 11.4% respectively.

4.3.4 Migratory Capacity of Lysate-pulsed Dendritic Cells

Dendritic cell migration is essential for the initiation of an adaptive immune response. After maturation, the chemokine receptor CCR7 is up-regulated on dendritic cells. Thus, activated DCs home to lymphoid organs, where the CXCR4 and CCR7 ligands CXCL12 and CCL19 are expressed and the T cell stimulation takes place. In conclusion, the homing of DCs from peripheral tissues to the draining lymph nodes is crucial for the stimulation of T cells. Therefore, the migratory capacity of lysate-pulsed matured DCs was analyzed by surface protein expression of CXCR4 and CCR7 and a chemotaxis assay towards the chemokines CXCL12 (SDF-1α) and CCL19 (MIP-3β).

The chemotaxis towards the chemokines CXCL12 ($250\frac{ng}{mL}$) and CCL19 ($100\frac{ng}{mL}$) was investigated using a transwell assay ($6\mu m$ pore size). As negative control a chamber without chemokines was used. $1 \cdot 10^5$ cells were incubated at $37°C$ and counted after $2h$ of incubation.

As demonstrated in figure 4.27 on page 85, matured DCs expressed both the receptors CXCR4 and CCR7. MCF-7 and MDA-MB-231 lysate-pulsed DCs showed efficient migration towards the indicated chemokines (figure 4.26 on page 85). However, MCF-7 lysate-pulsed DCs demonstrated an about 20% lower migratory capacity towards CCL19 (MIP-3β).

Scanning electron microscopy (SEM) image analysis demonstrated the effective migration of matured dendritic cells towards chemokines. Figure 4.28 and 4.29 on page 86 show the top of the membrane after $2h$ of incubation with the chemokine CCL19. Cells were assembling around the pores of the membrane towards highest chemokine concentration and demonstrated a stretched shape. Figure 4.30 illustrates a part of a dendritic cell in a pore of the membrane.

4.3.5 Cryopreservation of Dendritic Cells

The development of a protocol for the cryopreservation of dendritic cells is a key step not only to facilitate the use of DCs for in vitro assays (for the restimulation of T cells), but also to assist vaccination strategies, in which DCs are repetitively re-infused into the patient. However, it is commonly accepted that PBMCs can be easily frozen by using fetal calf serum (FCS) with 10% dimethyl sulfoxide (DMSO). In this experiment the development of an optimized protocol for the cryopreservation of dendritic cells was carried out, to avoid the usage of non-autologous components, which are not in accordance to GMP requirements.

For cryopreservation, lysate-pulsed dendritic cells matured with the adjuvants TNF-α and PGE_2 were frozen in autologous plasma (fluid phase of blood that is not coagulated), which had been heat-inactivated, centrifuged and filtered before usage, with 10% DMSO. Notably, the freezing of immature DCs was also a possible option. Nevertheless, readily matured and antigen loaded DCs facilitate the handling and were therefore preferred to immature DCs. Dendritic cells were frozen at cell densities varying between $1.1 \cdot 10^6$ and $3.5 \cdot 10^6$ $\frac{1}{mL}$ and thawed after one week. The mean yield was $85.3 \pm 9.4\%$ and the viability $89.3 \pm 6.0\%$, which is shown in table 4.10 on page 88. The flowcytometrical analysis demonstrated no difference of surface protein expression between frozen and freshly prepared dendritic cells (data not shown).

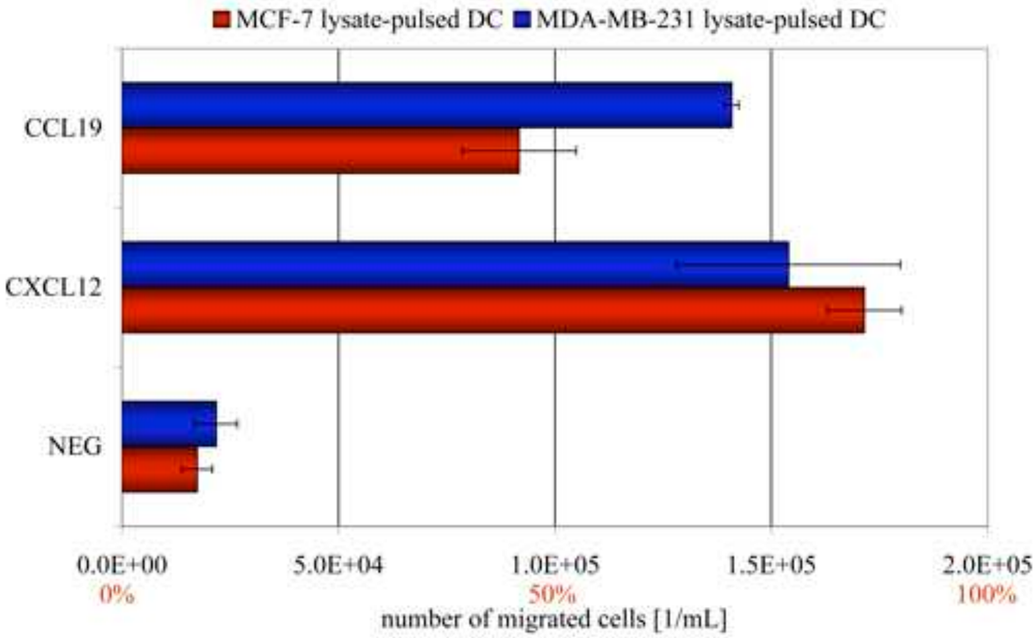


Figure 4.26: Migration of lysate-pulsed DCs towards the chemokines CCL19 (MIP-3β), CXCL12 (SDF-1α) or without chemokines (NEG). Data represent the means $\pm$ standard deviation of experiments from 3 different donors. From (Bohnenkamp et al., in preparation)

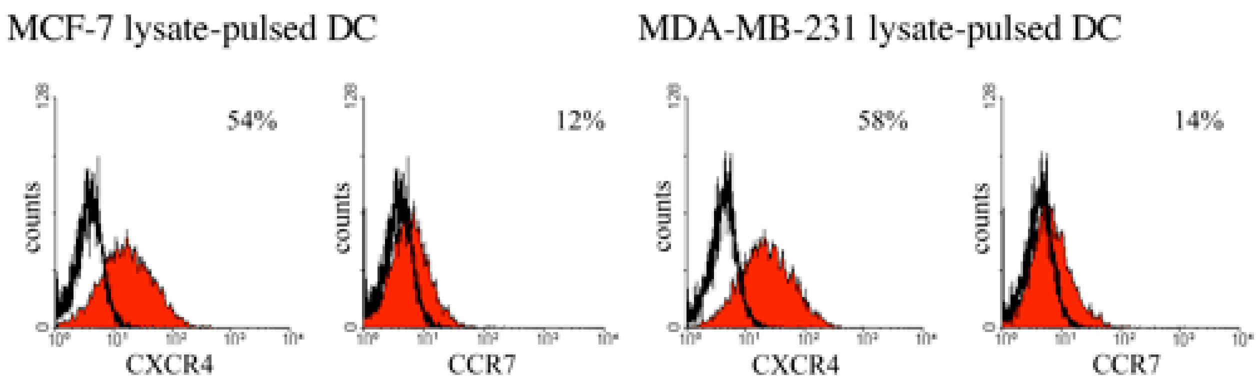


Figure 4.27: Analysis of CXCR4 and CCR7 expression on MCF-7 or MDA-MB-231 lysate-pulsed DCs in the presence of TNF-α and PGE_2. Data are representative of 3 separate experiments. From (Bohnenkamp et al., in preparation)

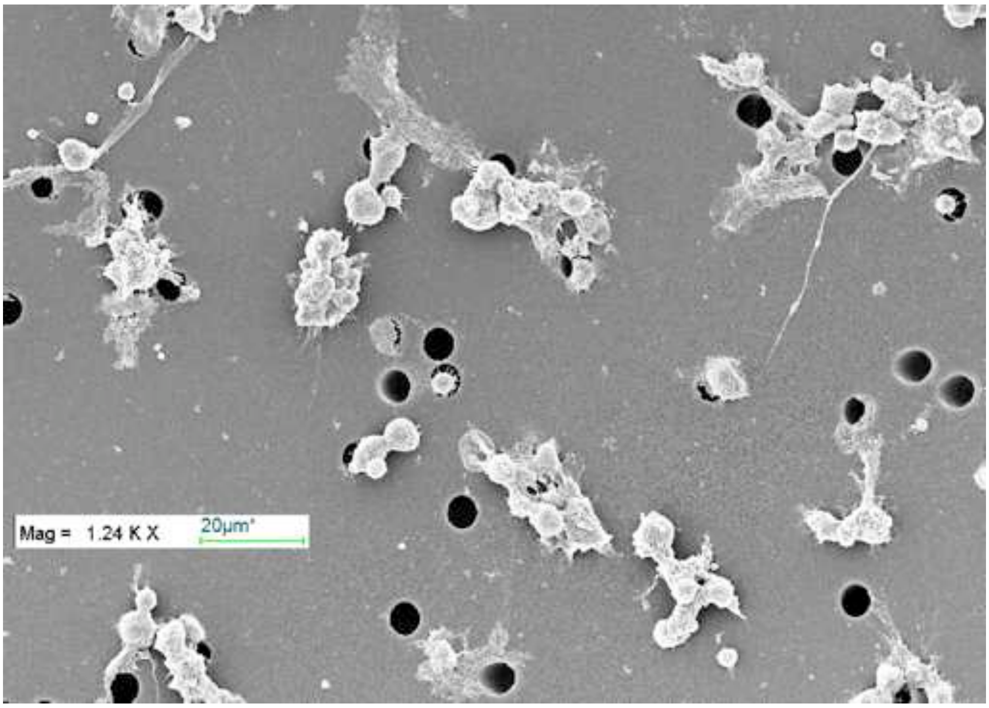


Figure 4.28: Electron microscopy image (magnification: 1,024x) of the top of the membrane after the chemotaxis assay. Dendritic cells migrated towards CCL19.

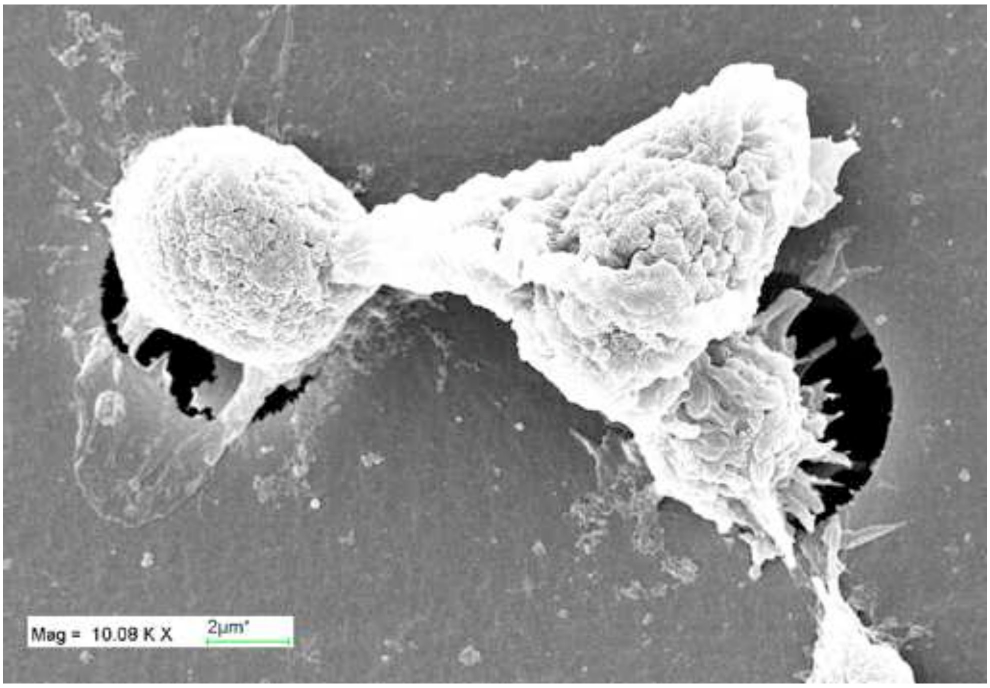


Figure 4.29: Electron microscopy image (magnification: 10,080x) of the top of the membrane after the chemotaxis assay towards CCL19.

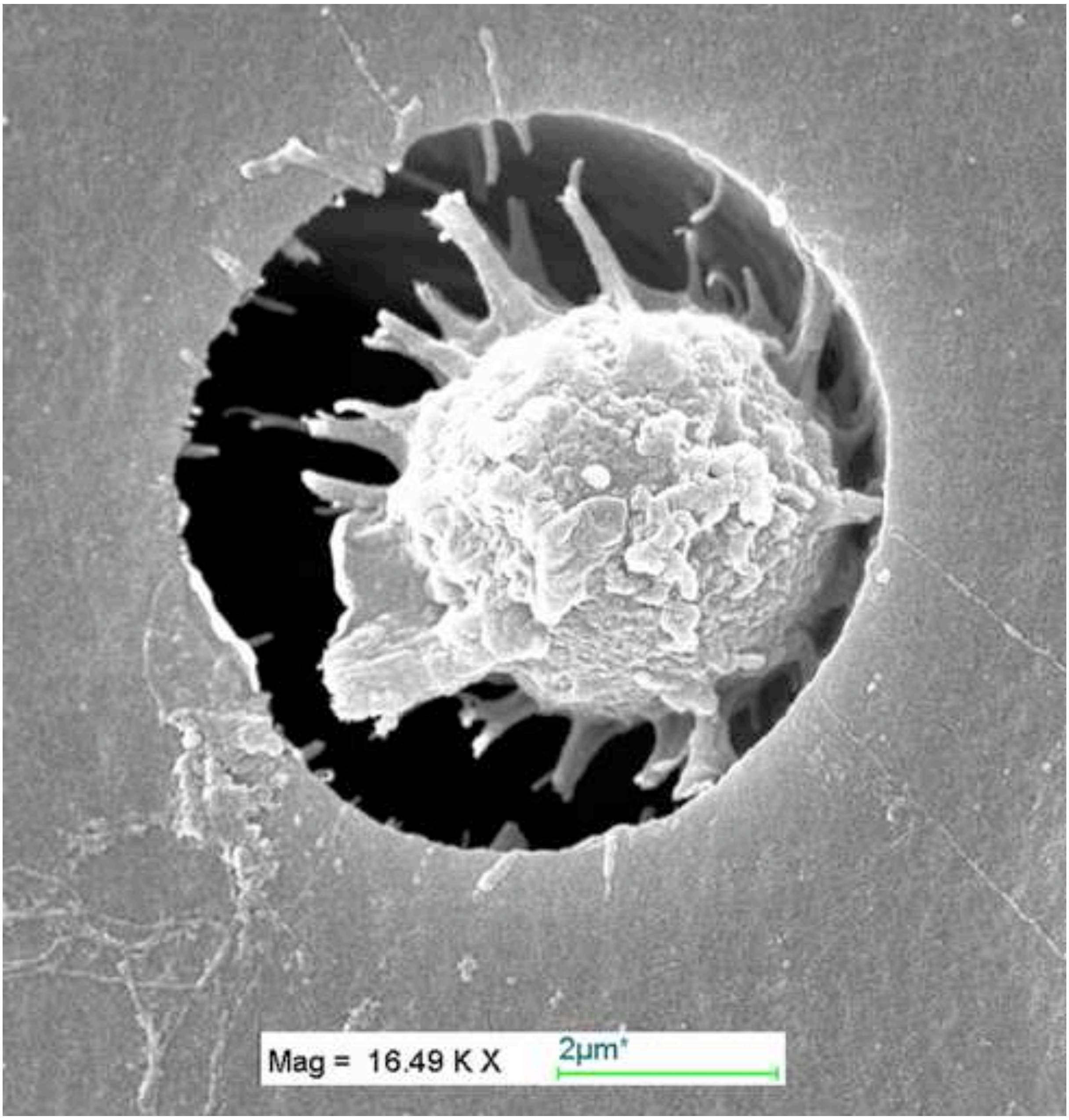


Figure 4.30: SEM image after the chemotaxis assay. Shown is a high magnification (16,490x) picture of a dendritic cell in a pore of the membrane.

Table 4.10: Cryopreservation of lysate-pulsed dendritic cells generated from 6 different donors

	Frozen DCs [-]	Thawed DCs [-]	Yield [%]	Viability [%]
Donor1	$1.1 \cdot 10^6$	$9.0 \cdot 10^5$	82.1	87.2
	$1.1 \cdot 10^6$	$1.1 \cdot 10^6$	95.6	88.3
Donor 2	$1.3 \cdot 10^6$	$1.2 \cdot 10^6$	92.3	79.4
	$1.3 \cdot 10^6$	$1.1 \cdot 10^6$	82.8	89.5
Donor 3	$3.0 \cdot 10^6$	$2.3 \cdot 10^6$	76.6	83.3
	$3.0 \cdot 10^6$	$3.0 \cdot 10^6$	98.8	83.1
Donor 4	$3.3 \cdot 10^6$	$3.3 \cdot 10^6$	98.7	87.6
	$3.3 \cdot 10^6$	$2.5 \cdot 10^6$	75.2	86.0
Donor 5	$1.7 \cdot 10^6$	$1.6 \cdot 10^6$	92.3	94.0
	$1.7 \cdot 10^6$	$1.2 \cdot 10^6$	71.8	98.0
Donor 6	$3.5 \cdot 10^6$	$2.7 \cdot 10^6$	75.7	98.0
	$3.5 \cdot 10^6$	$2.9 \cdot 10^6$	81.7	97.1
Mean			85.3	89.3
Standard deviation			9.4	6.0

4.3.6 Matured DCs Induce MHC Class I Restricted T Cell Responses

The goal of the following experiments was to demonstrate the functionality of the generated dendritic cells to launch an MHC class I restricted T cell response. Furthermore, this stimulation protocol was also developed to setup and optimize the stimulation of T cells with lysate-pulsed dendritic cells (section 4.3.7). The parameters used for these experiments are listed in table 4.11 on page 93. Additionally, the results of this section will be compared with the autologous stimulation with lysate-pulsed DCs for qualitative and quantitative estimation of the functionality and effects of the generated DCs on T cells.

To investigate an MHC class I restricted T cell response, the immunodominant HLA-A*0201-restricted peptide FLU M1 of the human influenza virus matrix protein (Gotch et al., 1987; Pogue et al., 1995) and the Melan-A / Mart-1_{26-35} analogue ELAGIGILTV A27L (Kawakami et al., 1994; Romero et al., 1997; Valmori et al., 1998) were used. These antigens were chosen because FLU M1 elicits a classical memory T cell response and the frequency of Melan-A / Mart-1 specific T cells in the blood of HLA-A2 individuals was reported to be considerably higher (10^{-3}) compared to the mean CTL frequency of approximately 10^{-6} (Coulie et al., 2003). Furthermore, it was shown that most of the anti-Melan-A / Mart-1-A2 lymphocytes present in the blood of individuals without melanoma have a naive phenotype (Zippelius et al., 2002). In sum, it was aimed to induce a memory T cell response by stimulating with FLU M1 peptide-pulsed DCs and a naive T cell response by priming with Melan-A / Mart-1 peptide-pulsed DCs.

Dendritic cells were loaded with the appropriate peptide, β_2-microglobulin and the helper peptide PADRE (Alexander et al., 1994, Brossart et al., 1999), which is shown in figure 4.32 on page 92. Thereby, the correct conformation of MHC class I molecules, which are loaded with peptides, is induced by β_2-microglobulin (see also Background - Tetramer analysis). Additionally, the PADRE helper peptide binds to MHC class II molecules and activates $CD4^+$ helper T cells resulting in cytokine production and further support for primed cytotoxic T cells. T lymphocytes were co-cultivated with DCs at a ratio of 10:1 in the presence of $2400\frac{U}{mL}$ IL-7 for preventing apoptosis of T cells. After 7 days most of the $CD4^+$ T cells were depleted with CD4 mAb coated tissue culture dishes and remaining cells were co-stimulated with DCs (without PADRE) at a ratio of 20:1 in fresh medium containing $20\frac{U}{mL}$ IL-2, the T cell growth factor. The culture was fed every 2^{nd} day according to the proliferation of T cells.

In figure 4.33 on page 93 the proliferation of FLU M1 and Melan-A / Mart-1 stimulated T cells is illustrated. As negative control TNF-α and PGE_2 matured, but unpulsed dendritic cells were used. The high expansion of FLU M1 T cells was evident, whereby Melan-A / Mart-1 induced T cells proliferated slower (see table 4.12). Matured, but unpulsed DCs did not induce proliferation in this autologous stimulation.

Already, after two stimulations 19.7% of T cells were shown to be FLU M1 peptide specific as determined by tetramer staining (figures 4.34 and 4.35 on page 94) and 45.9% were specific to Melan-A / Mart-1 peptide (figures 4.36 and 4.37 on page 95). Due to a stronger proliferation of FLU M1 stimulated T cells, the expansion factors were determined to 276-fold (Melan-A / Mart-1) and 4369-fold (FLU M1) respectively.

Background - analyzing T lymphocytes: Typically, inactive T cells are small featureless cells with few cytoplasmic organelles and much of the nuclear chromatin inactive (Janeway et al., 2001). These small lymphocytes have no functional activity until they recognize antigen for the induction of proliferation and their differentiation to specialized effector T cells.

In fact, T cells are important mediators of the effector mechanisms after dendritic cell stimulation and possess distinct immunobiological functions (see also chapter 2.2.3). The outcome of this is the necessity of functional distinction of immune responses and the assessment of the quantitative and qualitative nature of a T cell response. Therefore, different in vitro assays were established to investigate the phenotype, proliferation and activation of T cells and their specificity by tetramer staining:

T cell subsets: T cells ($CD3^+$) can be subdivided into several populations: T-helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$). Additionally, T-helper cells can be assigned to one of the several subsets including Th1 and Th2 cells (see also figure 2.10 on page 16). Theses cell are classified by the types of cytokines that are secreted: Th1 cells produce IL-2, IFN-γ and TNF-α, Th2 cells secrete IL-4, IL-5 and IL-10.

Proliferation of T cells: For the evaluation of proliferation of T cells that occurs following stimulation, various methods have been established (see also chapter 4.1.6). However, simple enumeration of T cells is laborious, and in most cases not possible because cells that respond represent only a small percentage of the total cell population (Hickling, 1998). For that reason, DNA synthesis (BrdU incorporation) or expression of 'proliferation markers' like CD25, the high affinity IL-2 receptor, and CD71, the transferrin receptor, are useful tools for the evaluation of proliferation.

Expression of activation markers: The analysis of activation markers on the cell surface of T cells can be correlated with the proliferation of T cells. Especially, CD25 and CD71 show a good correlation (Nguyen et al., 2003). Another activation marker, CD69, is up-regulated during $24h$ after activation and is therefore a good indicator for the induction of T cells (Craston et al., 1997; Rutella et al., 1999). However, proliferation does not correlate with the percentage of $CD69^+$ positive T cells because this surface protein is downregulated after the induction phase.

BACKGROUND - TETRAMER ANALYSIS: This technique enables the identification of individual T cells on the basis of their specific binding to the MHC:peptide complex. Due to the low affinity of one MHC class I molecule to the T cell receptor (TCR) (fast 'off-rate'), rapid dissociation of the TCR from the MHC:peptide complex can be observed. Therefore, multimeres (tetramer) of the MHC:peptide complex are built to overcome these limitations.

The restricting MHC molecule is synthesized in a soluble form by *E. coli*. These peptides can be biotinylated using the enzyme BirA, which recognizes a specific amino acid sequence. At this stage, MHC molecules are not correctly folded. The correct conformation is induced by the addition of β_2-microglobulin and the peptide that represents the appropriate epitope (compare the method for the peptide loading of DCs - both β_2-microglobulin and peptides are loaded on DCs). Next, the biotinylated and correctly folded MHC:peptide complex is mixed with streptavidin, which contains four binding sites for biotin and has previously been tagged with a fluorochrome. This results in the formation of an MHC:peptide tetramer - four specific MHC:peptide complexes bound to a single molecule streptavidin, which is labeled by a fluorescent dye (see figure 4.31).

Indeed, the four binding sites of a tetramer result in a greater affinity for T cells compared with a monomeric MHC class I molecule (HICKLING, 1998). With this method, $CD8^+$ T cell responses can be analyzed accurately. It is quantitative, can be combined with the phenotypical analysis of T cells and large numbers of samples can be processed. The lowest frequency that can be detected is one CTL in 50,000 (HICKLING, 1998).

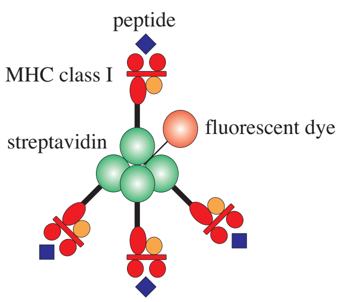


Figure 4.31: MHC:peptide complexes are formed to tetramers by coupling to streptavidin

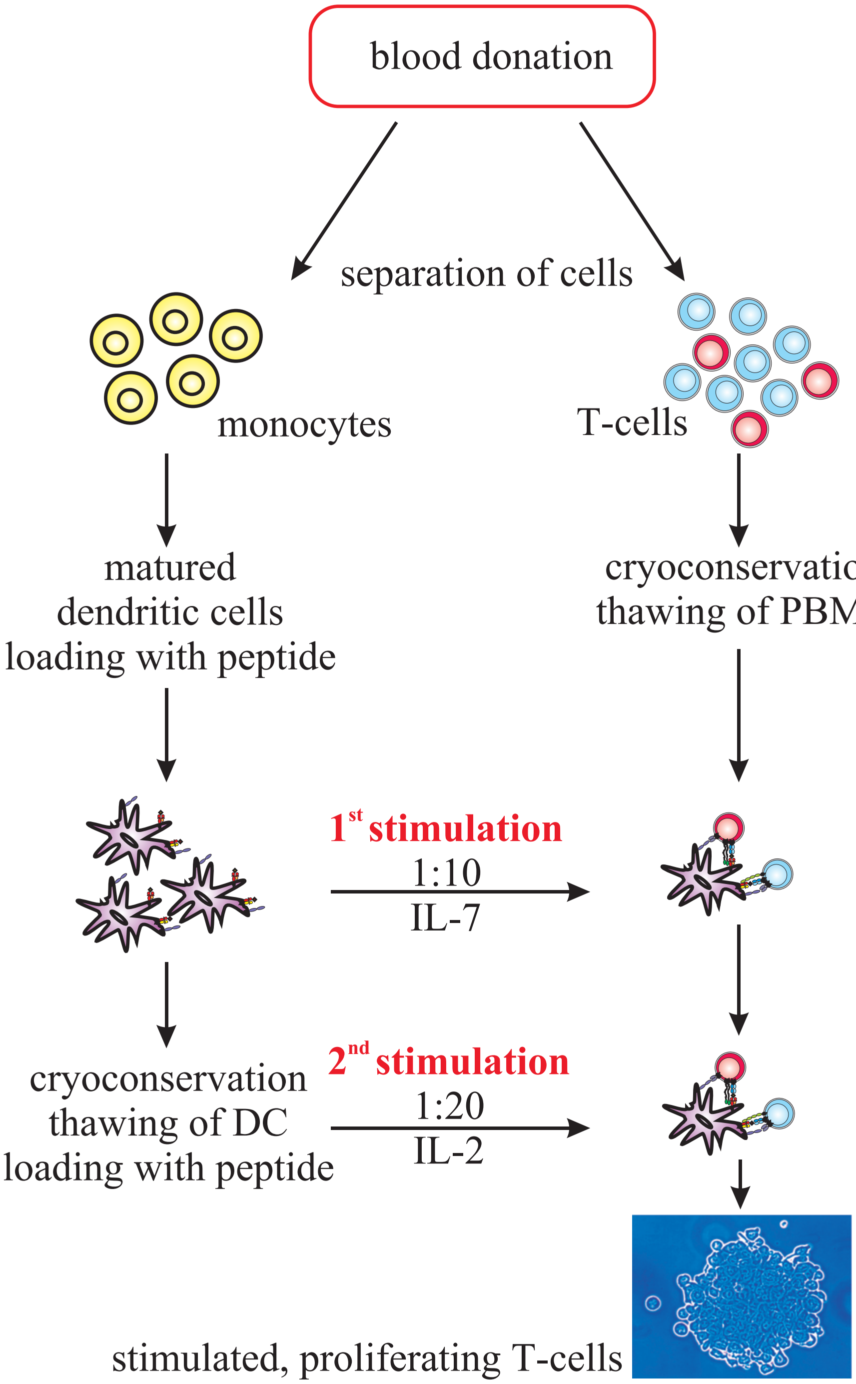


Figure 4.32: The scheme of the autologous stimulation of T cells with peptide-pulsed dendritic cells. Before stimulation, matured DCs were loaded with the appropriate peptide, β_2-microglobulin and the helper peptide PADRE and co-cultivated for 7 days. This was followed by an additional stimulation. Seven days after the 2^{nd} stimulation T cells were analyzed for their specificity by tetramer staining.

Table 4.11: The parameters of the experiments for the autologous stimulation of T cells

Cell density	$1.0 \cdot 10^6 \ \frac{1}{mL}$
Ratio DCs to T cells	1:10 (1^{st} stimulation), 1:20 (2^{nd} stimulation)
Pulsing of DCs	$40\frac{\mu g}{mL}$ peptide (1^{st} stimulation), $20\frac{\mu g}{mL}$ peptide (2^{nd} stimulation)
Volume	$12mL$
Medium	AIM V, serum-free
Donors	two
Cultivation system	Tissue culture flasks
IL-7	$2400\frac{U}{mL}$, 1^{st} day
IL-2	$20\frac{U}{mL}$, 7^{th} day
Feeding	every 2^{nd} day (medium + $20\frac{U}{mL}$ IL-2)
Cultivation time	14 days

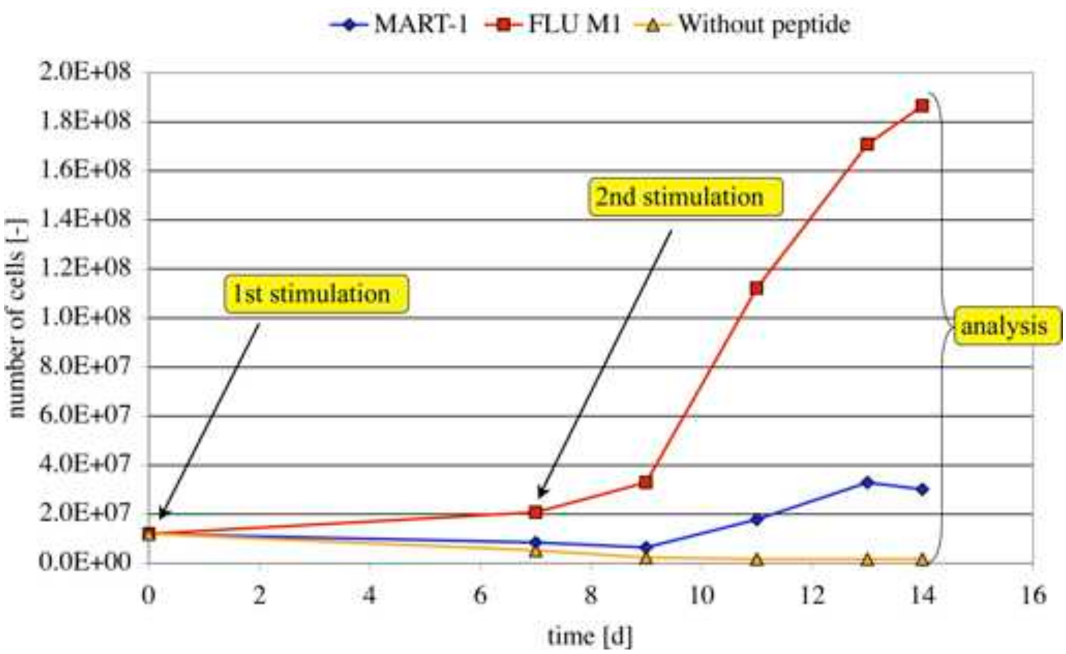


Figure 4.33: Autologous PBMCs were induced with peptide-pulsed DCs with IL-7 for 7 days and restimulated for additional 7 days with $20\frac{U}{mL}$ IL-2. Analysis of specificity was assessed by tetramers folded around the corresponding peptide. Shown is one representative donor out of two.

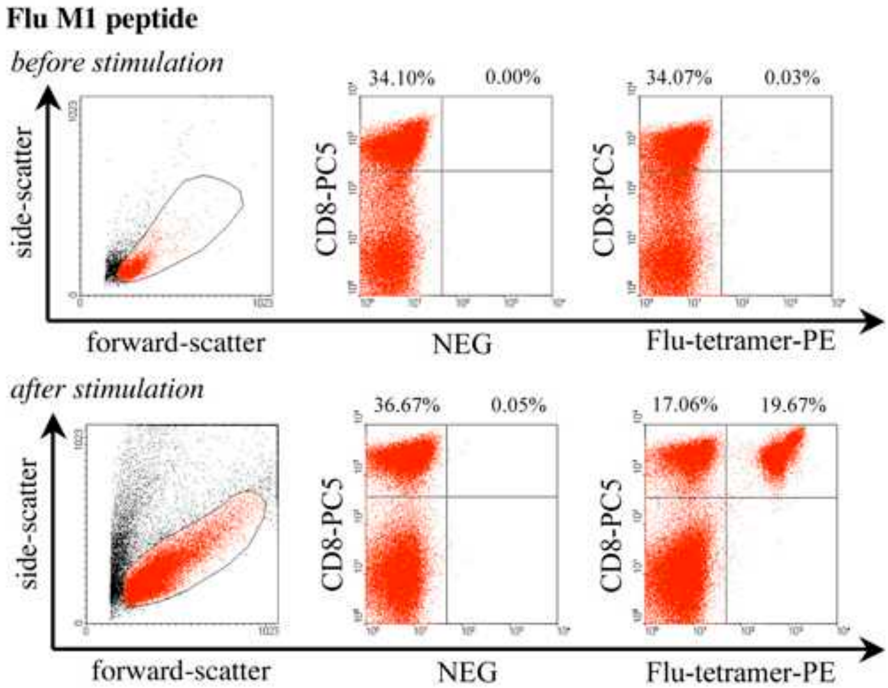


Figure 4.34: Tetramer analysis of FLU M1 stimulated T cells. Dot plots correspond to gated cells. From (Bohnenkamp et al., submitted)

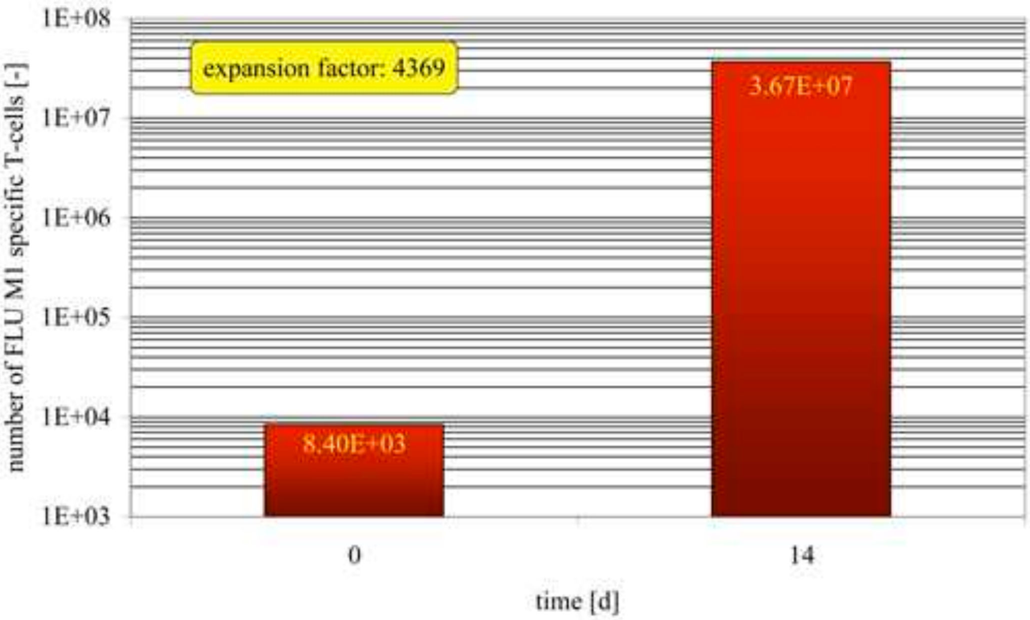


Figure 4.35: Number and expansion of FLU M1 specific T cells before and after stimulation. Inoculated were $1.0 \cdot 10^6 \frac{1}{mL}$ PBMC in $4mL$ AIM V medium (triplicate). From (Bohnenkamp et al., submitted)

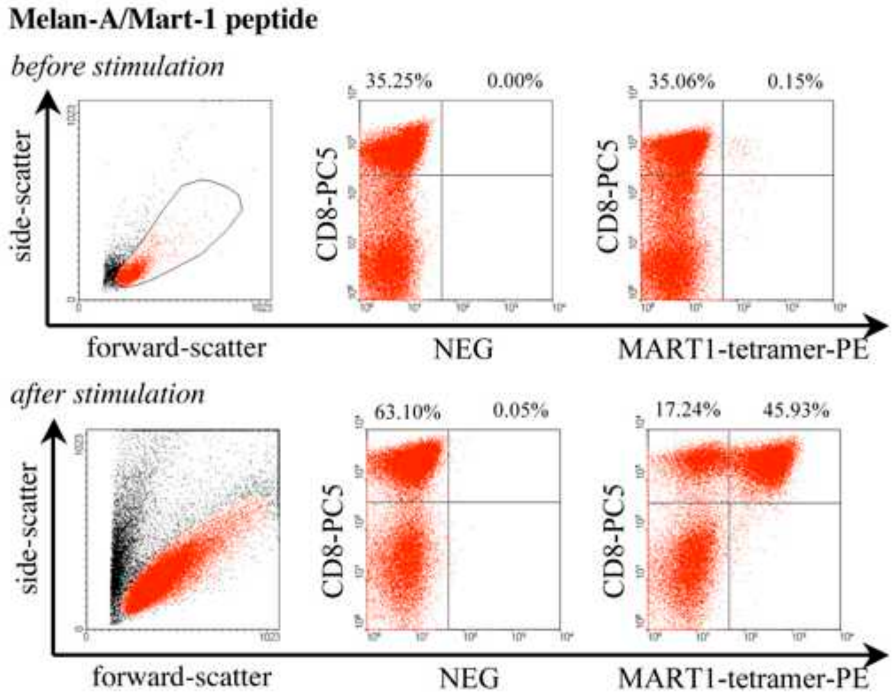


Figure 4.36: Melan-A / Mart-1 specific T cells analysed by corresponding tetramer. Dot plots indicate gated cells. From (Bohnenkamp et al., submitted)

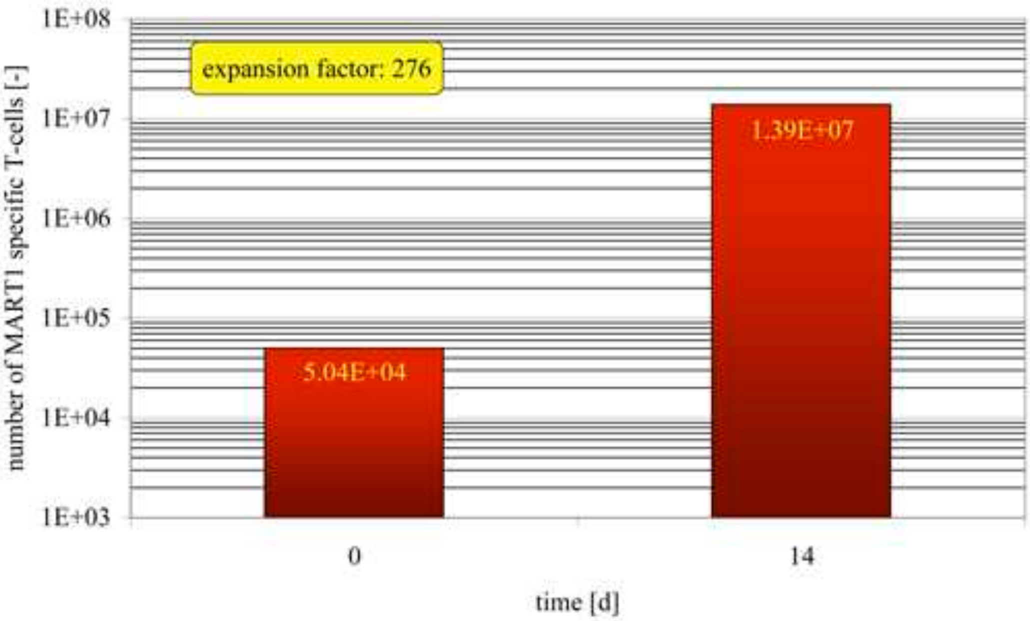


Figure 4.37: Number and expansion of Melan-A / Mart-1 specific T cells on day 0 and day 14. From (Bohnenkamp et al., submitted)

Table 4.12: Autologous stimulation of T cells with DCs loaded with different peptides

DCs pulsed with	FLU M1	Melan-A / Mart-1	Unpulsed
Inoculation $[\frac{1}{mL}]$	$1.0 \cdot 10^6$	$1.0 \cdot 10^6$	$1.0 \cdot 10^6$
Total cell number d0 [-]	$1.2 \cdot 10^7$	$1.2 \cdot 10^7$	$1.2 \cdot 10^7$
Total cell number d14 [-]	$1.9 \cdot 10^8$	$3.0 \cdot 10^7$	$1.8 \cdot 10^6$
Maximum growth rate μ_{max} $[\frac{1}{h}]$	$2.6 \cdot 10^{-2}$	$2.1 \cdot 10^{-2}$	-
Minimal generation time t_{min} [h]	27.2	32.5	-
Total $CD8^+$ T cells [%]	36.7	63.2	-
Specific $CD8^+$ T cells [%]	19.7	45.9	-
Expansion [x-fold]	4369	276	-

4.3.7 Autologous T Cell Response to Lysate-pulsed Dendritic Cells

In the previous experiments it has been demonstrated that fully matured lysate-pulsed dendritic cells can be obtained from a highly purified monocyte population. In addition, these TNF-α and PGE_2 matured DCs showed a high migratory capacity in several chemotaxis experiments. Furthermore, a cryopreservation method of lysate-pulsed DCs in autologous plasma has been established. Finally, peptide pulsed DCs efficiently stimulated autologous T cells in an MHC class I restricted manner. In this section, the autologous T cell response to lysate-pulsed dendritic cells is described, in which the previously established results are combined. The parameters of this experiment are shown in table 4.13 on page 98.

The aim of this experiment was to demonstrate that lysate-pulsed dendritic cells can stimulate T cells in an MHC class II restricted manner and cross-prime T cells by presenting antigens via the MHC class I pathway. Therefore, MCF-7 and MDA-MB-231 lysate-pulsed DCs were utilized and co-cultered with autologous CD14-depleted PBMCs in the presence of IL-7 (first week). Restimulation was performed weekly with addition of $20\frac{U}{mL}$ IL-2 and cells were adjusted to a cell density between $5 \cdot 10^5$ and $1 \cdot 10^6$ $\frac{1}{mL}$ according to their proliferation. As a negative control, TNF-α and PGE_2 matured DCs without lysate pulsing were used. The scheme of the autologous stimulation is shown in figure 4.38 on page 97.

A strong proliferative response in $CD4^+$ and $CD8^+$ T cells stimulated with lysate-pulsed DCs was observed, whereas no proliferation was detected in PBMCs co-cultivated with unpulsed DCs (see figure 4.39 on page 99). Two days after the 3^{rd} stimulation the expression of CD25 (α-

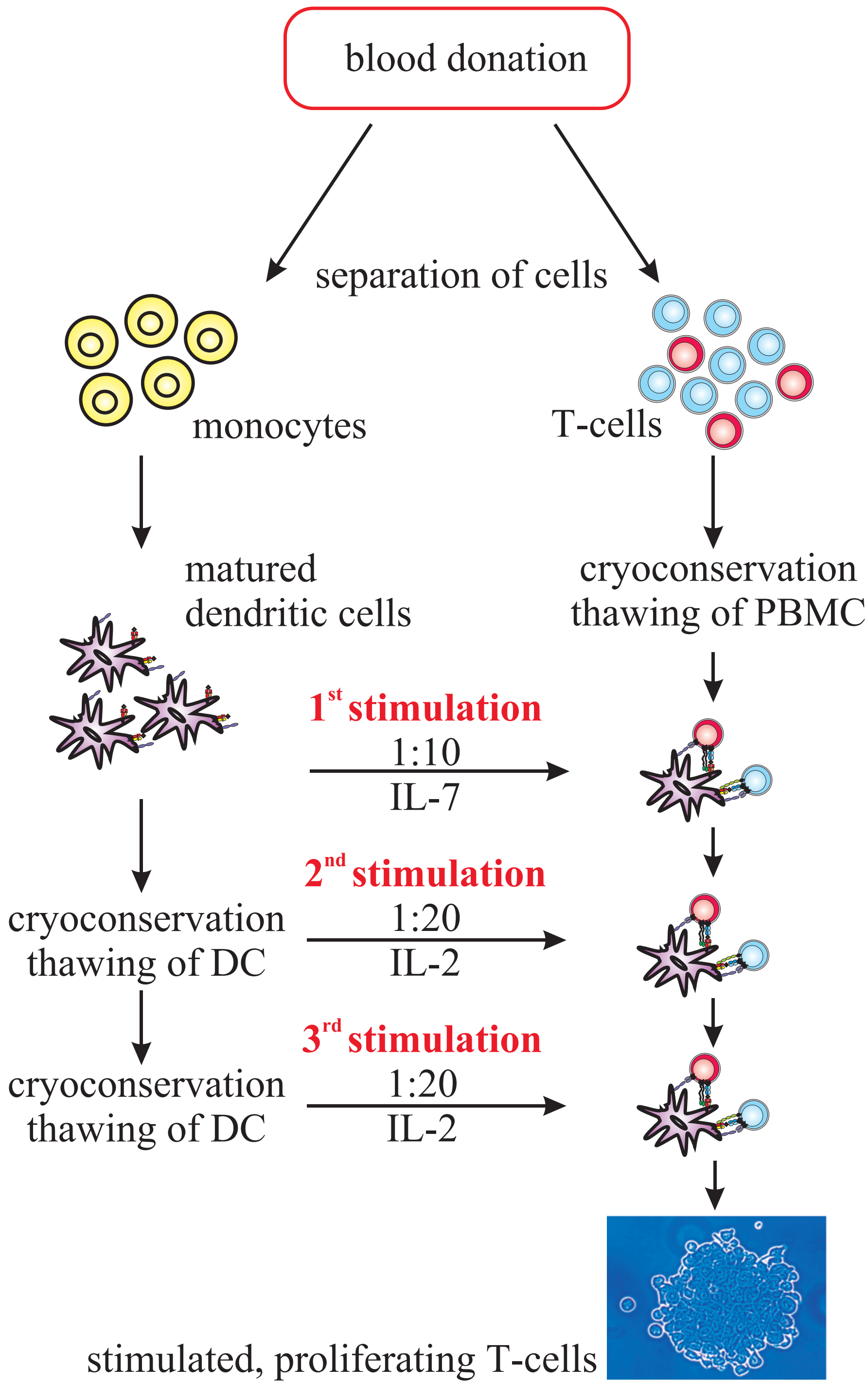


Figure 4.38: The scheme of the autologous stimulation of T cells with lysate-pulsed dendritic cells. Dendritic cells were loaded with 100$\frac{\mu g}{mL}$ tumor cell lysate and matured with TNF-α and PGE_2. Autologous T cells were co-cultivated for 7 days with DCs at a ratio of 10:1. Two re-stimulations were performed after 7 and 14 days respectively (ratio T cells to DCs 20:1). T cells were harvested on day 16 and analyzed as described.

Table 4.13: The parameters of the experiments for the autologous stimulation of T cells

Cell density	$1.0 \cdot 10^6 \frac{1}{mL}$
Ratio DCs to T cells	1:10 (1^{st} stimulation), 1:20 (2^{nd} and 3^{rd} stimulation)
Pulsing of DCs	$100\frac{\mu g}{mL}$ lysate
Volume	$12mL$
Medium	AIM V, serum-free
Donors	three
Cultivation system	Tissue culture flasks
IL-7	$2400\frac{U}{mL}$, 1^{st} day
IL-2	$20\frac{U}{mL}$, 7^{th} day
Feeding	every 2^{nd} day (medium + $20\frac{U}{mL}$ IL-2)
Cultivation time	16 days

chain of the IL-2 receptor), CD69 (c-type-lectin, early activation marker) and CD71 (transferrin receptor) on T cells was analyzed. As demonstrated in figure 4.40 on page 100, $CD4^+$ and $CD8^+$ T lymphocytes were strongly activated with MCF-7 and MDA-MB-231 lysate-pulsed dendritic cells. PBMCs co-cultured with TNF-α and PGE_2 matured DCs without lysate pulsing showed no expression of these surface proteins (data not shown). An IFN-γ secretion assay evidenced the activation of $CD4^+$ and $CD8^+$ subsets of T cells. However, the population of IFN-γ secreting cells was lower than the population of CD25, CD69 and CD71 expressing T cells.

MUC1 Specific T Cells Can be Recognized by Peptide MHC Class I Tetramers

Two days after the 3^{rd} stimulation $CD8^+$ T cells were analyzed with the F7 and M1.2 peptide MHC class I tetramers. As a control, the irrelevant FLU M1 peptide MHC class I tetramer was used. In the figures 4.41 (page 101), 4.42 and 4.43 (page 102) the frequency and the corresponding expansion factor for one representative experiment out of three are shown. In these experiments a selective expansion for the M1.2 epitope specific T cells and a higher induction of these T lymphocytes by MCF-7 lysate-pulsed DCs compared with MDA-MB-231 lysate-pulsed DCs were observed. The expansion factor for M1.2 epitope specific T cells ranged from 12-fold (MDA-MB-231 lysate) to 19-fold (MCF-7 lysate) respectively. The expansion of all $CD8^+$ T cells stimulated either with MCF-7 or MDA-MB-231 lysate pulsed dendritic cells

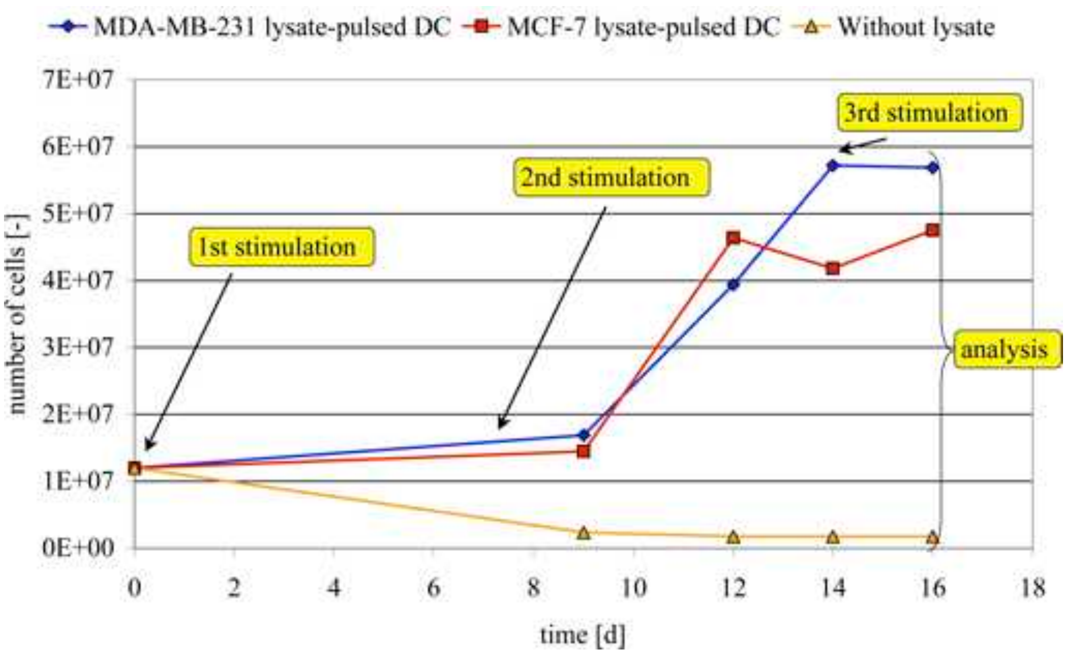


Figure 4.39: Proliferation of T cells stimulated with MCF-7 or MDA-MB-231 lysate-pulsed DCs. As negative control matured DCs (unloaded) were used.

was similar (see also table 4.14 on page 101). As demonstrated, the expansion of irrelevant FLU M1 specific T cells was comparably low.

Lysate-pulsed DCs Induce Strong Th1 and Low Th2 Cytokine Profile

After 14 days of co-cultivation of T cells with matured DCs, peptide pulsed DCs and breast carcinoma cell lysate-pulsed DCs, supernatants were harvested and analyzed for IL12p70, IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-10. IL-12p70 was not found in any of the supernatants (data not shown). Nevertheless, a strong Th1 response was observed in all experiments (figure 4.44 on page 103). Melan-A / Mart-1, MDA-MB-231 lysate and MCF-7 lysate-pulsed DCs induced 17,232$\frac{pg}{mL}$, 15,740$\frac{pg}{mL}$ and 10,737$\frac{pg}{mL}$ IFN-γ respectively. In contrast, a much stronger T cell response to the FLU M1 peptide was observed (51,100$\frac{pg}{mL}$ IFN-γ). Control TNF-α and PGE_2 matured DCs induced a very low IFN-γ response (960$\frac{pg}{mL}$). Interestingly, the Pan-DR binding epitope PADRE alone induced an IFN-γ response similar to that of Melan-A / Mart-1 pulsed DCs (17,232$\frac{pg}{mL}$ IFN-γ), which were accompanied by the PADRE peptide. Low amounts of TNF-α were detected in lysate-pulsed DC supernatants. The IL-2 concentrations correspond to the level of cytokine supplement.

Analysis of the Th2 cytokines showed low amounts of IL-4 and IL-10, ranging between 10

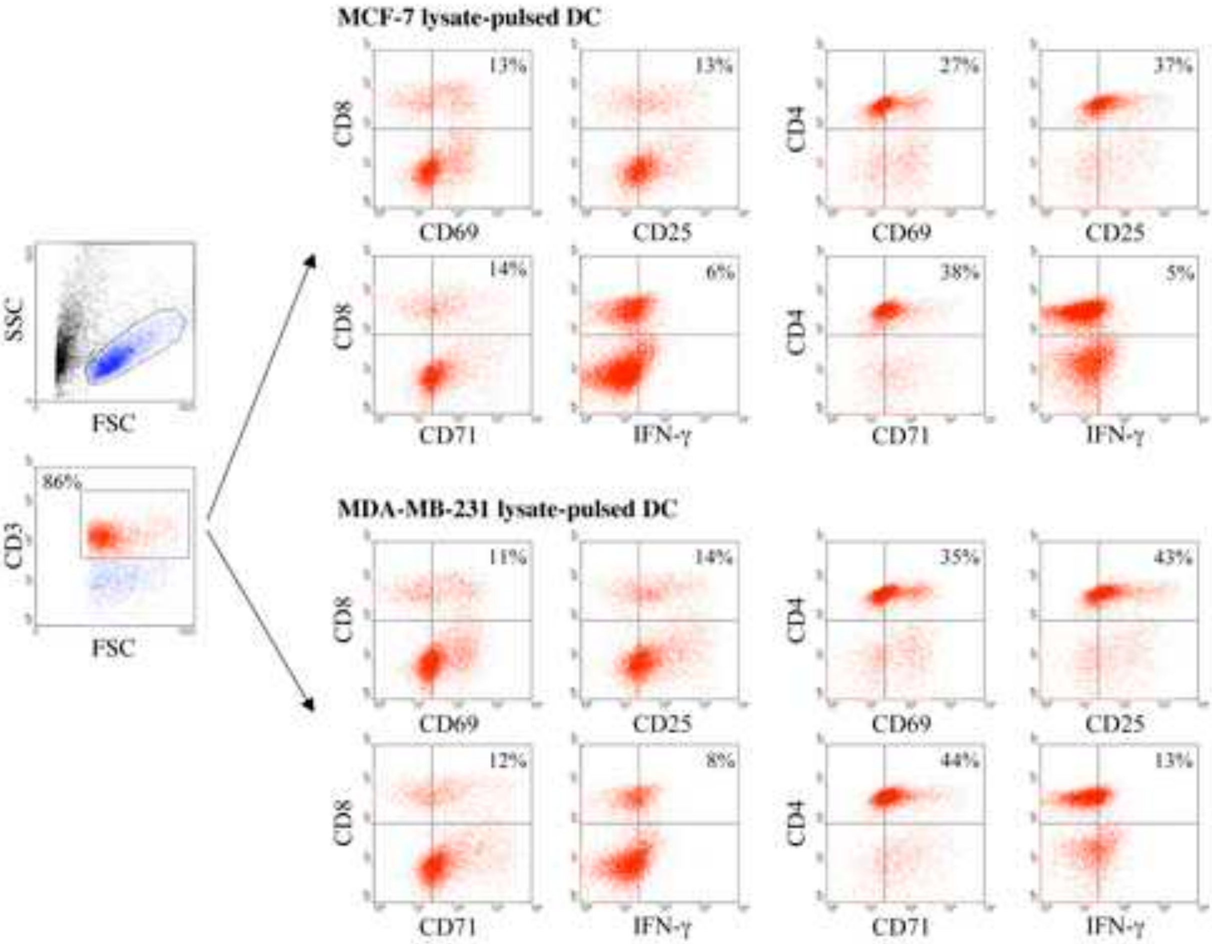


Figure 4.40: Breast carcinoma cell lysate-pulsed DCs induce the expression of CD25, the activation marker CD69, CD71 and IFN-γ secretion by T-helper cells and CTLs. Autologous T cells were stimulated with DCs pulsed with either MCF-7 or MDA-MB-231 lysate and cultivated for 7 days with IL-7. T cells were restimulated twice in the presence of IL-2 and analyzed 48h after the last stimulation. One representative experiment out of three is shown. Dot plots correspond to gated CD3$^+$ T cells. From (Bohnenkamp et al., in preparation)

Table 4.14: Autologous stimulation of T cells with lysate-pulsed DCs

DCs loaded with	MCF-7 lysate	MDA-MB-231 lysate	Unpulsed
Inoculation $[\frac{1}{mL}]$	$1.0 \cdot 10^6$	$1.0 \cdot 10^6$	$1.0 \cdot 10^6$
Total cell number d0 [-]	$1.2 \cdot 10^7$	$1.2 \cdot 10^7$	$1.2 \cdot 10^7$
Total cell number d16 [-]	$4.8 \cdot 10^8$	$5.7 \cdot 10^7$	$1.8 \cdot 10^6$
Maximum growth rate μ_{max} $[\frac{1}{h}]$	$1.6 \cdot 10^{-2}$	$1.2 \cdot 10^{-2}$	-
Minimum generation time t_{min} [h]	42.9	58.9	-
$CD8^+$ T cells d0 [%]	17.6	17.6	-
$CD8^+$ T cells d16 [%]	20.3	18.8	-
Expansion $CD8^+$ T cells [x-fold]	4.6	5.1	-
Expansion F7 $CD8^+$ T cells [x-fold]	8.1	7.5	-
Expansion M1.2 $CD8^+$ T cells [x-fold]	18.9	12.0	-

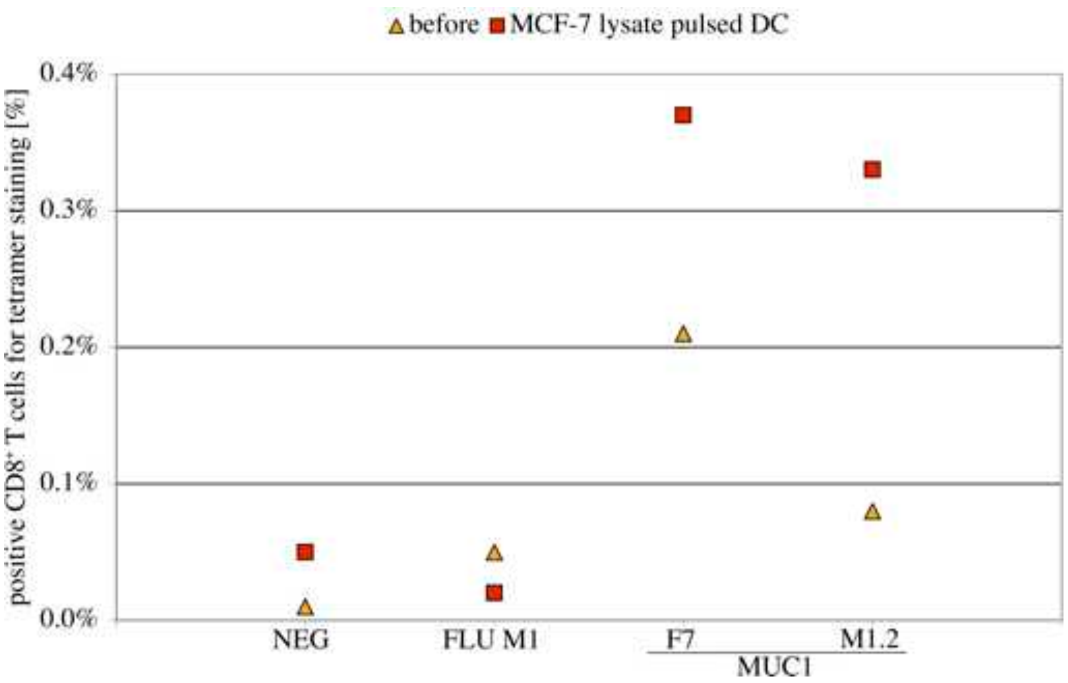


Figure 4.41: Tumor lysate-pulsed DCs activate MUC1 specific T cells. Forty-eight hours after the third stimulation T lymphocytes were stained with tetramers folded around the F7 and M1.2 peptide. FLU M1 was used as an irrelevant peptide. The negative control shows no staining. Frequency of MUC1 and FLU M1 specific T cells after stimulation with MCF-7 pulsed DCs. From (Bohnenkamp et al., in preparation)

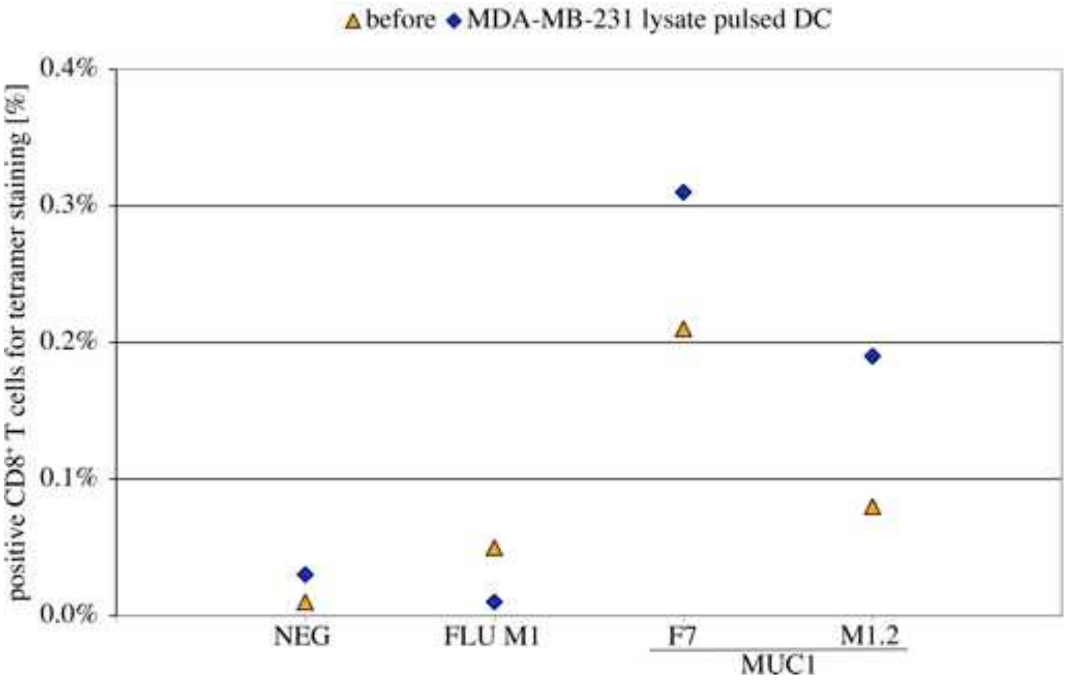


Figure 4.42: Frequency of MUC1 and FLU M1 specific T cells after stimulation with MDA-MB-231 lysate-pulsed DCs. From (Bohnenkamp et al., in preparation)

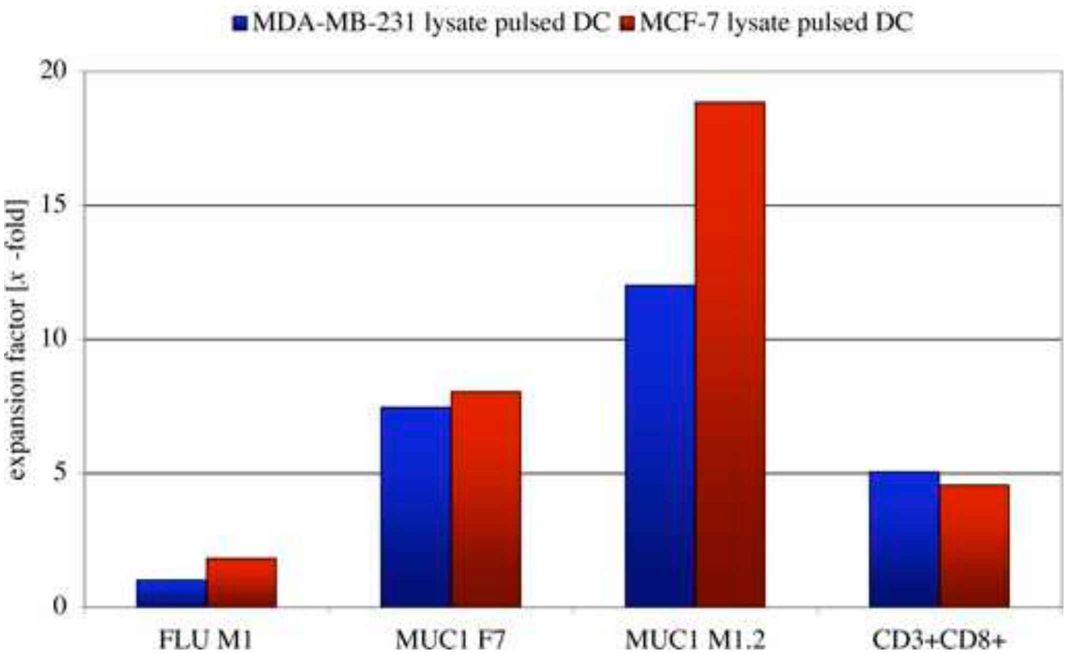


Figure 4.43: Frequency corresponds to shown expansion factors. One representative donor out of three is illustrated. From (Bohnenkamp et al., in preparation)

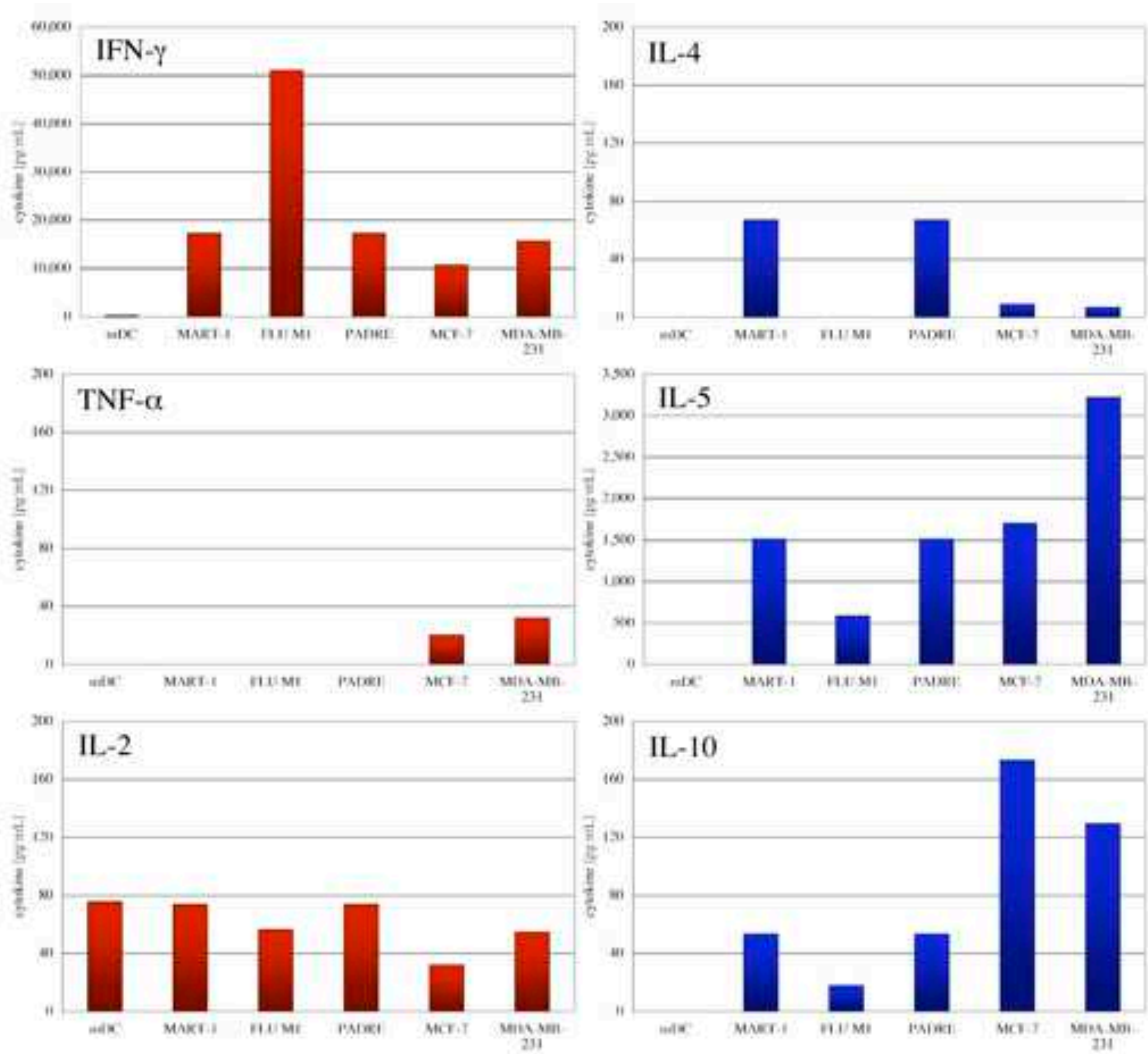


Figure 4.44: Th1 (IFN-γ, TNF-α, IL-2) and Th2 (IL-4, IL-5, IL-10) cytokine profile analyzed with the cytometric bead array after 14 days of co-cultivation of T cells with FLU M1 peptide (in the presence of PADRE), Melan-A / Mart-1 (in the presence of PADRE), PADRE and lysate-pulsed (MCF-7 and MDA-MB-231) DCs. Matured, unpulsed DCs did not induce cytokine production of T cells. From (Bohnenkamp et al., in preparation)

and 180$\frac{pg}{mL}$. No IL-4 was detected in FLU M1 stimulated experiments. Furthermore, high levels of IL-5 were measured. FLU M1, Melan-A / Mart-1, MDA-MB-231 lysate and MCF-7 lysate-pulsed DCs induced 585$\frac{pg}{mL}$, 1,516$\frac{pg}{mL}$, 3,221$\frac{pg}{mL}$ and 1,705$\frac{pg}{mL}$ IL-5 respectively. Interestingly, the lowest amounts for IL-4, IL-5 and IL-10 were observed in FLU M1 stimulated experiments. The supernatants of Melan-A / Mart-1, PADRE (1,516$\frac{pg}{mL}$ IL-5) and lysate-pulsed DCs showed similar amounts of measured Th2 cytokines.

4.4 Transfer to Clinical Application

4.4.1 Cultivation of Dendritic Cells in a Fully Closed System

The developed protocol for the reproducible generation of dendritic cells in a high yield uses standard tissue culture flasks for the cultivation. While this cultivation system yields dendritic cells with a high purity and stimulatory capacity, it is labour intensive (requirement for four tissue culture flasks per buffy coat) and bears the risk for contamination at various steps. Therefore, a closed semi-automated system for the generation of monocyte-derived dendritic cells is highly desirable.

The aim of this section is to describe the generation of DCs in a fully closed system. Monocytes can be enriched by immunomagnetic beads utilizing the CliniMacs system from Miltenyi, which can be operated semi-automated for the purification of cells from blood or leukapheresis products. Subsequently, a cultivation bag is connected and filled with cells and the required cultivation medium and supplements. Finally, the bag is centrifuged, cells are resuspended and directly re-infused into the patient. The concept of the fully closed system is illustrated in figure 4.45.

Monocytes were enriched and inoculated into tissue culture flasks and hydrophobic teflon bags with a cell density of $1.3 \cdot 10^6\ \frac{1}{mL}$ in $30mL$. After 6 days of differentiation, cells were incubated with 100$\frac{\mu g}{mL}$ lysate, TNF-α (1000$\frac{U}{mL}$) and PGE_2 (1$\frac{\mu g}{mL}$) for additional two days. Table 4.15 summarizes the parameters for the comparison of the two different cultivation systems.

After 8 days of cultivation, dendritic cells were harvested and analyzed. The yield of mature DCs (as defined by size of cells, morphology, surface antigen expression and apoptosis) cultivated in tissue culture flasks and hydrophobic teflon bags was $90.2 \pm 16.6\%$ ($n = 8$) and $57.0 \pm 8.1\%$ ($n = 4$) respectively and the number of apoptotic cells was $8.8 \pm 9.3\%$ ($n = 8$) and $25.8 \pm 13.6\%$ ($n = 4$) respectively (figure 4.46, see also figure 4.18 on page 74). Without apoptosis analysis

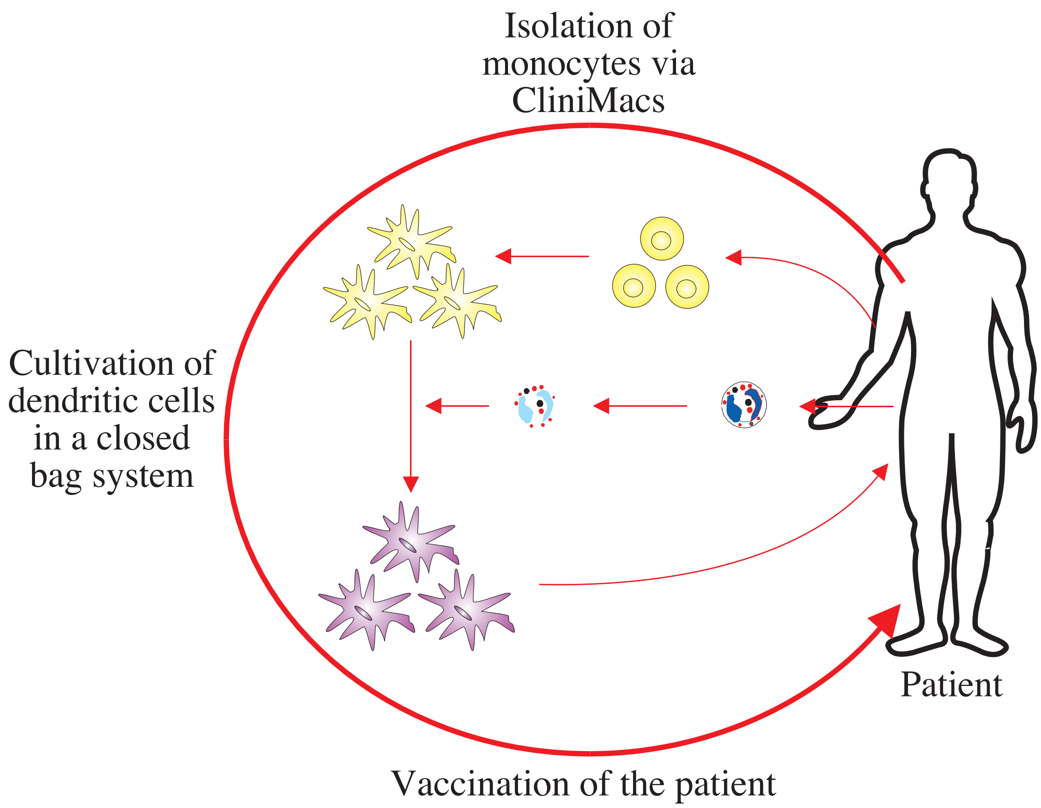


Figure 4.45: The concept of the generation of dendritic cells in a fully closed system.

Table 4.15: Experimental parameters for the generation of DCs in different cultivation systems

Cell densitiy	$1.3 \cdot 10^6 \ \frac{1}{mL}$
Donors	four
Cultivation system	tissue culture flask, teflon bag, $30mL$ volume
Medium	X-VIVO 15
GM-CSF	$400\frac{U}{mL}$
IL-4	$2000\frac{U}{mL}$
Maturation stimulus	$100\frac{\mu g}{mL}$ lysate, TNF-α ($1000\frac{U}{mL}$), PGE_2 ($1\frac{\mu g}{mL}$)
Time (differentiation / maturation)	6 days / 2 days

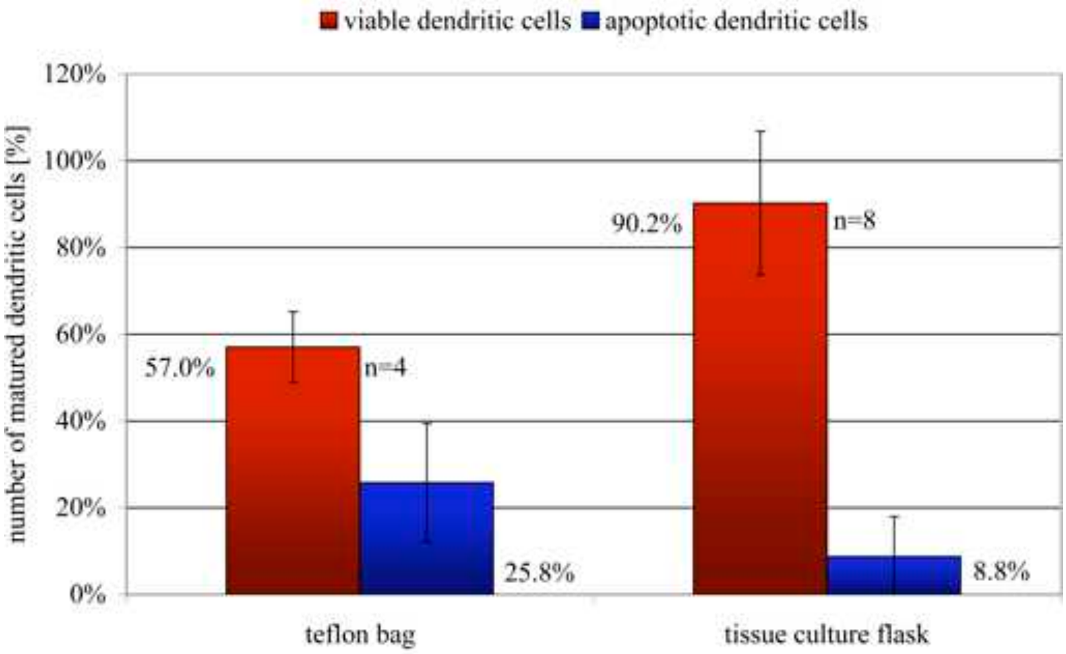


Figure 4.46: Yield of lysate-pulsed and TNF-α and PGE_2 matured dendritic cells cultivated either in tissue culture flasks or teflon bags. Results are from 8 (tissue culture flasks) or 4 (teflon bags) different donors respectively and presented as mean ± standard deviation. Shown are viable (AV^- / PI^-) and apoptotic (AV^+ / PI^-) cells.

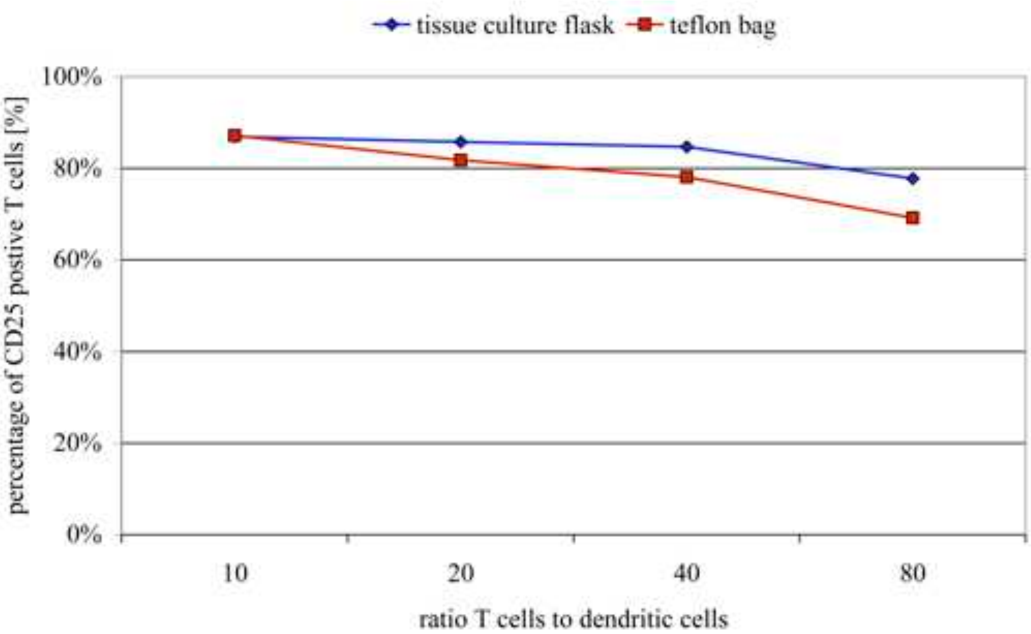


Figure 4.47: Activation of T cells (CD3) was assessed by CD25 (α-chain of the IL-2 receptor) staining after 4 days of co-cultivation.

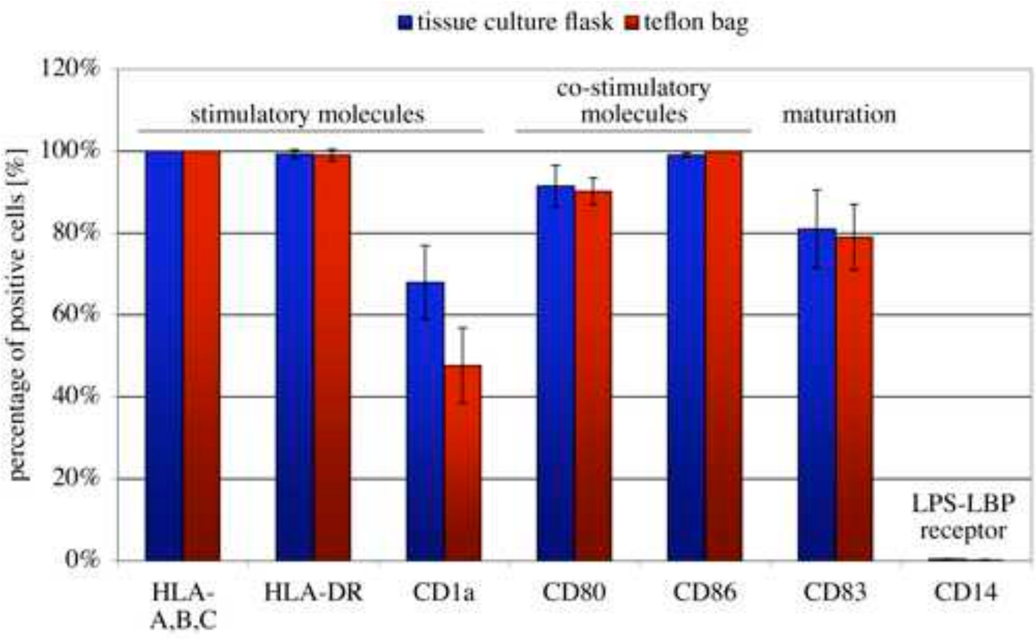


Figure 4.48: Phenotypical analysis of matured lysate-pulsed DCs either cultivated in tissue culture flasks or teflon bags. Shown are results of surface antigen expression as indicated as mean ± SD from 4 independent experiments.

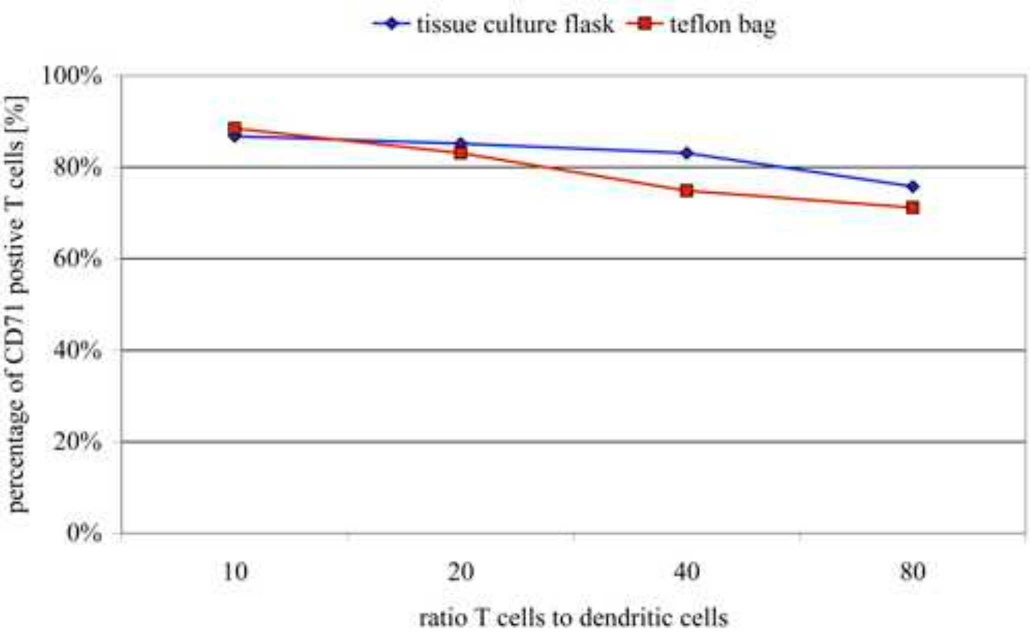


Figure 4.49: Activation of T cells (CD3) was assessed by CD71 (transferrin receptor) staining after 4 days of co-cultivation

the yield of matured DCs cultivated in tissue culture flasks and hydrophobic teflon bags was $66.3 \pm 13.6\%$ and $41.9 \pm 6.0\%$ respectively.

Nevertheless, the phenotypical analysis of both tissue culture flask and teflon bag DCs demonstrated no difference in surface marker expression. The stimulatory molecules HLA-A,B,C (MHC class I) and HLA-DR (MHC class II), the co-stimulatory surface proteins CD80 and CD86 and the maturation marker CD83 were highly up-regulated, which is shown in figure 4.48 on page 107. The surface protein CD1a was expressed in a medium level and CD14 was not found on matured dendritic cells.

In contrast, slight differences were found in an allostimulatory T cell response in a mixed leukocyte reaction. Both the analysis of CD25 and CD71 activation marker expression on T cells after 4 days of co-cultivation with DCs at different ratios (1:10, 1:20 and 1:40 respectively) indicated small differences in the stimulatory capacity of DCs cultivated in tissue culture flasks or teflon bags (figures 4.47 on page 106 and 4.49 on page 107). The number of positive T cells stimulated with DCs cultivated in tissue culture flasks were superior to the teflon bag DCs.

4.4.2 Dendritic Cells from Breast Cancer Patients

So far, in all described experiments of this thesis blood from healthy donors were used. Consequently, an important question has to be addressed: Can fully matured dendritic cells be obtained from breast cancer patients? For that reason, several experiments were performed utilizing patients' blood for the generation of monocyte-derived dendritic cells with the standardized protocol.

From six different patients who were under treatment in the Breast Cancer Unit at Guy's Hospital in London, UK, written informed consent was obtained after the nature and consequences of the study were fully explained. Blood donations (volume of $25mL$) were taken from each patient and directly processed. The history from each patient is summarized in table 4.16 on page 109. Furthermore, the collected number of PBMCs and the yield of matured dendritic cells are illustrated.

The number of matured dendritic cells generated from $25mL$ of blood from breast cancer patients was $3.5 \cdot 10^6 \pm 1.0 \cdot 10^6$ from 6 different donors, which correspond to a yield (as defined by size of cells, morphology and surface antigen expression) from PBMCs and monocytes of $7.6 \pm 1.8\%$ and $59.4 \pm 17.0\%$ respectively (compare table 4.6 on page 68, the yield of matured DCs from healthy donors was $66.3 \pm 13.6\%$). The apoptosis of monocytes or dendritic cells was

Table 4.16: The history from breast carcinoma patients who participated in the study for testing the quality and quantity of matured DCs generated with the standardized protocol

	LOG FILE [extracts]	Time of treatment [years]	Number of PBMCs [-]	Number of matured DCs [-]
Patient 1	Mastectomy Capecitabine[1] Docetaxel[1] Tamoxifen[2]	12	$4.0 \cdot 10^7$	$3.2 \cdot 10^6$
Patient 2	Radiotherapy / Biopsy Tamoxifen[2] Vaccinia MUC1[3] Docetaxel[1]	30	$4.0 \cdot 10^7$	$2.8 \cdot 10^6$
Patient 3	Radiotherapy Tamoxifen[2]	11	$5.0 \cdot 10^7$	$5.4 \cdot 10^6$
Patient 4	Primary Breast Cancer FEC[1,4]	0	$5.0 \cdot 10^7$	$3.7 \cdot 10^6$
Patient 5	Irridium Inplant Mastectomy Tamoxifen[2] Docetaxel[1]	18	$5.0 \cdot 10^7$	$3.4 \cdot 10^6$
Patient 6	Radiotherapy Biopsy Tamoxifen[2]	9	$3.9 \cdot 10^7$	$2.1 \cdot 10^6$
MEAN ± SD			$46.0 \pm 5.6 \cdot 10^6$	$3.5 \pm 1.0 \cdot 10^6$
Yield from				
PBMCs [%]				7.6 ± 1.8
Monocytes [%]				59.4 ± 17.0
Viability of DCs [%]				95.0 ± 3.0

[1]Chemotherapy, [2]Steroid endocrine therapy, [3]Immunotherapy, [4]S-Fluorouracil Epirubicin Cyclophosphamide

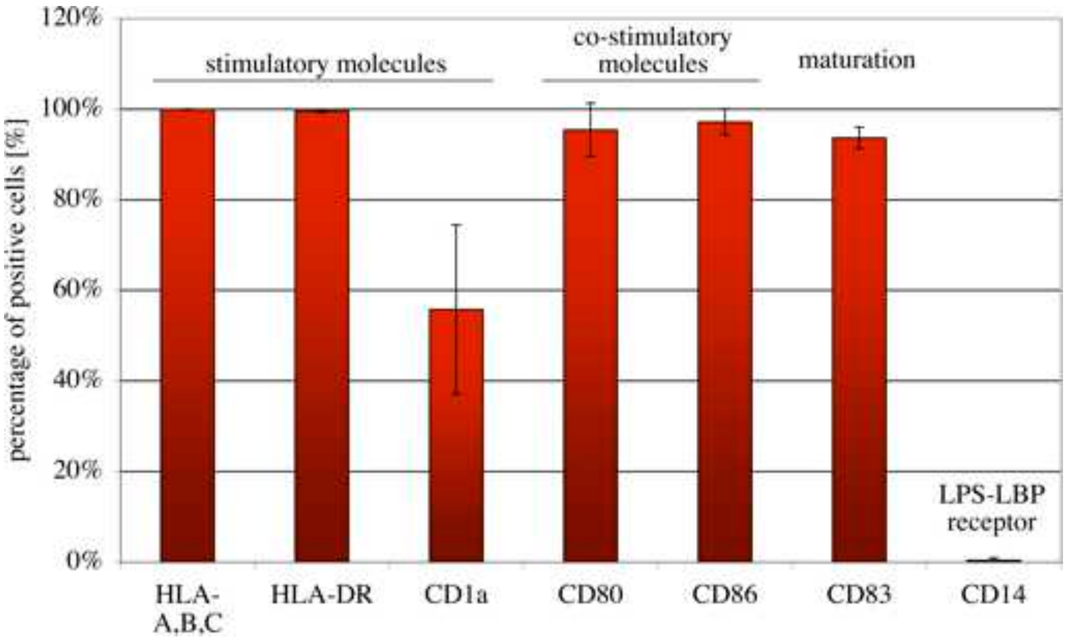


Figure 4.50: Phenotypical analysis of TNF-α and PGE_2 matured dendritic cells from breast cancer patients. Shown are results of surface antigen expression as indicated as mean ± SD from 6 independent experiments.

not determined during this experiment.

After harvesting the dendritic cell population on day 8, the phenotypical analysis showed no differences of DCs from breast cancer patients compared to the cells from healthy donors (see also figure 4.14 on page 68). As illustrated in figure 4.50, the stimulatory molecules HLA-A,B,C (MHC class I) and HLA-DR (MHC class II), the co-stimulatory surface proteins CD80 and CD86 and the maturation marker CD83 were highly up-regulated. The surface molecule CD1a was weakly expressed, while the receptor for LPS-LBP CD14 was not found on DCs.

Chapter 5

Discussion

5.1 The Generation of Dendritic Cells: Clinical Applicability

The aim of this study was to determine optimal conditions for the generation of monocyte-derived dendritic cells by developing a standardized, easy to use and reproducible protocol that yields in a sufficient number of DCs for immunotherapeutical vaccination trials. Within this study a non-feeding protocol in serum-free conditions was accomplished that is in accordance with GMP requirements and therefore applicable for clinical use. The results of the development of the optimized protocol are illustrated in table 5.1 on page 112.

Culture of immunomagnetic bead enriched monocytes using serum-free conditions for 6 days resulted in immature dendritic cells with low levels of CD83 expression. Final maturation was induced for additional 2 days by the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 supported by PGE_2 (Jonuleit et al., 1997) or TNF-α and PGE_2 (Kalinski et al., 1998), both of which resulted in the up-regulation of high levels of the stimulatory molecules HLA-A,B,C (MHC class I) and HLA-DR (MHC class II), the co-stimulatory molecules CD40, CD80 and CD86, the intercellular adhesion molecule ICAM-1, the maturation marker CD83 and the chemokine receptors CXCR4 and CCR7. CD1a, an MHC class I analogue molecule that presents lipids, showed medium levels of expression, whereby CD14 (receptor for the LPS-LBP complex) was not found on matured DCs.

Using this protocol a yield of $66.3 \pm 13.6\%$ of fully matured dendritic cells from inoculated monocytes (viable and apoptotic) from healthy donors with a viability of $93.4 \pm 5.9\%$ were

Table 5.1: The results of the development of the optimized protocol for the generation of monocyte-derived dendritic cells

Enrichment of monocytes	Immunomagnetic beads
Cell densitiy	$1.3 \cdot 10^6 \frac{1}{mL}$ monocytes
Cultivation system	$75cm^2$ tissue culture flasks, $30mL$ volume
Fully closed system	Hydrophobic teflon bag, up to $500mL$ volume
Medium	X-VIVO 15, serum-free
GM-CSF	$400\frac{U}{mL}$
IL-4	$2000\frac{U}{mL}$
Maturation stimulus	TNF-α ($1000\frac{U}{mL}$), PGE_2 ($18\frac{\mu g}{mL}$)
Lysate pulsing	Lysate ($100\frac{\mu g}{mL}$), TNF-α ($1000\frac{U}{mL}$), PGE_2 ($1\frac{\mu g}{mL}$)
Time (differentiation / maturation)	6 days / 2 days
In accordance with GMP requirements	YES

obtained. It was demonstrated that feeding of the cells is not necessary and that it is sufficient to add only $400\frac{U}{mL}$ GM-CSF and $2000\frac{U}{mL}$ IL-4 at the beginning of the cultivation. The comparison of the results described in this thesis with published data from various reports in literature is shown in table 5.2 on page 113. Nearly all published protocols used either feeding or serum and resulted in a lower yield.

Moreover, the data clearly demonstrate that under serum-free conditions the cytokine-cocktails consisting of either TNF-α ($1000\frac{U}{mL}$), IL-1β ($1000\frac{U}{mL}$), IL-6 ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$) or TNF-α ($1000\frac{U}{mL}$) and PGE_2 ($18\frac{\mu g}{mL}$), a simplified cocktail with just two components, are sufficient to induce the final maturation of immature DCs into mature, homogenous and immunostimulatory dendritic cells.

For the generation of dendritic cells under GMP requirements the usage of a fully closed system from the blood donation until the re-infusion of cells into the patient would facilitate the handling and decrease potential contamination sources. For that reason, dendritic cells were generated in teflon bags and compared with DCs cultivated in tissue culture flasks. The yield of DCs from teflon bags and tissue culture flasks was $41.9 \pm 6.0\%$ and $66.3 \pm 13.6\%$ respectively (yield of DCs from all viable monocytes without apoptosis analysis; for comparison

Table 5.2: Comparison of the results described in this thesis with data of the generation of DCs reported in various publications

Name	Year	Enrichment	Serum	Feeding	Yield*
Babatz et al.	2003	Magnetic beads	1%	YES	12.1%
Dietz et al.	2000	Magnetic beads	2%	YES	18.3 ± 6.3%
Felzmann et al.	2003	Magnetic beads	NO	YES	8.0 ± 3.0%
Meyer-Wentrup et al.	2003	Magnetic beads	1%	YES	41.0 ± 4.0%
Motta et al.	2003	Magnetic beads	1%	YES	20.7 ± 4.6%
Padley et al.	2001	Magnetic beads	1%	YES	30.3 ± 6.4%
Pullarkat et al.	2002	Magnetic beads	NO	NO	9.2 ± 5.2%
Ratta et al.	2000	Magnetic beads	10%	YES	4.8 ± 1.3%
Berger et al.	2002	Attachment	2%	YES	19.9 ± 9.6%
Goxe et al.	2000	Elutriation	2%	NO	48.3 %
Tissue culture flask	**Thesis**	**Magnetic beads**	**NO**	**NO**	**66.3 ± 13.6%**
Teflon bag	**Thesis**	**Magnetic beads**	**NO**	**NO**	**41.9 ± 6.0%**

*Yield: Yield of viable dendritic cells from inoculated monocytes

with the results reported in various publications; see table 5.2). At first sight, the decrease in yield of about 37% might be disappointing, which was caused by the induction of apoptosis. Nevertheless, it was proven that the quality of matured and lysate-pulsed DCs were nearly similar to tissue culture flask DCs, as analyzed by phenotype and mixed leukocyte reaction. By comparison, Pullarkat et al. (2002) generated dendritic cells in gas-permeable culture bags, which yielded in $9.2 \pm 5.2\%$ matured DCs. Thus, even in teflon bags, the generation of DCs using the protocol developed in this study is highly effective compared with published data.

For immunotherapeutical trials using dendritic cell based vaccines various amounts of matured dendritic cells have been used for different numbers of infusions into the patient. Nevertheless, the average total number of utilized dendritic cells was $3.2 \cdot 10^7$ that have been supplied in 3 up to 10 infusions. Therefore, the number of $5.8 \pm 1.2 \cdot 10^7$ (average amount of DCs generated from $500 mL$ of blood, see table 4.6 on page 68) of fully matured dendritic cells from healthy donors provide the required number for vaccination trials. In table 5.3 on page 115 recently

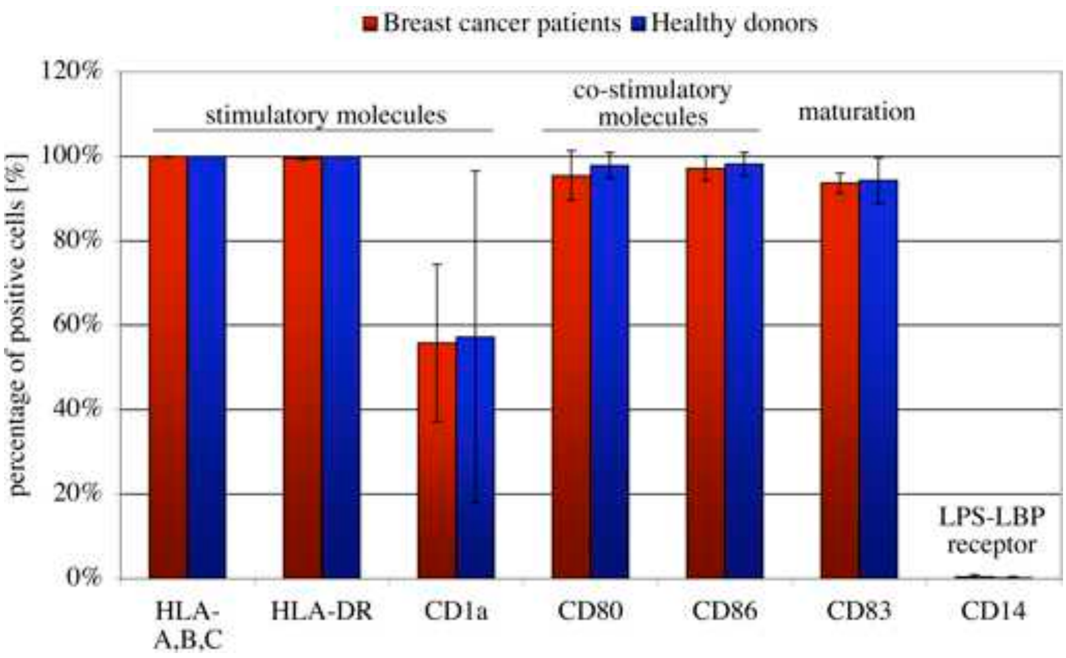


Figure 5.1: The phenotype of matured dendritic cells from either breast cancer patients' blood or blood from healthy donors. Shown are results from 6 (breast cancer patients) and 8 (healthy donors) different experiments and illustrated as mean ± standard deviation.

published data from several clinical trials using dendritic cell based vaccines are summarized and compared with the results of this thesis. Interestingly, the number of dendritic cells from breast cancer patients out of $25mL$ of blood was $3.5 \pm 1.0 \cdot 10^6$, which corresponds to a theoretical number of $7.0 \pm 2.0 \cdot 10^7$ from $500mL$ of blood. The higher number of fully matured DCs from patients' blood might be due to the usage of fresh blood compared with $12h$ to $18h$ old buffy coats in the case of healthy donors.

Additionally, the flowcytometrical analysis of matured dendritic cells from healthy donors and breast cancer patients demonstrated no difference in the level of expression of important surface molecules, such as the stimulatory molecules MHC class I (HLA-A,B,C), MHC class II (HLA-DR) and CD1a, the co-stimulatory molecules CD80 and CD86, the maturation marker CD83 and the receptor for the LPS-LBP complex CD14 (see figure 5.1). In sum, a high yield of fully matured monocyte-derived dendritic cells from patients' blood were obtained regardless the type of previous cancer therapies and the duration of treatment.

Stimulation of allogeneic naive T cells in a mixed leukocyte reaction led to high proliferation with either cytokine cocktail. Production of IFN-γ was significantly induced, while no effect on the production of IL-4 or IL-12p70 was observed. These findings are in agreement with

Table 5.3: Published clinical trials and the number of total dendritic cells used for vaccination

Name	Year	Antigen	Average No. of DCs	No. of vacc.*	Total No. of DCs
Brossart et al.	2000	Her-2 / neu MUC1	$5.9 \cdot 10^6$	5	$3.0 \cdot 10^7$
Chang et al.	2002	Lysate* / KLH*	$1.0 \cdot 10^7$	6	$6.0 \cdot 10^7$
Dhodapkar et al.	2000	FLU M1 KLH* / TT*	$4.0 \cdot 10^6$	3	$1.2 \cdot 10^7$
Geiger et al.	2001	Lysate* / KLH*	$1.0 \cdot 10^7$	3	$3.0 \cdot 10^7$
Gitlitz et al.	2003	Lysate*	$6.0 \cdot 10^6$	4	$2.4 \cdot 10^7$
Hoeltl et al.	2002	Lysate* / KLH* Cell line A-498	$1.0 \cdot 10^7$	6	$6.0 \cdot 10^7$
Nestle et al.	1998	Lysate* / KLH* Peptides / KLH*	$1.0 \cdot 10^6$	10	$1.0 \cdot 10^7$
Thurner et al.	1999a	Mage-3A1 peptide TT* / turberculin	$5.5 \cdot 10^6$	5	$2.8 \cdot 10^7$
Mean					$3.2 \cdot 10^7$
Thesis (Buffy coat, $500mL$)	2004	-	-	-	$5.8 \pm 1.2 \cdot 10^7$
Thesis (Patients, $25mL$)	2004	-	-	-	$3.5 \pm 1.0 \cdot 10^6$
Theoretically (Patients, $500mL$)	2004	-	-	-	$7.0 \pm 2.0 \cdot 10^7$

*Vacc.: vaccination; helper antigens: KLH: keyhole limpet hemocyanin; TT: tetanus toxoid; Lysate: autologous tumor cell lysate

Jonuleit et al. (1997), who demonstrated that the addition of PGE_2 to a cocktail of TNF-α, IL-1β and IL-6 led to higher IFN-γ production in an allogeneic primary stimulation. They also observed, that neither $CD4^+$ nor $CD8^+$ T cells produced IL-4 or IL-10, indicating that these dendritic cells could not support the development of Th2 cells. Moreover, three studies showed recently that PGE_2 regulates the migratory capacity of monocyte-derived dendritic cells (Luft et al., 2002; Scandella et al., 2002, 2004) and that these migratory-type dendritic cells produce lower levels of cytokines (including IL-12p70) and induce IFN-γ production of T cells in mixed leukocyte reactions (see also chapter 5.3). These conclusions were confirmed by several clinical investigations using monocyte-derived dendritic cells matured with the cytokine-cocktail utilized in this study (TNF-α, IL-1β, IL-6, PGE_2), which induced potent T cell immune responses in patients undergoing immunotherapy (Schuler-Thurner et al., 2002; Dhodapkar et al., 2001). In contrast, Kalinski et al. (1998) reported that DCs matured in the additional presence of PGE_2 bias naive T-helper cell development towards Th2 cells. However, this may be caused by different cultivation parameters including FCS in the cultivation setup.

In conclusion, it has been shown that this simplified, easy to use protocol, which circumvents the necessity of feeding and the usage of serum, results in a sufficient number of dendritic cells for immunotherapeutical vaccine trials. Furthermore, this protocol can be used in a fully closed system, which results in a decreased number of DCs but facilitates the handling and reduces the risk of contaminations. Moreover, this procedure, developed with blood from healthy donors, is usable with breast cancer patients' blood without compromises regarding yield or quality of fully matured dendritic cells.

5.2 Apoptosis of Monocytes

Currently, monocyte-derived dendritic cells are becoming widely used as cell vaccines for cancer treatment (Gabrilovich 2002; Schuler et al., 2003). The requirement for optimized and standardized protocols for the generation of dendritic cells in a large-scale has led to several protocols (see chapter 2.4). The present study was designed to determine why the yield of monocyte-derived dendritic cells is lower than 70%. Additionally, it focusses on the fate of monocytes during differentiation to matured dendritic cells.

Buffy coats from healthy donors were used, processed by immunomagnetic-bead selection to obtain highly enriched $CD14^+$ cells, which were analyzed for apoptosis. In agreement with previous observations, monocytes were partly apoptotic after enrichment. Fahy et al. (1999) reported $25 \pm 1.1\%$ ($n = 3$) of annexin V positive cells after enrichment by clumping. In the present study an average of $37.8 \pm 11.1\%$ ($n = 8$) of the monocyte population was found to be positive for annexin V. The higher percentage might be caused by the quality or age of the utilized buffy coats ($12h$ to $18h$) or the method of enrichment of the cells. However, no difference after the immunomagnetic-bead selection compared with unselected cells was seen (data not shown).

These data were based on the flow cytometric detection of PS expression by discriminating intact cells (annexin V^-/PI^-), early apoptotic cells (annexin V^+/PI^-) and necrotic cells (annexin V^+/PI^+), which has been shown to be a reliable method (Vermes et al., 1995; Lecoeur et al., 1997). Under viable conditions the molecular architecture of biological membranes is highly asymmetric (Bevers et al., 1989). Typically, phospatidylserine is almost completely absent in the outer leaflet of the plasma membrane. During programmed cell death various biological alterations take place including phospatidylserine redistribution on the outer leaflet (Vermes et al., 2000). Van Engeland et al. (1998) reported that at the early stage of apoptosis membrane integrity has not been compromised and no nuclear alterations can be observed but PS exposure is detectable.

To further characterize the fate of cells during differentiation to matured dendritic cells the non-attached population was analyzed. After $24h$ more than 80% of the suspension cells (53% of initial apoptotic cell number) were annexin V positive, which correlated with most of the viable cells becoming attached to the surface of the tissue culture flask. In contrast, after 6 days almost all immature dendritic cells, now in suspension, were viable and represented an average yield of $91.2 \pm 26.8\%$ ($n = 8$) of inoculated, non-apoptotic monocytes. These results indicate that nearly all viable, non-apoptotic monocytes differentiated to immature dendritic cells. The yield of matured dendritic cells was $90.2 \pm 16.6\%$ ($n = 8$) after 8 days (see figure 5.2). Cells, still attached on day 6, became suspension cells during maturation, resulting in a higher total cell number (viable and apoptotic cells).

An overall cell loss of 40% of highly purified monocytes during the first $24h$ has been observed when differentiating these cells to macrophages (Andreesen et al., 1983, Brugger et al., 1991, Lund et al., 2001). Lund et al. (2001) speculated that monocytes undergo programmed cell death as a combined effect of in vivo aging and in vitro stress condi-

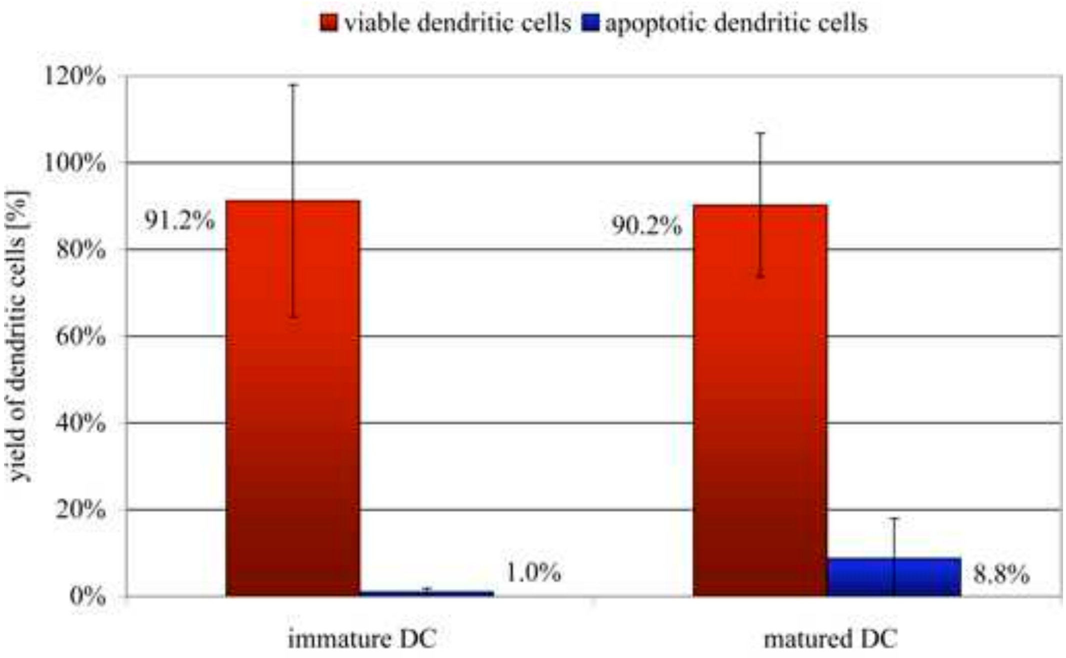


Figure 5.2: Yield of immature and TNF-α and PGE_2 matured dendritic cells. Results are from 8 different donors and presented as mean ± standard deviation. Shown are viable (AV^- / PI^-) and apoptotic (AV^+ / PI^-) cells. From (Bohnenkamp et al., submitted)

tions. The first $6h$ seemed to be critical for the choice between death and survival, with only the survivors developing into macrophages (Lund et al., 2001). Furthermore PS exposure, which is one of the 'eat me' signals at the surface of apoptotic cells, results in phagocytosis (Reutelingsperger et al., 2002). In agreement with the results of this thesis, Lund et al. (2002) demonstrated that only a few signs of cell remnants are found. The results of this study also showed that GM-CSF and IL-4 protected monocytes from further apoptosis. Fahy et al. (1999) concluded that monocytes undergo spontaneous programmed cell death in the absence of inflammatory stimulation.

Maturation of monocyte-derived dendritic cells generated by the previously described protocol (Bohnenkamp and Noll, 2003) was monitored by up-regulation of stimulatory and co-stimulatory molecules as well as the CD83 maturation marker (Zhou and Tedder, 1995). The CD83 expression in all donors was higher than 85% ($n = 8$). Analysis of the stimulatory capacity in an allogeneic T cell response (Nguyen et al., 2003) and in the MHC class I restricted autologous stimulation demonstrated the immunological functionality of the generated matured dendritic cells.

In conclusion, it was shown that apoptosis plays an important role in the generation of monocyte-derived dendritic cells. For calculation of the yield of any cell population only viable, non-apoptotic cells should be evaluated. It was confirmed that more than 90% of viable monocytes differentiated to fully matured dendritic cells. Thus, the starting population of monocytes determines the yield of DCs, which is consistently 90% of viable monocytes. Further investigation could focus on the effect of dendritic cell generation in the absence of apoptotic monocytes. Additionally, the analysis of apoptosis of dendritic cells used in clinical vaccination approaches should be generally considered because cells, presenting PS on their surface, would be eliminated and less effective.

5.3 Induction of MUC1 Specific T Lymphocytes

The goal of this study was to evaluate a potential breast carcinoma cell vaccine for controlling and enhancing tumor associated antigen presentation, which is a critical regulatory element for the induction of a cellular immune response (GUNZER AND GRABBE, 2001). A reproducible preparation method for tumor cell lysates by sonication at low temperatures was developed to avoid protein alteration by proteases or phosphatases ensuring that the tumor associated proteins that are loaded onto dendritic cells for processing were unaffected by the preparation method. For induction of maturation, DCs were pulsed with $100\frac{\mu g}{mL}$ of protein combined with the adjuvants TNF-α and PGE_2. These PGE_2 matured DCs migrated towards the chemokines CXCL12 and CCL19, which is critical for induction of T cells in lymph nodes. Moreover, it has been shown that lysate-pulsed DCs can be cryopreserved without affecting stimulatory properties. The exogenous proteins were effectively processed by DCs and presented in the context of MHC class I and MHC class II, which was evidenced by cross-priming of $CD8^+$ cytotoxic T cells and a strong $CD4^+$ Th1 response. The activation of MUC1 specific cytotoxic T cells was demonstrated by tetramer analysis with the epitopes M1.2 and F7. The results of this study are presented in table 5.4 on page 120.

The up-regulation of the chemokine receptor CCR7 on dendritic cells is crucial for their homing to secondary lymphoid organs, e.g. lymph nodes where the CCR7 ligand CCL19 is expressed. During infections, high levels of TNF-α, IL-1β, GM-CSF and PGE_2 are secreted by monocytes, macrophages and endothelial cells (LUFT ET AL., 2002). These pro-inflammatory mediators directly act on immature dendritic cells being present in inflamed tissues, resulting in

Table 5.4: The results of the study investigating the induction of MUC1 specific T lymphocytes primed by breast carcinoma cell lysate-loaded dendritic cells.

Chapter	Results
4.3.1	A reproducible and simple lysate preparation method was developed, which avoids proteolytic reactions to solubilize proteins without degradation
4.3.2	Immature DCs efficiently endocytoze dextran, LPS and microspheres
4.3.3	Fully matured DCs were obtained after maturation with $100\frac{\mu g}{mL}$ of protein combined with the adjuvants TNF-α and PGE_2 Dendritic cells demonstrated a high allostimulatory capacity in MLR Yield of lysate-pulsed DCs: $75.6 \pm 12.3\%$ ($n = 3$) and $91.8 \pm 8.2\%$ ($n = 6$) (MDA-MB-231 and MCF-7 lysate-pulsed DCs respectively)
4.3.4	TNF-α and PGE_2 matured DCs up-regulated CXCR4 and CCR7 Matured DCs showed high migration towards CXCL12 and CCL19
4.3.5	Lysate-loaded DCs can be cryopreserved in autologous plasma + 10% DMSO Yield: $85.3 \pm 9.4\%$, Viability: $89.3 \pm 6.0\%$, ($n = 6$)
4.3.6	Matured DCs effectively induce MHC class I restricted T cell responses Expansion factors: 4369-fold (FLU M1), 276-fold (Melan-A / Mart-1) Induction of IFN-γ producing Th1 cells: $51{,}100\frac{pg}{mL}$ (FLU M1), $17{,}232\frac{pg}{mL}$ (Melan-A / Mart-1) Induction of IL-5 producing Th2 cells: $585\frac{pg}{mL}$ (FLU M1), $1{,}516\frac{pg}{mL}$ (Melan-A / Mart-1)
4.3.7	Lysate-pulsed DCs cross-prime autologous T cells analyzed by MUC1 epitopes Expansion factors (MDA-MB-231): 7.5-fold (F7), 12.0-fold (M1.2) Expansion factors (MCF-7): 8.1-fold (F7), 18.9-fold (M1.2) Induction of IFN-γ producing Th1 cells: $15{,}740\frac{pg}{mL}$ (MDA-MB-231), $10{,}737\frac{pg}{mL}$ (MCF-7) Induction of IL-5 producing Th2 cells: $3{,}221\frac{pg}{mL}$ (MDA-MB-231), $1{,}705\frac{pg}{mL}$ (MCF-7)

the expression of CCR7. In several studies it has been reported that PGE_2 is crucial for the up-regulation of CCR7 and the migration towards its ligands (Luft et al., 2002; Scandella et al., 2002, 2004). Furthermore Scandella and co-workers (2004) demonstrated that CXCR4 mediates migration of monocyte-derived dendritic cells to CXCL12 is dependent on the presence of PGE_2. Neither LPS nor CD40 ligand induces migratory dendritic cells (Luft et al., 2002; Scandella et al., 2002). Additionally, immature DCs expressing CXCR4 do not migrate substantially to the CXCR4 binding pro-inflammatory chemokine CXCL12 (Luft et al., 2002). Thus, the presence of PGE_2 for the maturation seems to be essential to induce migratory dendritic cells.

Exogenous antigens are internalized by different pathways: dendritic cells endocytose antigen by receptor mediated phagocytosis and receptor-independent macropinocytosis (Guermonprez et al., 2002). Wether lysate-pulsed dendritic cells efficiently cross-present antigens via the MHC class I pathway is a matter of discussion. However, it has been shown using antigens bound to latex beads that they can be processed by both phagocytosis and macropinocytosis and then cross-presented on dendritic cells (Shen et al, 1997; Reis e Sousa and Germain, 1995). Recently, it was suggested that cross-presentation is controlled by the existence of phagosomal compartments, generated through fusion of the endoplasmatic reticulum with endolysosomal vesicles (Lizée et al., 2003). DC uptake of soluble antigen is only via macropinocytosis (Watts, 1997). Comparative studies using apoptotic cells or necrotic cell-lysates as a source of whole tumor cell antigens, have demonstrated no difference in the efficiency of either method to prime an anti-tumor immune response (Lambert et al., 2001; Kotera et al., 2001). Here, it was validated that tumor associated MUC1 was effectively processed and cross-presented by mature dendritic cells by their ability to generate MUC1 specific T cells. The expansion of M1.2 specific T cells was higher compared with T cells activated by the F7 epitope, which was probably caused by the phenomenon of dominance between different epitopes (Kedl et al., 2003). Thus, the rather simple method of lysate preparation in contrast to apoptotic cell generation seems to be the technique of choice to deliver tumor associated antigens to the MHC class I and MHC class II pathway. More importantly, the obtainment of viable tumor cells from primary breast carcinomas could prove to be difficult.

Tumor cell lysate is generally prepared by repetitive freeze and thaw cycles. However, the amount of protein, the equivalent ratio of tumor cells to dendritic cells and the maturation inducing adjuvant, which promotes the distinct phenotype, are currently under extensive investigation. In this study it was confirmed that $100\frac{\mu g}{mL}$ of tumor cell lysate, which is equivalent to a ratio of one tumor cell to one DC, with the adjuvants TNF-α ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$) administered for two days resulted in a fully mature phenotype with effective migratory, allostimulatory, MHC class I and MHC class II stimulatory capacity. However, the maturation adjuvants direct DCs to a non-cytokine producing type, as no IL-12p70, IL-4 or IL-10 was detected after two days of activation. Furthermore, no IL-12p70 was detected during stimulation of T cells. Phenotypically identical DCs may differ in their ability to produce cytokines to induce either Th1 or Th2 cells, depending on the inflammatory cytokine environment that induces their maturation (Hilkens et al., 1997; Kalinski et al., 1998). The presence of PGE_2 during the maturation development of DCs led to the generation of IL-12p70 deficient cells (Kalinski et al., 1997). After triggering of DCs by bacterial stimuli, IL-12p70 is produced only transiently, during a narrow time window of about $10h$ to $18h$ and is refractory to further stimulation (Langenkamp et al., 2000). Therefore, for efficient IL-12p70 production dendritic cells have to be vaccinated at a time point when they become mature cells, in other words these dendritic cells would be not fully mature with regard to up-regulation of surface molecules like co-stimulatory signals and the maturation marker CD83.

In contrast, Schnurr et al. (2001) showed that DCs pulsed with $120\frac{\mu g}{mL}$ protein (equivalent to 1.3 tumor cells to DCs) and matured with TNF-α ($1000\frac{U}{mL}$) and PGE_2 ($1\frac{\mu g}{mL}$) produced IL-12 during stimulation of T cells. It was not clear whether the monomeric IL-12p40 or the bioreactive IL-12p70 was analyzed. Although it was reported that PGE_2 induces the inactive IL-12p40 (Rieser et al., 1997), it is not accompanied by the production of the bioreactive heterodimer IL-12p70 (Kalinski et al., 1998). However, several studies have reported the induction of anti-tumor immunity by DCs loaded with tumor lysate at various concentrations in the absence of IL-12p70. Bachleitner-Hofmann et al. (2002) used $100\frac{\mu g}{mL}$ and TNF-α for $12h$, Herr et al. (2000) incubated lysate and the cytokines TNF-α, IL-1β, IL-6 and PGE_2 for 2 days, Kass et al. (2003) pulsed matured DCs (TNF-α, IL-1β and PGE_2) with lysate and the cationic lipid DOTAP for protein uptake, Thumann et al. (2003) pulsed DCs with up to $1\frac{mg}{mL}$ ovalbumin and the cytokines TNF-α, IL-1β, IL-6 and PGE_2 for 2 days and Wen et al. (2002) triggered DCs with $100\frac{\mu g}{mL}$ protein for 6 days. In contrast, Vegh and Mazumber (2003) utilized protein (equivalent to one tumor cell to one DC), LPS and IFN-γ and was able to induce IL-12p70 during the first $24h$ following maturation of DCs.

The selective differentiation of either subset of T helper cells is established during priming and depends on various factors, namely the cytokine environment, the dose of the antigen, strength of antigenic stimulation, duration of the T cell receptor engagement and the nature and quantity of co-stimulatory molecules (Constant and Bottomly, 1997; Kapsenberg, 2003; Langenkamp et al., 2000; O'Garra and Arai, 2000; Sallusto and Lanzavecchia, 2002). The T cell stimulation and Th1 and Th2 polarization require three dendritic cell derived signals. The first signal is the antigen-specific signal, the second is given by co-stimulatory molecules and the third, the polarizing signal, is mediated by soluble or membrane-bound factors (Kapsenberg, 2003). The breast carcinoma lysate-pulsed dendritic cells generated did not deliver a third signal by soluble mediators such as the analyzed cytokines. As a consequence, the polarization of the strong Th1 cell response may be mediated by the dose of antigen presented on the surface of the DCs and the quantity of co-stimulatory signals. In mouse experiments it was demonstrated that high doses of soluble antigen favored a Th1 cell response (reviewed in Constant and Bottomly, 1997). However, as it was demonstrated by induction of MHC class I restricted T cell responses with the FLU M1 and Melan-A / Mart-1 peptide, the kind of antigen and response (memory or naive) determines the polarization.

In the current study several important parameters were tested to evaluate the effective pulsing with breast carcinoma cell lysate of dendritic cells. The migratory and immunostimulatory capacity of matured dendritic cells were demonstrated and confirmed that lysate loaded DCs are able to effectively cross-prime $CD8^+$ cytotoxic T cells. The cytokine profile and the polarization of T helper cells suggest that a high antigen dose and quantity of co-stimulatory molecules on DCs is able to induce a strong Th1 T cell response, even in the absence of a polarizing third signal.

5.4 Final Observation

Dendritic cells were first discovered in 1973 by Ralph Steinman and are now known for over 30 years. The concept that DCs are the initiators of primary immune responses were proven and it has become evident that these unique cells have a dual role as controllers of both immunity and tolerance.

Currently, many in vitro studies and in vivo clinical trials have reported promising results, which includes monitored T cell responses and tumor regressions in some cancer patients.

Nevertheless, as long as the tumor cell escape from immune surveillance and the active role of dendritic cells in tolerance is not fully understood, effective immunotherapeutical vaccine strategies need still to be developed.

One crucial factor for getting new insights into the immunobiological function of the body's defense system may be a standardized administration of dendritic cell based cancer vaccines, which is followed by close immunomonitoring of patients. Perhaps this work may contribute to the further understanding of the concerted interplay of immunity and tolerance.

Chapter 6

Summary

Monocyte-derived dendritic cells (DCs) are currently under extensive evaluation as cell vaccines for cancer treatment. The requirement for large-scale cell products demands optimized and standardized protocols. This study demonstrates that highly viable DCs ($93.4 \pm 5.9\%$) can be produced from $CD14^+$ monocytes enriched via immunomagnetic beads in a high yield ($66.3 \pm 13.6\%$) and purity ($95.4 \pm 2.1\%$) with X-VIVO 15, $400\frac{U}{mL}$ GM-CSF and $2000\frac{U}{mL}$ IL-4 without serum and feeding. For the maturation of DCs different cytokine combinations (TNF-α, IL-1β, IL-6, PGE_2 and TNF-α, PGE_2) were compared. In both cases cells expressed typical surface molecules of mature DCs and induced high proliferative responses in mixed leukocyte reactions which led to IFN-γ producing T lymphocytes. Comparison of dendritic cells from blood from healthy donors with breast cancer patients' blood demonstrated no differences regarding yield or quality of fully matured dendritic cells.

Furthermore, this study aimed to investigate if the yield is determined by the properties of the starting population of inoculated monocytes. $CD14^+$ cells were enriched by immunomagnetic-bead selection and analyzed for apoptosis by an annexin V / propidiumiodide assay. It was demonstrated that $37.8{\pm}11.1\%$ ($n = 8$) of freshly isolated monocytes from buffy coats of healthy donors underwent programmed cell death. Further analysis of the fate of apoptotic cells during differentiation suggested phagocytosis. The yield of viable matured dendritic cells from non-apoptotic monocytes was calculated to be $90.2{\pm}16.7\%$. These results indicate that the yield of dendritic cells is mainly influenced by the percentage of apoptotic cells in the inoculum, and impact on DC generation for clinical application.

For cancer immunotherapy the loading of DCs with whole tumor cell lysate preparations represents a simple and promising approach to utilize all potential known and unknown tumor-associated antigens (TAAs) and to circumvent the disadvantages like HLA-mismatching and the requirements for known TAAs. For this reason it is important whether lysate-pulsed DCs efficiently cross-prime cytotoxic T cells (CTLs) and induce a strong Th1 cell response. Additionally, this was compared to FLU M1 and Melan-A / Mart-1 peptide pulsed DCs. As a model system breast carcinoma cell lysate from either MCF-7 or MDA-MB-231 cells expressing the TAA MUC1 were chosen. The epithelial mucin MUC1 is a large molecular weight *O*-glycosylated protein, which is overexpressed in the majority of breast, ovarian and other epithelial malignancies and represents a promising target in cancer immunotherapy. A simple lysate preparation method was developed to solubilize all cell proteins without degradation. For loading of monocyte-derived dendritic cells, $100\frac{\mu g}{mL}$ of breast carcinoma cell lysate was used, accompanied by an adjuvant consisting of tumor necrosis factor-α and Prostaglandin-E_2. T cells were co-cultivated with lysate or peptide pulsed DCs and were restimulated weekly. Before cultivation, and after the 3^{rd} stimulation, tetramer frequencies for the MUC1 epitopes F7 and M1.2 as well as for the FLU M1 and Melan-A / Mart-1 epitopes were determined. After stimulation with lysate, higher frequencies for M1.2 specific T cells were observed compared with the F7 epitope. Furthermore, the expansion factor for M1.2 specific T cells that had been stimulated with MCF-7 lysate-pulsed DCs were found to be up to 19-fold. The analysis of typical Th1 / Th2 cytokines (IFN-γ, TNF-α, IL-12p70, IL-2, IL-4, IL-5, IL-10) revealed a strong Th1 response: up to $50{,}000\frac{pg}{mL}$ IFN-γ were determined in the supernatants of the stimulations with either lysate or peptide pulsed DCs. These results provide evidence for a strong Th1 polarization and cross-priming of MUC1 specific CTLs and demonstrate the feasibility of using lysate-pulsed dendritic cells in breast cancer immunotherapy.

Chapter 7

Outlook

The development of the optimized protocol for the reproducible and serum-free generation of fully mature dendritic cells provides the number of cells necessary for immunotherapeutical vaccine strategies either cultivated in tissue culture flasks or hydrophobic teflon bags. Nevertheless, it would be useful to investigate the influence of different materials on the yield and apoptosis induction to further improve the differentiation in a fully closed bag system.

The investigation of apoptosis of monocytes and their fate during differentiation gave new insight in the process of the generation of dendritic cells. Further analysis of the influence of cytokines with apoptosis inhibitor effects like IL-7 would be interesting.

A more detailed immunobiological analysis of dendritic cells is pivotal for understanding of both the stimulatory and regulatory functions of lysate-pulsed dendritic cells. Therefore, following in vitro experiments seem to have impact on the interplay of dendritic cells with the immune system:

- A fully autologous system with T cells, dendritic cells and tumor cells from breast cancer patients needs to be carried out with extensive analysis of the used tumor cells. For expressed known tumor-associated antigens matching tetramers need to be utilized.

- For the tetramer analysis a broad spectrum of antigens would be useful, e.g. epitopes from PLU-1, MUC1 and Her-2 / neu.

- Tetramer analysis of MHC class II specific T cells would give new insights in the activation of T-helper cells by lysate-pulsed dendritic cells.

- A cytotox assay against primary tumor cells and tumor cell lines should be used to proove the cytotoxic functionality of activated T cells.

- A more detailed phenotypical analysis of stimulated T cells: Distinction between naive, memory effector and regulatory T cells by using CD45RA, CD45RO and CCR7 expression levels and specific cytokine staining for IFN-γ, IL-4, IL-10 and TGF-β.

- Investigation of Th1 type maturation stimuli, which induce the cytokine IL-12p70, on the influence to result in migratory type dendritic cells.

Analysis of the glycoprotein MUC1 on the surface of mature dendritic cells demonstrated expression of a normal, non breast cancer associated form of the protein (personal observation). However, it would be useful to evaluate the influence on stimulation of T cells by non-aberrantly glycosilated protein in comparison with the breast cancer related form. Additionally, it would be of great interest to investigate whether aberrantly glycosilated MUC1 can be taken up efficiently by dendritic cells.

Appendix A

Mathematical Calculations

Expansion Factor

$$\text{Expansion factor} = \frac{\text{Cell number day}_x}{\text{Cell number day}_0} \quad (A.1)$$

with:

day_x: day of sample

day_0: day of inoculum

Growth Rate

The growth rate was determined using the following term:

$$\mu = \frac{ln(c_2) - ln(c_1)}{\triangle t} \quad (A.2)$$

with:

μ: Growth rate $[\frac{1}{h}]$

c_1: Number of cells on day 0 or day of last sample

c_2: Number of cells of sample 2

t_1: Time of sample 1

t_2: Time of sample 2

Generation Time

The generation time was calculated using the following formula:

$$t = \frac{ln2}{\mu} \quad (A.3)$$

with:

t: Generation time $[h]$

μ: Growth rate $[\frac{1}{h}]$

Appendix B

Patient Information Sheet

Imperial Cancer Research Fund
Academic Oncology Unit

Guy's Hospital
3rd Floor Thomas Guy House
London SE1 9RT
Telephone: 0207-955-4542
Fax: 0207-955-2027

Centre: Guy's Hospital
Patient Identification Number for this research:

PATIENT INFORMATION SHEET

Title of Project: **Investigation of methods of fusing malignant cells with dendritic cells**

Pleural Fluid

Name of Researcher: **Dr D W Miles**

What is the purpose of donating this fluid sample?
You are being invited to participate in a research project which involves a sample of the pleural fluid which is being drained from your chest being taken to the laboratory rather than discarded so that it can then be analysed in the Imperial Cancer Research Laboratory at Guy's Hospital. The scientists will process the fluid to remove any malignant cells that are present They will be then attempt to fuse the malignant cells from the fluid sample with dendritic cells. Dendritic cells are immune cells that play a vital role in assisting antigens (cells which cause an immune response) to stimulate the immune system. It is hope that in the future an immunotherapy treatment may be developed using this approach.

What does the test involve?
If you consent to this test the fluid that is drained from your chest will be transferred into sterile bottles and will then be taken to the ICRF lab where it will be processed and analysed.
It is possible that we may also ask you for a blood sample, either 50mls (approx 4 tablespoons), on a maximum of two occasions or 20mls (approx 1.5 tablespoons) on a maximum of five occasions. The blood will be drawn from a vein in your hand or arm. The blood will then be taken to the ICRF laboratory where it will be processed and any dendritic cells present will be used to fuse with the cells obtained from the fluid removed from your chest (pleural fluid).

What are the possible benefits?
You will not receive any personal benefit from having this test.

Will my test result be kept confidential?
The results of these tests will remain strictly confidential

Obtaining further information
If you have any questions about this test please contact Dr D. W. Miles on 020-7955-4542.

Basis of participation
Your participation in this research is entirely voluntary. You can withdraw your consent at any stage and without giving a reason and this will not affect your current or future treatment.

I (name) ______________________________

of (address) ______________________________

____

hereby consent to take part in the above investigation, the nature and purpose of which have been explained to me. Any questions I wished to ask have been answered to my satisfaction. I understand that I may withdraw from the investigation at any stage without necessarily giving a reason for doing so and that this will in no way affect the care I receive as a patient.

SIGNED (Volunteer) ____________________ Date ____________

(Doctor) ____________________ Date ____________

(Witness, where appropriate) ____________________ Date ____________

ICRF/BCBG/Version3/12.09.2001

[illegible]

Bibliography

Acuto, O. and Michel, F. (2003) CD28-mediated co-stimulation: a quantitative support for TCR signalling. Nat Rev Immunol 3, 939-51.

Alberts, B., Bray, D., Johnson, A., Lewis, L., Raff, M., Roberts, K. and Walter, P. (1999) Lehrbuch der molekularen Zellbiologie. Wiley-VCH, Weinheim.

Alexander, J., Sidney, J., Southwood, S., Ruppert, J., Oseroff, C., Maewal, A., Snoke, K., Serra, H.M., Kubo, R.T., Sette, A. and et al. (1994) Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. Immunity 1, 751-61.

Andree, H.A., Reutelingsperger, C.P., Hauptmann, R., Hemker, H.C., Hermens, W.T. and Willems, G.M. (1990) Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. J Biol Chem 265, 4923-8.

Andree, H.A., Willems, G.M., Hauptmann, R., Maurer-Fogy, I., Stuart, M.C., Hermens, W.T., Frederik, P.M. and Reutelingsperger, C.P. (1993) Aggregation of phospholipid vesicles by a chimeric protein with the N-terminus of annexin I and the core of annexin V. Biochemistry 32, 4634-40.

Andreesen, R., Picht, J. and Lohr, G.W. (1983) Primary cultures of human blood-born macrophages grown on hydrophobic teflon membranes. J Immunol Methods 56, 295-304.

Araki, H., Katayama, N., Mitani, H., Suzuki, H., Nishikawa, H., Masuya, M., Ikuta, Y., Hoshino, N., Miyashita, H., Nishii, K., Minami, N. and Shiku, H. (2001) Efficient ex vivo generation of dendritic cells from CD14+ blood monocytes in the presence of human serum albumin for use in clinical vaccine trials. Br J Haematol 114, 681-9.

Babatz, J., Rollig, C., Oelschlagel, U., Zhao, S., Ehninger, G., Schmitz, M. and Bornhauser, M. (2003) Large-scale immunomagnetic selection of CD14+ monocytes to generate dendritic cells for cancer immunotherapy: a phase I study. J Hematother Stem Cell Res 12, 515-23.

Bachleitner-Hofmann, T., Stift, A., Friedl, J., Pfragner, R., Radelbauer, K., Dubsky, P., Schuller, G., Benko, T., Niederle, B., Brostjan, C., Jakesz, R. and Gnant, M. (2002) Stimulation of autologous antitumor T-cell responses against medullary thyroid carcinoma using tumor lysate-pulsed dendritic cells. J Clin Endocrinol Metab 87, 1098-104.

Banchereau, J. and Steinman, R.M. (1998) Dendritic cells and the control of immunity. Nature 392, 245-52.

Bell, D., Young, J.W. and Banchereau, J. (1999) Dendritic cells. Adv Immunol 72, 255-324.

Bender, A., Sapp, M., Schuler, G., Steinman, R.M. and Bhardwaj, N. (1996) Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. J Immunol Methods 196, 121-35.

Berger, C.L., Xu, A.L., Hanlon, D., Lee, C., Schechner, J., Glusac, E., Christensen, I., Snyder, E., Holloway, V., Tigelaar, R. and Edelson, R.L. (2001) Induction of human tumor-loaded dendritic cells. Int J Cancer 91, 438-47.

Berger, T.G., Feuerstein, B., Strasser, E., Hirsch, U., Schreiner, D., Schuler, G. and Schuler-Thurner, B. (2002) Large-scale generation of mature monocyte-derived dendritic cells for clinical application in cell factories. J Immunol Methods 268, 131-40.

Bernard, J., Ittelet, D., Christoph, A., Potron, G., Adjizian, J.C., Kochman, S. and Lopez, M. (1998) Adherent-free generation of functional dendritic cells from purified blood monocytes in view of potential clinical use. Hematol Cell Ther 40, 17-26.

Bevers, E.M., Tilly, R.H., Senden, J.M., Comfurius, P. and Zwaal, R.F. (1989) Exposure of endogenous phosphatidylserine at the outer surface of stimulated platelets is reversed by restoration of aminophospholipid translocase activity. Biochemistry 28, 2382-7.

Blockzjil, A., Nilsson, K. and Nilsson, O. (1998) Epitope characterization of MUC1 antibodies. Tumour Biol 19 Suppl 1, 46-56.

Bohnenkamp, H.R., Burchell, J., Taylor-Papadimitriou, J. and Noll, T. Apoptosis of monocytes and the influence on yield of monocyte-derived dendritic cells. Submitted.

Bohnenkamp, H.R., Coleman, J., Burchell, J., Taylor-Papadimitriou, J. and Noll, T. Breast carcinoma cell lysate-pulsed dendritic cells cross-prime MUC1 specific cytotoxic T cells identified by peptide-MHC-class-I tetramers. In preparation.

Bohnenkamp, H.R., Hilbert, U. and Noll, T. (2002) Bioprocess development for the cultivation of human T-lymphocytes in a clinical scale. Cytotechnology 38, 135-145.

Bohnenkamp, H.R. and Noll, T. (2003) Process development for standardized generation of mature monocyte-derived dendritic cells suitable for clinical application. Cytotechnology 42, 121-31.

Bremers, A.J., Kuppen, P.J. and Parmiani, G. (2000a) Tumour immunotherapy: the adjuvant treatment of the 21st century? Eur J Surg Oncol 26, 418-24.

Bremers, A.J. and Parmiani, G. (2000b) Immunology and immunotherapy of human cancer: present concepts and clinical developments. Crit Rev Oncol Hematol 34, 1-25.

Brossart, P. and Bevan, M.J. (1997) Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. Blood 90, 1594-9.

Brossart, P., Heinrich, K.S., Stuhler, G., Behnke, L., Reichardt, V.L., Stevanovic, S., Muhm, A., Rammensee, H.G., Kanz, L. and Brugger, W. (1999) Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. Blood 93, 4309-17.

Brossart, P., Wirths, S., Stuhler, G., Reichardt, V.L., Kanz, L. and Brugger, W. (2000) Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. Blood 96, 3102-8.

Browning, M. and Krausa, P. (1996) Genetic diversity of HLA-A2: evolutionary and functional significance. Immunol Today 17, 165-70.

Brugger, W., Kreutz, M. and Andreesen, R. (1991) Macrophage colony-stimulating factor is required for human monocyte survival and acts as a cofactor for their terminal differentiation to macrophages in vitro. J Leukoc Biol 49, 483-8.

Bryant, P. and Ploegh, H. (2004) Class II MHC peptide loading by the professionals. Curr Opin Immunol 16, 96-102.

Buchler, T., Hajek, R., Bourkova, L., Kovarova, L., Musilova, R., Bulikova, A., Doubek, M., Svobodnik, A., Mareschova, I., Vanova, P., Tuzova, E., Vidlakova, P., Vorlicek, J. and Penka, M. (2003) Generation of antigen-loaded dendritic cells in a serum-free medium using different cytokine combinations. Vaccine 21, 877-82.

Burchell, J., Durbin, H. and Taylor-Papadimitriou, J. (1983) Complexity of expression of antigenic determinants, recognized by monoclonal antibodies HMFG-1 and HMFG-2, in normal and malignant human mammary epithelial cells. J Immunol 131, 508-13.

Burchell, J., Gendler, S., Taylor-Papadimitriou, J., Girling, A., Lewis, A., Millis, R. and Lamport, D. (1987) Development and characterization of breast cancer reactive monoclonal antibodies directed to the core protein of the human milk mucin. Cancer Res 47, 5476-82.

Burnet, F.M. (1970) The concept of immunological surveillance. Prog Exp Tumor Res 13, 1-27.

Cailleau, R., Young, R., Olive, M. and Reeves, W.J., Jr. (1974) Breast tumor cell lines from pleural effusions. J Natl Cancer Inst 53, 661-74.

Calabi, F. and Bradbury, A. (1991) The CD1 system. Tissue Antigens 37, 1-9.

Calabi, F. and Milstein, C. (2000) The molecular biology of CD1. Semin Immunol 12, 503-9.

Carreno, B.M. and Collins, M. (2002) The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu Rev Immunol 20, 29-53.

Caux, C., Dezutter-Dambuyant, C., Schmitt, D. and Banchereau, J. (1992) GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. Nature 360, 258-61.

Chang, J.F., Zhao, H.L., Phillips, J. and Greenburg, G. (2000) The epithelial mucin, MUC1, is expressed on resting T lymphocytes and can function as a negative regulator of T cell activation. Cell Immunol 201, 83-8.

Chang, A.E., Redman, B.G., Whitfield, J.R., Nickoloff, B.J., Braun, T.M., Lee, P.P., Geiger, J.D. and Mule, J.J. (2002) A phase I trial of tumor lysate-pulsed dendritic cells in the treatment of advanced cancer. Clin Cancer Res 8, 1021-32.

Constant, S.L. and Bottomly, K. (1997) Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. Annu Rev Immunol 15, 297-322.

Cornelissen, M., Philippe, J., De Sitter, S. and De Ridder, L. (2002) Annexin V expression in apoptotic peripheral blood lymphocytes: an electron microscopic evaluation. Apoptosis 7, 41-7.

Correa, I., Plunkett, T., Vlad, A., Mungul, A., Candelora-Kettel, J., Burchell, J.M., Taylor-Papadimitriou, J. and Finn, O.J. (2003) Form and pattern of MUC1 expression on T cells activated in vivo or in vitro suggests a function in T-cell migration. Immunology 108, 32-41.

Costello, R.T., Gastaut, J.A. and Olive, D. (1999) Tumor escape from immune surveillance. Arch

Immunol Ther Exp (Warsz) 47, 83-8.

Coulie, P.G. and van der Bruggen, P. (2003) T-cell responses of vaccinated cancer patients. Curr Opin Immunol 15, 131-7.

Craston, R., Koh, M., Mc Dermott, A., Ray, N., Prentice, H.G. and Lowdell, M.W. (1997) Temporal dynamics of CD69 expression on lymphoid cells. J Immunol Methods 209, 37-45.

Deutsches Rotes Kreuz (2004) Wissenswertes ueber Blut. www.drk.de.

Dhodapkar, M.V. and Bhardwaj, N. (2000) Active immunization of humans with dendritic cells. J Clin Immunol 20, 167-74.

Dhodapkar, M.V., Steinman, R.M., Krasovsky, J., Munz, C. and Bhardwaj, N. (2001) Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med 193, 233-8.

Dietz, A.B., Bulur, P.A., Erickson, M.R., Wettstein, P.J., Litzow, M.R., Wyatt, W.A., Dewald, G.W., Tefferi, A., Pankratz, V.S. and Vuk-Pavlovic, S. (2000) Optimizing preparation of normal dendritic cells and bcr-abl+ mature dendritic cells derived from immunomagnetically purified CD14+ cells. J Hematother Stem Cell Res 9, 95-101.

Duperrier, K., Eljaafari, A., Dezutter-Dambuyant, C., Bardin, C., Jacquet, C., Yoneda, K., Schmitt, D., Gebuhrer, L. and Rigal, D. (2000) Distinct subsets of dendritic cells resembling dermal DCs can be generated in vitro from monocytes, in the presence of different serum supplements. J Immunol Methods 238, 119-31.

Dzionek, A., Piechaczek, C., Campbell, J., Zysk, M., Winkels, G., Huppert, V. and Schmitz, J. (2002) Clinical-scale magnetic sorting and multiparameter analysis of dendritic cells aubsets and precursers. In: 7th international symposium on dendritic cells, Bamberg.

Encyclopedia Britannica (2004) Ultimative Reference Suite. Merriam-Webster, London.

Fahy, R.J., Doseff, A.I. and Wewers, M.D. (1999) Spontaneous human monocyte apoptosis utilizes a caspase-3-dependent pathway that is blocked by endotoxin and is independent of caspase-1. J Immunol 163, 1755-62.

Falk, K., Rotzschke, O., Stevanovic, S., Gnau, V., Sparbier, K., Jung, G., Rammensee, H.G. and Walden, P. (1994) Analysis of a naturally occurring HLA class I-restricted viral epitope. Immunology 82, 337-42.

Felzmann, T., Witt, V., Wimmer, D., Ressmann, G., Wagner, D., Paul, P., Huttner, K. and Fritsch, G. (2003) Monocyte enrichment from leukapharesis products for the generation of DCs by plastic adherence, or by positive or negative selection. Cytotherapy 5, 391-8.

Feuerstein, B., Berger, T.G., Maczek, C., Roder, C., Schreiner, D., Hirsch, U., Haendle, I., Leisgang, W., Glaser, A., Kuss, O., Diepgen, T.L., Schuler, G. and Schuler-Thurner, B. (2000) A method for the production of cryopreserved aliquots of antigen-preloaded, mature dendritic cells ready for clinical use. J Immunol Methods 245, 15-29.

Fong, L., Hou, Y., Rivas, A., Benike, C., Yuen, A., Fisher, G.A., Davis, M.M. and Engleman, E.G. (2001) Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. Proc Natl Acad Sci U S A 98, 8809-14.

Gabrilovich, D.I. (2002) Dendritic cell vaccines for cancer treatment. Curr Opin Mol Ther 4, 452-8.

Galli-Stampino, L., Meinjohanns, E., Frische, K., Meldal, M., Jensen, T., Werdelin, O. and Mouritsen, S. (1997) T-cell recognition of tumor-associated carbohydrates: the nature of the glycan moiety plays a decisive role in determining glycopeptide immunogenicity. Cancer Res 57, 3214-22.

Geiger, J.D., Hutchinson, R.J., Hohenkirk, L.F., McKenna, E.A., Yanik, G.A., Levine, J.E., Chang, A.E., Braun, T.M. and Mule, J.J. (2001) Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression. Cancer Res 61, 8513-9.

Gendler, S., Taylor-Papadimitriou, J., Duhig, T., Rothbard, J. and Burchell, J. (1988) A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. J Biol Chem 263, 12820-3.

Gendler, S.J., Lancaster, C.A., Taylor-Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Lalani, E.N. and Wilson, D. (1990) Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. J Biol Chem 265, 15286-93.

Girling, A., Bartkova, J., Burchell, J., Gendler, S., Gillett, C. and Taylor-Papadimitriou, J. (1989) A core protein epitope of the polymorphic epithelial mucin detected by the monoclonal antibody SM-3 is selectively exposed in a range of primary carcinomas. Int J Cancer 43, 1072-6.

Gitlitz, B.J., Belldegrun, A.S., Zisman, A., Chao, D.H., Pantuck, A.J., Hinkel, A., Mulders, P., Moldawer, N., Tso, C.L. and Figlin, R.A. (2003) A pilot trial of tumor lysate-loaded dendritic cells for the treatment of metastatic renal cell carcinoma. J Immunother 26, 412-9.

Gong, J., Chen, D., Kashiwaba, M. and Kufe, D. (1997) Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. Nat Med 3, 558-61.

Gong, J., Koido, S., Chen, D., Tanaka, Y., Huang, L., Avigan, D., Anderson, K., Ohno, T. and Kufe, D. (2002) Immunization against murine multiple myeloma with fusions of dendritic and plasmacytoma cells is potentiated by interleukin 12. Blood 99, 2512-7.

Gotch, F., Rothbard, J., Howland, K., Townsend, A. and McMichael, A. (1987) Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. Nature 326, 881-2.

Goxe, B., Latour, N., Bartholeyns, J., Romet-Lemonne, J.L. and Chokri, M. (1998) Monocyte-derived dendritic cells: development of a cellular processor for clinical applications. Res Immunol 149, 643-6.

Goxe, B., Latour, N., Chokri, M., Abastado, J.P. and Salcedo, M. (2000) Simplified method to generate large quantities of dendritic cells suitable for clinical applications. Immunol Invest 29, 319-36.

Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C. and Amigorena, S. (2002) Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol 20, 621-67.

Gunzer, M. and Grabbe, S. (2001) Dendritic cells in cancer immunotherapy. Crit Rev Immunol 21, 133-45.

Guyre, C.A., Fisher, J.L., Waugh, M.G., Wallace, P.K., Tretter, C.G., Ernstoff, M.S. and Barth, R.J., Jr. (2002) Advantages of hydrophobic culture bags over flasks for the generation of monocyte-derived dendritic cells for clinical applications. J Immunol Methods 262, 85-94.

Hart, D.N. (1997) Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 90, 3245-87.

Hauptmann, R., Maurer-Fogy, I., Krystek, E., Bodo, G., Andree, H. and Reutelingsperger, C.P. (1989) Vascular anticoagulant beta: a novel human Ca2+/phospholipid binding protein that inhibits coagulation and phospholipase A2 activity. Its molecular cloning, expression and comparison with VAC-alpha. Eur J Biochem 185, 63-71.

Herr, W., Ranieri, E., Olson, W., Zarour, H., Gesualdo, L. and Storkus, W.J. (2000) Mature dendritic cells pulsed with freeze-thaw cell lysates define an effective in vitro vaccine designed to elicit EBV-specific CD4(+) and CD8(+) T lymphocyte responses. Blood 96, 1857-64.

Heukamp, L.C., van der Burg, S.H., Drijfhout, J.W., Melief, C.J., Taylor-Papadimitriou, J. and Offringa, R. (2001) Identification of three non-VNTR MUC1-derived HLA-A*0201-restricted T-cell epitopes that induce protective anti-tumor immunity in HLA-A2/K(b)-transgenic mice. Int J Cancer 91, 385-92.

Hickling, J.K. (1998) Measuring human T-lymphocyte function. Expert Rev Mol Med 1998, 1-20.

Hilkens, C.M., Kalinski, P., de Boer, M. and Kapsenberg, M.L. (1997) Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. Blood 90, 1920-6.

Holtl, L., Rieser, C., Papesh, C., Ramoner, R., Herold, M., Klocker, H., Radmayr, C., Stenzl, A., Bartsch, G. and Thurnher, M. (1999) Cellular and humoral immune responses in patients with metastatic renal cell carcinoma after vaccination with antigen pulsed dendritic cells. J Urol 161, 777-82.

Imanishi, T., Akaz, T., Kimura, A., Tokunaga, K. and Gojobori, T. (1991) Allele and haplotype frequencies for HLA and Complement loci in various ethnic groups. In HLA 1991, Vol. 1. Tsuji, K., Aizawa, M. and Sasazuki, T., eds. Oxford University Press, 1065.

Janeway, C.A., Travers, P., Walport, M. and Shlomchik, M. (2001) Immunobiology - The immune system in health and disease. Garland Publishing, New York.

Jenne, L., Arrighi, J.F., Jonuleit, H., Saurat, J.H. and Hauser, C. (2000) Dendritic cells containing apoptotic melanoma cells prime human CD8+ T cells for efficient tumor cell lysis. Cancer Res 60, 4446-52.

Jonuleit, H., Kuhn, U., Muller, G., Steinbrink, K., Paragnik, L., Schmitt, E., Knop, J. and Enk, A.H. (1997) Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur J Immunol 27, 3135-42.

Kalinski, P., Hilkens, C.M., Snijders, A., Snijdewint, F.G. and Kapsenberg, M.L. (1997) IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. J Immunol 159, 28-35.

Kalinski, P., Schuitemaker, J.H., Hilkens, C.M. and Kapsenberg, M.L. (1998) Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. J Immunol 161, 2804-9.

Kapsenberg, M.L. (2003) Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol 3, 984-93.

Kass, R., Bellone, S., Palmieri, M., Cane, S., Bignotti, E., Henry-Tillman, R., Hutchins, L., Cannon, M.J., Klimberg, S. and Santin, A.D. (2003) Restoration of tumor-specific HLA class I restricted cytotoxicity in tumor infiltrating lymphocytes of advanced breast cancer patients by in vitro stimulation with tumor antigen-pulsed autologous dendritic cells. Breast Cancer Res Treat 80, 275-85.

Kawakami, Y., Eliyahu, S., Sakaguchi, K., Robbins, P.F., Rivoltini, L., Yannelli, J.R., Appella, E. and Rosenberg, S.A. (1994) Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J Exp Med 180, 347-52.

Kedl, R.M., Kappler, J.W. and Marrack, P. (2003) Epitope dominance, competition and T cell affinity maturation. Curr Opin Immunol 15, 120-7.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26, 239-57.

Kikuchi, T., Akasaki, Y., Irie, M., Homma, S., Abe, T. and Ohno, T. (2001) Results of a phase I clinical trial of vaccination of glioma patients with fusions of dendritic and glioma cells. Cancer Immunol Immunother 50, 337-44.

Kobayashi, T., Shinohara, H., Toyoda, M., Iwamoto, S. and Tanigawa, N. (2001) Regression of lymph node metastases by immunotherapy using autologous breast tumor-lysate pulsed dendritic cells: report of a case. Surg Today 31, 513-6.

Koch, N. and Stockinger, B. (1991) Molecules that modify antigen recognition. Curr Opin Immunol 3, 10-5.

Koch, N., van Driel, I.R. and Gleeson, P.A. (2000) Hijacking a chaperone: manipulation of the MHC class II presentation pathway. Immunol Today 21, 546-50.

Kondo, M., Wagers, A.J., Manz, M.G., Prohaska, S.S., Scherer, D.C., Beilhack, G.F., Shizuru, J.A. and Weissman, I.L. (2003) Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol 21, 759-806.

Kotera, Y., Shimizu, K. and Mule, J.J. (2001) Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. Cancer Res 61, 8105-9.

Kugler, A., Stuhler, G., Walden, P., Zoller, G., Zobywalski, A., Brossart, P., Trefzer, U., Ullrich, S., Muller, C.A., Becker, V., Gross, A.J., Hemmerlein, B., Kanz, L., Muller, G.A. and Ringert, R.H. (2000) Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat Med 6, 332-6.

Kugler, A., Stuhler, G., Walden, P., Zoller, G., Zobywalski, A., Brossart, P., Trefzer, U., Ullrich, S., Muller, C.A., Becker, V., Gross, A.J., Hemmerlein, B., Kanz, L., Muller, G.A. and Ringert, R.H. (2003) Retraction: Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat Med 9, 1221.

Labeur, M.S., Roters, B., Pers, B., Mehling, A., Luger, T.A., Schwarz, T. and Grabbe, S. (1999) Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. J Immunol 162, 168-75.

Lambert, L.A., Gibson, G.R., Maloney, M. and Barth Jr, R.J. (2001) Equipotent Generation of Protective Antitumor Immunity by Various Methods of Dendritic Cell Loading With Whole Cell Tumor Antigens. J Immunother 24, 232-236.

Lancaster, C.A., Peat, N., Duhig, T., Wilson, D., Taylor-Papadimitriou, J. and Gendler, S.J. (1990) Structure and expression of the human polymorphic epithelial mucin gene: an expressed VNTR unit. Biochem Biophys Res Commun 173, 1019-29.

Langenkamp, A., Messi, M., Lanzavecchia, A. and Sallusto, F. (2000) Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol 1, 311-6.

Langerhans, P. (1868) Ueber die Nerven der menschlichen Haut. Virchows Arch 44, 325.

Lecoeur, H., Ledru, E., Prevost, M.C. and Gougeon, M.L. (1997) Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V and 7-AAD cytofluorometric staining methods. J Immunol Methods 209, 111-23.

Lizee, G., Basha, G., Tiong, J., Julien, J.P., Tian, M., Biron, K.E. and Jefferies, W.A. (2003) Control of dendritic cell cross-presentation by the major histocompatibility complex class I cytoplasmic domain. Nat Immunol 4, 1065-73.

Luft, T., Jefford, M., Luetjens, P., Toy, T., Hochrein, H., Masterman, K.A., Maliszewski, C., Shortman, K., Cebon, J. and Maraskovsky, E. (2002) Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. Blood 100, 1362-72.

Lund, P.K., Westvik, A.B., Joo, G.B., Ovstebo, R., Haug, K.B. and Kierulf, P. (2001) Flow cytometric evaluation of apoptosis, necrosis and recovery when culturing monocytes. J Immunol Methods 252, 45-55.

Lund, P.K., Namork, E., Brorson, S.H., Westvik, A.B., Joo, G.B., Ovstebo, R. and Kierulf, P. (2002) The fate of monocytes during 24 h of culture as revealed by flow cytometry and electron microscopy. J Immunol Methods 270, 63-76.

Luster, A.D. (2002) The role of chemokines in linking innate and adaptive immunity. Curr Opin Immunol 14, 129-35.

Mackensen, A., Herbst, B., Chen, J.L., Kohler, G., Noppen, C., Herr, W., Spagnoli, G.C., Cerundolo, V. and Lindemann, A. (2000) Phase I study in melanoma patients of a vaccine with peptide-pulsed dendritic cells generated in vitro from CD34(+) hematopoietic progenitor cells. Int J Cancer 86, 385-92.

Mangan, D.F., Welch, G.R. and Wahl, S.M. (1991) Lipopolysaccharide, tumor necrosis factor-alpha, and IL-1 beta prevent programmed cell death (apoptosis) in human peripheral blood monocytes. J Immunol 146, 1541-6.

Manz, R., Assenmacher, M., Pfluger, E., Miltenyi, S. and Radbruch, A. (1995) Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix. Proc Natl Acad Sci U S A 92, 1921-5.

McGuirk, P. and Mills, K.H. (2002) Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. Trends Immunol 23, 450-5.

McIlroy, D. and Gregoire, M. (2003) Optimizing dendritic cell-based anticancer immunotherapy: maturation state does have clinical impact. Cancer Immunol Immunother 52, 583-91.

Meidenbauer, N., Marienhagen, J., Laumer, M., Vogl, S., Heymann, J., Andreesen, R. and Mackensen, A. (2003) Survival and tumor localization of adoptively transferred Melan-A-specific T cells in melanoma patients. J Immunol 170, 2161-9.

Meyer-Wentrup, F. and Burdach, S. (2003) Efficacy of dendritic cell generation for clinical use: recovery and purity of monocytes and mature dendritic cells after immunomagnetic sorting or adherence selection of CD14+ starting populations. J Hematother Stem Cell Res 12, 289-99.

Michal, G. (1999) Biochemical Pathways. Spektrum Academisher Verlag, Heidelberg.

Moldenhauer, A., Nociari, M.M., Dias, S., Lalezari, P. and Moore, M.A. (2003) Optimized culture conditions for the generation of dendritic cells from peripheral blood monocytes. Vox Sang 84, 228-36.

Morse, M.A., Vredenburgh, J.J. and Lyerly, H.K. (1999) A comparative study of the generation of dendritic cells from mobilized peripheral blood progenitor cells of patients undergoing high-dose chemotherapy. J Hematother Stem Cell Res 8, 577-84.

Motta, M.R., Castellani, S., Rizzi, S., Curti, A., Gubinelli, F., Fogli, M., Ferri, E., Cellini, C., Baccarani, M. and Lemoli, R.M. (2003) Generation of dendritic cells from CD14+ monocytes positively selected by immunomagnetic adsorption for multiple myeloma patients enrolled in a clinical trial of anti-idiotype vaccination. Br J Haematol 121, 240-50.

Nestle, F.O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G. and Schadendorf, D. (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 4, 328-32.

Nguyen, X.D., Eichler, H., Sucker, A., Hofmann, U., Schadendorf, D. and Kluter, H. (2002) Collection of autologous monocytes for dendritic cell vaccination therapy in metastatic melanoma patients. Transfusion 42, 428-32.

Nguyen, X.D., Eichler, H., Dugrillon, A., Piechaczek, C., Braun, M. and Kluter, H. (2003) Flow cytometric analysis of T cell proliferation in a mixed lymphocyte reaction with dendritic cells. J Immunol Methods 275, 57-68.

Noto, H., Takahashi, T., Makiguchi, Y., Hayashi, T., Hinoda, Y. and Imai, K. (1997) Cytotoxic T lymphocytes derived from bone marrow mononuclear cells of multiple myeloma patients recognize an underglycosylated form of MUC1 mucin. Int Immunol 9, 791-8.

O'Garra, A. and Arai, N. (2000) The molecular basis of T helper 1 and T helper 2 cell differentiation. Trends Cell Biol 10, 542-50.

Ochsenbein, A.F., Klenerman, P., Karrer, U., Ludewig, B., Pericin, M., Hengartner, H. and Zinkernagel, R.M. (1999) Immune surveillance against a solid tumor fails because of immunological ignorance. Proc Natl Acad Sci U S A 96, 2233-8.

Padley, D.J., Dietz, A.B., Gastineau, D.A. and Vuk-Pavlovic, S. (2001) Mature myeloid dendritic cells for clinical use prepared from CD14+ cells isolated by immunomagnetic adsorption. J Hematother Stem Cell Res 10, 427-9. Pardoll, D. (2003) Does the immune system see tumors as foreign or self? Annu Rev Immunol 21, 807-39.

Parmiani, G., Castelli, C., Dalerba, P., Mortarini, R., Rivoltini, L., Marincola, F.M. and Anichini, A. (2002) Cancer immunotherapy with peptide-based vaccines: what have we achieved? Where are we going? J Natl Cancer Inst 94, 805-18.

Patel, S.D., Papoutsakis, E.T., Winter, J.N. and Miller, W.M. (2000) The lactate issue revisited: novel feeding protocols to examine inhibition of cell proliferation and glucose metabolism in hematopoietic cell cultures. Biotechnol Prog 16, 885-92.

Pickl, W.F., Majdic, O., Kohl, P., Stockl, J., Riedl, E., Scheinecker, C., Bello-Fernandez, C. and Knapp, W. (1996) Molecular and functional characteristics of dendritic cells generated from highly purified CD14+ peripheral blood monocytes. J Immunol 157, 3850-9.

Pogue, R.R., Eron, J., Frelinger, J.A. and Matsui, M. (1995) Amino-terminal alteration of the HLA-A*0201-restricted human immunodeficiency virus pol peptide increases complex stability and in vitro immunogenicity. Proc Natl Acad Sci U S A 92, 8166-70.

Pospisilova, D., Borovickova, J., Polouckova, A., Spisek, R., Sediva, A., Hrusak, O., Stary, J. and Bartunkova, J. (2002) Generation of functional dendritic cells for potential use in the treatment of acute lymphoblastic leukemia. Cancer Immunol Immunother 51, 72-8.

Psychyrembel (1998) Klinisches Woerterbuch. de Gruyter, Berlin.

Pullarkat, V., Lau, R., Lee, S.M., Bender, J.G. and Weber, J.S. (2002) Large-scale monocyte enrichment coupled with a closed culture system for the generation of human dendritic cells. J Immunol Methods 267, 173-83.

Ratta, M., Curti, A., Fogli, M., Pantucci, M., Viscomi, G., Tazzari, P., Fagnoni, F., Vescovini, R., Sansoni, P., Tura, S. and Lemoli, R.M. (2000) Efficient presentation of tumor idiotype to autologous T cells by CD83(+) dendritic cells derived from highly purified circulating CD14(+) monocytes in multiple myeloma patients. Exp Hematol 28, 931-40.

Reis e Sousa, C. and Germain, R.N. (1995) Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. J Exp Med 182, 841-51.

Reutelingsperger, C.P., Dumont, E., Thimister, P.W., van Genderen, H., Kenis, H., van de Eijnde, S., Heidendal, G. and Hofstra, L. (2002) Visualization of cell death in vivo with the annexin A5 imaging protocol. J Immunol Methods 265, 123-32.

Rieser, C., Bock, G., Klocker, H., Bartsch, G. and Thurnher, M. (1997) Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production. J Exp Med 186, 1603-8.

Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P.O., Steinman, R.M. and Schuler, G. (1994) Proliferating dendritic cell progenitors in human blood. J Exp Med 180, 83-93.

Romani, N., Reider, D., Heuer, M., Ebner, S., Kampgen, E., Eibl, B., Niederwieser, D. and Schuler, G. (1996) Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. J Immunol Methods 196, 137-51.

Romero, P., Gervois, N., Schneider, J., Escobar, P., Valmori, D., Pannetier, C., Steinle, A., Wolfel, T., Lienard, D., Brichard, V., van Pel, A., Jotereau, F. and Cerottini, J.C. (1997) Cytolytic T lymphocyte recognition of the immunodominant HLA-A*0201-restricted Melan-A/MART-1 antigenic peptide in melanoma. J Immunol 159, 2366-74.

Rutella, S., Rumi, C., Lucia, M.B., Barberi, T., Puggioni, P.L., Lai, M., Romano, A., Cauda, R. and Leone, G. (1999) Induction of CD69 antigen on normal CD4+ and CD8+ lymphocyte subsets and its relationship with the phenotype of responding T-cells. Cytometry 38, 95-101.

Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 179, 1109-18.

Sallusto, F. and Lanzavecchia, A. (1999) Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. J Exp Med 189, 611-4.

Sallusto, F. and Lanzavecchia, A. (2002) The instructive role of dendritic cells on T-cell responses. Arthritis Res 4 Suppl 3, S127-32.

Scandella, E., Men, Y., Gillessen, S., Forster, R. and Groettrup, M. (2002) Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. Blood 100, 1354-61.

Scandella, E., Men, Y., Legler, D.F., Gillessen, S., Prikler, L., Ludewig, B. and Groettrup, M. (2004) CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. Blood 103, 1595-601.

Scheffold, A., Lohning, M., Richter, A., Assenmacher, M., Manz, R., Austrup, F., Hamann, A. and Radbruch, A. (1998) Analysis and sorting of T cells according to cytokine expression. Eur Cytokine Netw 9, 5-11.

Schnurr, M., Galambos, P., Scholz, C., Then, F., Dauer, M., Endres, S. and Eigler, A. (2001) Tumor cell lysate-pulsed human dendritic cells induce a T-cell response against pancreatic carcinoma cells: an in vitro model for the assessment of tumor vaccines. Cancer Res 61, 6445-50.

Scholl, S., Squiban, P., Bizouarne, N., Baudin, M., Acres, B., Von Mensdorff-Pouilly, S., Shearer, M., Beuzeboc, P., Van Belle, S., Uzielly, B., Pouillart, P., Taylor-Papadimitriou, J. and Miles, D. (2003) Metastatic Breast Tumour Regression Following Treatment by a Gene-Modified Vaccinia Virus Expressing MUC1 and IL-2. J Biomed Biotechnol 2003, 194-201.

Schuler, G., Schuler-Thurner, B. and Steinman, R.M. (2003) The use of dendritic cells in cancer immunotherapy. Curr Opin Immunol 15, 138-47.

Schuler-Thurner, B., Schultz, E.S., Berger, T.G., Weinlich, G., Ebner, S., Woerl, P., Bender, A., Feuerstein, B., Fritsch, P.O., Romani, N. and Schuler, G. (2002) Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. J Exp Med 195, 1279-88.

Shen, Z., Reznikoff, G., Dranoff, G. and Rock, K.L. (1997) Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. J Immunol 158, 2723-30.

Shimizu, M. and Yamauchi, K. (1982) Isolation and characterization of mucin-like glycoprotein in human milk fat globule membrane. J Biochem (Tokyo) 91, 515-24.

Sorg, R.V., Ozcan, Z., Brefort, T., Fischer, J., Ackermann, R., Muller, M. and Wernet, P. (2003) Clinical-scale generation of dendritic cells in a closed system. J Immunother 26, 374-83.

Soule, H.D., Vazguez, J., Long, A., Albert, S. and Brennan, M. (1973) A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51, 1409-16.

Sparwasser, T., Koch, E.S., Vabulas, R.M., Heeg, K., Lipford, G.B., Ellwart, J.W. and Wagner, H. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol 28, 2045-54.

Spisek, R., Bretaudeau, L., Barbieux, I., Meflah, K. and Gregoire, M. (2001) Standardized generation of fully mature p70 IL-12 secreting monocyte-derived dendritic cells for clinical use. Cancer Immunol

Immunother 50, 417-27.

Steinman, R.M. and Cohn, Z.A. (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 137, 1142-62.

Syme, R.M., Duggan, P., Stewart, D. and Gluck, S. (2001) Generation of dendritic cells ex vivo: differences in steady state versus mobilized blood from patients with breast cancer, with lymphoma, and from normal donors. J Hematother Stem Cell Res 10, 621-30.

Taylor-Papadimitriou, J., Burchell, J.M., Plunkett, T., Graham, R., Correa, I., Miles, D. and Smith, M. (2002) MUC1 and the immunobiology of cancer. J Mammary Gland Biol Neoplasia 7, 209-21.

Tazbirkova, A., Okai, M., Horley, D.C., Crough, T.M., Maksoud, A., Nieda, M. and Nicol, A.J. (2003) Effects of leukapheresis protocol, cell processing and cryopreservation on the generation of monocyte-derived DC for immune therapy. Cytotherapy 5, 31-9.

Thiel, A., Scheffold, A. and Radbruch, A. (1998) Immunomagnetic cell sorting–pushing the limits. Immunotechnology 4, 89-96.

Thumann, P., Moc, I., Humrich, J., Berger, T.G., Schultz, E.S., Schuler, G. and Jenne, L. (2003) Antigen loading of dendritic cells with whole tumor cell preparations. J Immunol Methods 277, 1-16.

Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P., Brocker, E.B., Steinman, R.M., Enk, A., Kampgen, E. and Schuler, G. (1999a) Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med 190, 1669-78.

Thurner, B., Roder, C., Dieckmann, D., Heuer, M., Kruse, M., Glaser, A., Keikavoussi, P., Kampgen, E., Bender, A. and Schuler, G. (1999b) Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. J Immunol Methods 223, 1-15.

Tuyaerts, S., Noppe, S.M., Corthals, J., Breckpot, K., Heirman, C., De Greef, C., Van Riet, I. and Thielemans, K. (2002) Generation of large numbers of dendritic cells in a closed system using Cell Factories. J Immunol Methods 264, 135-51.

Valmori, D., Fonteneau, J.F., Lizana, C.M., Gervois, N., Lienard, D., Rimoldi, D., Jongeneel, V., Jotereau, F., Cerottini, J.C. and Romero, P. (1998) Enhanced generation of specific tumor-reactive CTL in vitro by selected Melan-A/MART-1 immunodominant peptide analogues. J Immunol 160, 1750-8.

van Engeland, M., Nieland, L.J., Ramaekers, F.C., Schutte, B. and Reutelingsperger, C.P. (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 31, 1-9.

Vasselon, T., Hailman, E., Thieringer, R. and Detmers, P.A. (1999) Internalization of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14. J Exp Med 190, 509-21.

Vegh, Z. and Mazumder, A. (2003) Generation of tumor cell lysate-loaded dendritic cells preprogrammed for IL-12 production and augmented T cell response. Cancer Immunol Immunother 52, 67-79.

Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184, 39-51.

Vermes, I., Haanen, C. and Reutelingsperger, C. (2000) Flow cytometry of apoptotic cell death. J Immunol Methods 243, 167-90.

Walsh, D., Luckie, S.M., Cummings, M.C., Antalis, T.M. and McGuckin, A. (2000) Heterogeneity of MUC1 expression by human breast carcinoma cell lines in vivo and in vitro. Breast Cancer Research and Treatment 58, 255-266.

Watts, C. (1997) Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunol 15, 821-50.

Weiner, G.J., Liu, H.M., Wooldridge, J.E., Dahle, C.E. and Krieg, A.M. (1997) Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. Proc Natl Acad Sci U S A 94, 10833-7.

Wen, Y.J., Min, R., Tricot, G., Barlogie, B. and Yi, Q. (2002) Tumor lysate-specific cytotoxic T lymphocytes in multiple myeloma: promising effector cells for immunotherapy. Blood 99, 3280-5.

Wiechelman, K.J., Braun, R.D. and Fitzpatrick, J.D. (1988) Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. Anal Biochem 175, 231-7.

Wong, E.C., Maher, V.E., Hines, K., Lee, J., Carter, C.S., Goletz, T., Kopp, W., Mackall, C.L., Berzofsky, J. and Read, E.J. (2001) Development of a clinical-scale method for generation of dendritic cells from PBMC for use in cancer immunotherapy. Cytotherapy 3, 19-29.

Yewdell, J.W., Reits, E. and Neefjes, J. (2003) Making sense of mass destruction: quantitating MHC class I antigen presentation. Nat Rev Immunol 3, 952-61.

Zhang, S., Zhang, H.S., Reuter, V.E., Slovin, S.F., Scher, H.I. and Livingston, P.O. (1998) Expression of potential target antigens for immunotherapy on primary and metastatic prostate cancers. Clin Cancer Res 4, 295-302.

Zhou, L.J. and Tedder, T.F. (1995a) A distinct pattern of cytokine gene expression by human CD83+ blood dendritic cells. Blood 86, 3295-301.

Zhou, L.J. and Tedder, T.F. (1995b) Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. J Immunol 154, 3821-35.

Zhou, L.J. and Tedder, T.F. (1996) CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. Proc Natl Acad Sci U S A 93, 2588-92.

Zippelius, A., Pittet, M.J., Batard, P., Rufer, N., de Smedt, M., Guillaume, P., Ellefsen, K., Valmori, D., Lienard, D., Plum, J., MacDonald, H.R., Speiser, D.E., Cerottini, J.C. and Romero, P. (2002) Thymic selection generates a large T cell pool recognizing a self-peptide in humans. J Exp Med 195, 485-94.

Zotter, S., Hageman, P.C., Lossnitzer, A., van den Tweel, J., Hilkens, J., Mooi, W.J. and Hilgers, J. (1988) Monoclonal antibodies to epithelial sialomucins recognize epitopes at different cellular sites in adenolymphomas of the parotid gland. Int J Cancer Suppl 3, 38-44.