Developing anti-tumor vaccines: Antigens and Antigen-presenting cells

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Zusammenfassung

Bei der Behandlung maligner hämatologischer Erkrankungen wurden in den letzten Jahren große Fortschritte erzielt. Trotzdem kann die Mehrzahl der Patienten bisher nicht geheilt werden. Dies verlangt nach neuen Therapiestrategien, wie z.B. der Immuntherapie, deren Hauptziel es ist, eine zytotoxische Immunantworten gegen Tumor-assoziierte Antigene zu induzieren.

Dendritische Zellen (DC) bieten aufgrund ihrer einzigartigen Fähigkeit zur Stimulation humoraler und zellulärer Immunantworten die besten Voraussetzungen über aktive Vakzinierung eine effiziente Anti-Tumor Immunantwort zu induzieren. Die Herstellung einer erfolgreichen anti-Tumor-Vakzine verlangt einerseits eine genaue Kenntnis der Physiologie der DC, andererseits müssen geeignete Vakzinierungsantigene charakterisiert werden. Die vorliegende Arbeit beschäftigt sich mit beiden Aspekten der Vakzineherstellung.

Im ersten Teil der Arbeit werden MHC-Klasse-I Antigene (Peptide) aus den polymorphen Bereichen der HLA-DP-Allele sowie der konstanten Immunglobulin (A und D)-Regionen identifiziert und als mögliche Antigene zur Vakzinierung von Patienten mit hämatologischen Erkrankungen nach Stammzell-Transplantationen evaluiert. Dabei waren gegen eines der IgD-Peptide und gegen 11 der HLA-DP-Peptide zytotoxische T-Zellen (CTL) induzierbar. CTL-Klone konnten jedoch nur gegen das IgD-Peptid gewonnen werden. Mit deren Hilfe gelang der Nachweis, dass dieses Peptid nicht auf der Oberfläche von normalen und malignen B-Zellen exprimiert wird und sich somit nicht als Vakzinierungsantigen eignet. Hingegen konnte die natürliche Präsentation der HLA-DP-Antigene wegen der Nichtklonierbarkeit der CTL nicht untersucht werden. Aufgrund der Immunogenität der Peptide sollte ihre Relevanz im Kontext einer allogenen Stammzell-Transplantation weiter untersucht werden.

Im zweiten Teil der Arbeit wird die Rolle der Glykogen-Synthase-Kinase (GSK)-3 bei der Differenzierung und Aktivierung humaner DC untersucht. Dabei kann gezeigt werden, dass 1) GSK-3 für die Differenzierung von DC aus Monozyten notwendig ist, 2) GSK-3 konstitutiv in unreifen DC aktiv ist und deren spontane Reifung hemmt, und 3) GSK-3 im Kontext einer DC-Aktivierung eine neue, aktivierende Funktion aufgrund des sich ändernden intrazellulären Kontextes bekommt und die Sekretion großer Mengen inflammatorischer Zytokine (wie IL-12p70) vermittelt bzw. das anti-inflammatorische IL-10 hemmt.

Die Modulation der GSK-3-Aktivität im Rahmen einer Immuntherapie kann daher die Aktivierung Antigen-präsentierender DC verstärken und somit die Effizienz einer Vakzine verbessern.

Summary

The treatment of haematological malignancies has achieved a significant progress during the last years; however, many subtypes of these diseases still remain incurable. Therefore, new complementary therapeutic approaches have to be developed. One possibility to enhance the effects of conventional chemo- and radiotherapy is provided by immunotherapy. The major aim of immunotherapy is to induce immune responses against antigens expressed by tumor cells, resulting in a therapeutic anti-tumor effect.
Due to their unique capacity to stimulate both humoral and cell-mediated immune responses, dendritic cells (DC) are attractive candidates for active vaccination strategies. Intensive research is focused on improving the effectiveness of DC-based vaccines. The generation of successful anti-tumor vaccines requires both, a better understanding of the fundamental biology of DC, as well as the identification and characterization of tumor-associated antigens that can be used for vaccinations. The thesis presented here deals with both aspects of vaccine generation.
In the first part of this thesis, MHC class I antigens derived from HLA-DP polymorphic regions and from immunoglobulin A and D constant regions were evaluated as potential targets for vaccination of patients with haematological malignancies after stem cell transplantation (SCT). $CD8^+$ cytotoxic T-cell (CTL) responses could be generated *in vitro* against 1 IgD-derived peptide and 11 HLA-DP-derived synthetic peptides. The IgD-derived epitope was demonstrated to lack efficient natural presentation on the surface of either normal or malignant cells and is therefore not a suitable target for vaccination. The characterization of natural presentation of the HLA-DP-derived peptides was not possible as peptide-specific CTL clones could not be generated. However, as immunogenicity of HLA-DP-derived peptides was shown, further studies are proposed to investigate whether CTL recognizing these antigens have any impact on development of graft versus host disease in patients after HLA-DP mismatched allogeneic SCT.
The second part of this thesis is focused on fundamental biology of DC. The role of the glycogen synthase kinase-3 (GSK-3) pathway in the differentiation of DC and regulation of DC functions was investigated using an *in vitro* model of human monocyte-derived DC. The results presented in this work demonstrate that (1) GSK-3 is necessary for differentiation of monocytes into DC as in the absence of GSK-3 activity monocytes differentiate into macrophage-like cells; (2) GSK-3 is constitutively active in immature DC and suppresses spontaneous maturation; (3) in the context of DC activation, GSK-3 acquires a pro-inflammatory function mediating high levels of IL-12p70, IL-6 and TNF-α secretion, and partially inhibits IL-10. Therefore, GSK-3 might become a target for therapeutic interventions in clinical immune imbalances, such as autoimmune diseases and transplantation settings. Modulation of this pathway during the course of vaccine-based immunotherapy may enhance DC activation *in vivo* and thus may enhance vaccine-mediated immune responses.

List of abbreviations

AEC	3-amino-9-ethyl-carbazole
AP-1	Activator protein-1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BHK	Baby Hamster Kidney cell line
C/EBP	CCAAT enhancer-binding protein
cAMP	Cyclic adenosine monophosphate
CCR	CC chemokine receptor
CD40L	CD40 ligand
CD83L	CD83 ligand
CLIP	Class II-associated invariant chain peptide
CREB	cAMP response element binding protein
CsA	Cyclosporin A
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cells
DMSO	Dimethylsulfoxide
DRiP	Defective ribosome products
DTT	Dithiothreitol
EBV	Epstein-Barr virus
ELISA	Enzyme-linked Immunosorbent Assay
ELISPOT	Enzyme-linked Immunospot Assay
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence Activated Cell Sorting
FcR	Fc-receptor
FCS	Fetal calf serum
FI	Fluorescence index
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GSK-3	Glycogen synthase kinase 3
GvH	Graft versus Hematopoietic reaction
GvHD	Graft versus Host Disease
GvL	Graft vs Leukemia/lymphoma
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HRP	Horseradish peroxidase

ICAM-1	Intracellular adhesion molecule 1
iDC	Immature DC
IFN	Interferon
Ig	Immunoglobulin
Ii	Class II-associated chaperone invariant chain
IκB	Inhibitor of NF-κB
IKK	IκB kinases
IL	Interleukin
IP-10	IFNγ inducible protein
IRAK	IL-1R-associated kinase
IRF3	IFN-regulatory factor 3
IRS	Insulin receptor substrate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
Jak	Janus kinase family
LFA-3	Lymphocyte function-associated antigen 3
LPS	Llipopolysaccharide
mAB	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic peptide-1
MDC	Macrophage-derived Chemokine
MDP	Muramyldipeptide
mHA	Minor histocompatibility antigens
MHC	Major histocompatibility complex
MIP	Macrophage Inflammatory Protein
MoDC	Monocyte-derived DC
MyD88	Myeloid differentiation primary response protein 88
NF-AT	Nuclear factor of activated. T cells
NF-κB	Nuclear factor κB
NIK	NF-κB inducing kinase
NK cells	Natural killer cells
NKT cells	Natural killer T-cells
NOD	Nucleotide binding oligomerization domain
OX40L	OX40 ligand
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PI3K	Phoshatidylinositol-3 kinase

PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase-Cγ
PRR	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
RANTES	Regulated upon activation normally T expressed and secreted chemokine
rh	recombinant human
RIP	Receptor interacting protein
RT-PCR	Real-Time polymerase chain reaction
SCF	Stem cell factor
SCT	Stem cell transplantation
SDS	Sodium dodecylsulfate
SEM	Standard error of mean
SRM	Signal response module
Stdev	Standard deviation
TAA	Tumor-associated antigens
TAK1	TGF-β-activated kinase 1
TAP	Transporter associated with Antigen Presentation
TARC	Thymus and Activation-Regulated Chemokine
TCR	T-cell receptor
TGF-β	Tumor growth factor β
Th1	T-helper type 1 effector cells
Th2	T-helper type 2 effector cells
TIR	Toll/IL-1R domain
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNF-receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
WM	Wortmannin

1 INTRODUCTION

1.1 Immunotherapy against cancer

Despite the introduction of new cytotoxic drugs, a large proportion of malignant diseases remains incurable. There is still a great need to develop new complementary therapeutic modalities. One possibility to enhance the effects of conventional chemo- and radiotherapy is provided by immunotherapy. Anti-tumor immunotherapy can be divided into two major approaches: non-specific and antigen-specific immunotherapy (reviewed in [1]) Non-specific immunotherapy includes immunostimulating cytokines and adoptive transfer of non-specific immune effector cells. Antigen-specific immunotherapy can be classified into passive (monoclonal antibodies, adoptive transfer of specific immune effector cells) and active vaccination strategies. This work is focused mainly on the active vaccination strategies.

Vaccination against viral and bacterial pathogens represents a preventive treatment and is widely and successfully used in clinics. In contrast, vaccination against cancer aims at achieving therapeutic effect. In cancer patients, the immune system failed to recognize and fight the tumor cells. In this case, a successful vaccine should reverse the established tolerance to tumor antigens and overcome the tumor escape mechanisms. Therapeutic vaccination against an established malignancy still represents a great challenge for immunologists.

1.1.1 Classification of tumor associated antigens

Tumors express several antigens that allow the immune system to recognize the tumor as foreign. These antigens are called tumor-associated antigens (TAA). TAA can be recognized by T-cells as well as induce an antibody response. In order to direct the immune response against the tumor, TAA suitable for vaccinations have to be identified and characterized. Recent strategies of identifying TAA-derived T-cell epitopes' include:

1) Screening of tumor-derived cDNA libraries
2) Comparison of gene expression profiles between tumors and normal tissues using microarray technology
3) Theoretical epitope prediction on the basis of known HLA binding motifs

4) Elution and fractionation of peptides naturally presented on the MHC of tumor cells

TAA can be divided into several main groups according to their mutation- and expression patterns (reviewed in [2]):

1) The expression of ***cancer-testis antigens*** in normal tissues is restricted mainly to spermatocytes/spermatogonia of testis, but they are expressed in many histologically different tumor types. This group of antigens includes the MAGE, BAGE and GAGE family of antigens, NY-ESO and the recently identified LAGE, SAGE, HAGE and several other antigens.
2) ***Differentiation antigens*** are shared between the tumor and its normal parental tissue. The best studied differentiation antigens are melanoma-associated antigens, for instance Melan-A/MART1.
3) ***Overexpressed antigens*** are antigens found in normal tissues, but they are amplified or overexpressed in tumors. This is the largest group of antigens that includes anti-apoptotic proteins (e.g. survivin and livin) and tumor-supressor proteins (e.g. p53).
4) ***Unique TAA*** arise from point mutations, splicing aberrations as well as chromosome aberrations. The examples of unique TAA are the epitopes derived from mutated β-catenin, N-RAS and K-RAS.
5) ***Viral antigens*** are the antigens encoded by oncogenic viruses, e.g. Epstein-Barr virus (EBV) and human papilloma virus (HPV).

1.2 Dendritic cells (DC) as potential adjuvants for anti-cancer immunotherapy

DC are the most potent type of antigen presenting cells (APC) that play an important role in the induction of immune responses. Three main developmental stages can be distinguished – DC precursor, immature DC and mature DC. Immature DC (iDC) are distributed in tissues throughout the body and function as sentinels in the periphery. They sample the environment for presence of "danger signals" by multiple antigen uptake mechanisms. As soon as iDC detect a danger signal, they undergo a complex maturation process involving phenotypic and functional changes. In the process of

maturation, DC change their expression profile of surface markers resulting in an augmented capacity to activate naive and memory T-cell responses. They can also directly interact with other immune cell types, including natural killer (NK) cells, natural killer T-cells (NKT) and B-lymphocytes. Furthermore, DC may also secrete high levels of pro-inflammatory cytokines and acquire the capacity to migrate towards lymph nodes. These characteristics provide DC with a unique capacity of linking innate and adaptive immune responses.

1.2.1 Dendritic cell differentiation and subtypes

Dendritic cells originate from the hematopoietic precursors within the bone marrow. Two models have been proposed for the differentiation of DC from hematopoietic progenitor cells, one postulating that dendritic cells can be generated from either myeloid or lymphoid derived progenitors, the other postulating a single committed DC lineage that has functional plasticity (reviewed in [3]). Indeed, evidence generated by different experimental approaches in both mouse and human DC supports the notion that certain subsets of DC are of myeloid origin whereas others can be generated from lymphoid progenitors [3]. However, the concept of lymphoid versus myeloid DC progenitors was challenged by mouse experiments, showing that both myeloid and lymphoid DC ($CD8^-$ and $CD8^+$, respectively) could be generated from common lymphoid [4] as well as common myeloid progenitors [5, 6]. Moreover, del Hoyo and coworkers could identify a $CD11c^+$ MHC class II^-, DC-committed precursor population which was capable of generating different DC subpopulations including $CD8^-$ and $CD8^+$ DC [7]. This suggested the existence of a single committed DC lineage that has functional plasticity. Although mouse and human DC were shown to be generated from multiple precursor cells *in vitro*, it is unclear whether the generation of DC under different specific experimental conditions is relevant for *in vivo* situation. So far, it remains unknown how DC are generated *in vivo.*

The population of DC is heterogeneous. In mice, three main subsets have been defined according to the surface markers and functional properties: $CD8^-CD205^-$ $CD11b^+CD4^{+/-}$ myeloid DC, $CD8^+CD205^+CD11b^-CD4^-$ lymphoid DC and $CD11b^-$ $B220^+GR1^{+/-}$ plasmacytoid DC [8].

In humans, DC subtypes are less well characterized due to the limiting availability of DC derived from various tissues. The information about human DC subtypes derives

mainly from *in vitro* studies. DC and DC precursors from human blood are divided into two main populations: conventional $CD11c^{+}CD123^{lo}$ myeloid DC and $CD11c^{-}$ $CD123^{hi}$ plasmacytoid DC [9]. Plasmacytoid DC are the major type I interferon (IFNα/β) producers and play an important role in innate anti-viral immunity [9]. Myeloid DC are commonly differentiated *in vitro* from $CD14^{+}$ monocytes. Upon activation with different stimuli they can secrete pro-inflammatory cytokines IL-6, TNF and IL-12 and efficiently initiate adaptive immune responses [9].

Conventional human monocyte-derived myeloid DC are the main focus of this discussion.

1.2.2 Mechanisms of Antigen Processing and Presentation

Antigen presenting cells employ two major systems to present antigens to T-lymphocytes - MHC class I and II. MHC class I is recognized by $CD8^{+}$ cytotoxic T-lymphocytes and MHC class II is recognized by $CD4^{+}$ T-helper cells.

1.2.2.1 MHC class II presentation

MHC class II complex formation takes place in the endocytic pathway. The endocytic pathway is generally divided in 3 regions – early endosomes, late endosomes and lysosomes. Self or foreign endocytosed material moves predominantly from early endosomes towards lysosomes being exposed to increasingly acidic and proteolytic environment resulting in gradual release of polypeptides. Some of these peptides will occasionally have a proper sequence to bind MHC class II alleles.

The MHC class II molecule is a heterodimer consisting of polymorphic α and β-chains that form a peptide-binding groove open from both ends [10]. This allows for a certain variability in the length of the peptides that can bind to MHC class II. Most of the peptides contain 12-19 residues, although MHC class II binding peptides with a length up to 30 amino acids were identified, as reviewed in [11].

Newly synthesized MHC II molecules are cotranslationally translocated into the endoplasmic reticulum (ER) and assembled there with the chaperone invariant chain (Ii) (reviewed in [12]). Binding of MHC class II to Ii stabilizes the complex and prevents ER-resident polypeptides from binding to the peptide-binding grove of MHC class II [13]. Ii also contains the sorting signal for endosomal compartments [14, 15]. When the MHC class II-Ii complex reaches the endosomal compartment, Ii is

degraded stepwise by proteases, producing Ii-p22 and Ii-p10 intermediates and finally the CLIP fragment that occupies the peptide-binding cleft. This last step is performed by cathepsin S [16, 17]. The conversion of Ii-p10 into CLIP releases the MHC-II complex from the cytoplasmic part of Ii that contains the endosomal targeting motif. This motif acts as a retention signal that prevents MHC-II from escaping to the cell surface [18, 19]. Thus, after MHC class II is released from the Ii retention signal, the replacement of CLIP by the antigenic peptide has to occur rapidly in order to prevent surface expression of CLIP-loaded MHC molecules. The CLIP peptide is substituted by antigenic peptides with the help of the chaperones H-2DM and H-2DO (in humans, HLA-DM and HLA-DO) that destabilize the complex and facilitate the release of CLIP [20]. H-2DM has also been shown to execute a peptide editing function, promoting the exchange of low affinity for high affinity peptides [21].

1.2.2.2 MHC class I presentation

Antigens derived from endogenous proteins are predominantly presented to $CD8^+$ lymphocytes via the MHC class I presentation pathway. Endogenous proteins, including defective ribosome products (DRiPs) [22], are ubiquitinylated and thus targeted for degradation by proteasomes [23]. Proteasomes cleave these proteins to peptides of 3-22 residues [24]. These peptides can then be further processed by cytosolic peptidases, transported into the ER by the transporter associated with antigen presentation (TAP) and loaded on MHC-I molecules. MHC class I loading is a complex process which requires a macromolecular peptide loading complex comprised of MHC heavy chain, β2-microglobulin, TAP and chaperones, such as tapasin, calreticulin and ERp57 [25-27]. After peptide binding, MHC-class I is released from the peptide loading complex and transported via Golgi to the cell surface.

Although mammalian cells express thousands of different proteins, which can give rise to numerous antigenic peptide variants, only a minor part of these antigens is naturally presented on MHC class I molecules. There are several mechanisms that contribute to the restriction of the antigen repertoire, including:

1) Proteasomal cleavage specificity
2) Sequence- and size preferences for peptide transfer into the ER by TAP

3) Allele-specific binding affinity of antigens to MHC-class I

Proteasomes are believed to represent the main protease activity for the generation of MHC-class I peptides (reviewed in [28]). They contain few catalytic subunits with three distinct catalytic activities: chymotrypsin-like, trypsin-like and caspase-like (cleavage after hydrophobic residues, basic residues and acidic residues, respectively). Moreover, expression of several subunits of the proteasomal system (immunosubunits) [29] as well as proteasomal regulator PA28 [30] are controlled by IFN-γ. IFN-γ is produced by activated T-cells and NK-cells and was shown to enhance MHC-class I antigen presentation. Immunosubunits replace their constitutive counterparts within the active site, resulting in the formation of immunoproteasomes [31, 32]. Immunoproteasomes have altered cleavage site preferences and differ in cleavage rate [33, 34], resulting in the generation of different peptide pool for MHC-class I presentation [35-37].

The peptides generated by proteasomal degradation are transported into the ER in an ATP-dependent manner by TAP [38, 39]. TAP is composed of two homologous subunits: TAP1 and TAP2. Apart from the preference for 8-16-mer peptides [40], TAP was demonstrated to have a certain selectivity for sequence of transported peptides. The first 3 N-terminal and the C-terminal residues of the peptide are important for binding to TAP. Specifically, the human TAP complex is selective for hydrophobic or charged residues at the C-terminus and in the second position; moreover, strongly hydrophobic residues in P3 and P7 increase the binding affinity of the peptides to TAP, whereas proline in the first three positions, as well as acidic residues in P1, P3 and P7 or an aromatic amino acid in P1, have strong deleterious effects [41]. TAP-deficient cells have a significantly diminished MHC-class I surface expression [42]. Because in the absence of functional TAP, the majority of TAP-dependent MHC-class I antigens are not presented at cell surface, tumor cells lacking TAP function generally escape recognition and elimination by $CD8^+$ T-cells [43-45]. However, a TAP-independent MHC-class I presentation pathway has been reported [46]. The peptides derived from C-terminus of the transmembrane- and soluble proteins of the secretory pathway have access to MHC class I molecules in ER in the absence of TAP activity [46]. It was recently shown by van Hall and colleagues, that some of these peptides, although derived from self antigens, are specifically presented by TAP-deficient tumor cells but not by normal cells. Thus, these peptides could serve

as neoantigens inducing cytotoxic T-lymphocyte (CTL) responses against TAP-deficient tumor variants [47].

MHC class I molecules are composed of a membrane anchored heavy chain and the non-covalently bound small soluble protein β2-microglobulin. The heavy chain can be subdivided into 3 domains (α1-α3). Two distal domains α1 and α2 form a peptide-binding groove that can bind short antigenic peptides (8-11 amino acids). Human MHC-class I genes are organized in the class I region of chromosome 6, which contains 3 loci: HLA-A, -B and -C. Each locus encodes the heavy chain of MHC class I. These genes are highly polymorphic. The amino acid variability is clustered within three main regions of α1 and α2, which are involved in the formation of the peptide-binding groove. MHC class I molecules bind N- and C- terminal residues (anchor positions) of antigenic peptides which flank a variable sequence that is recognized by the T-cell receptor (TCR). Depending on the polymorphic amino acids in the peptide-binding site, different class I alleles will bind different peptide motifs [11, 48]. For example, human HLA-A2.1 selects for Met, Leu or Ile in position P2 of the antigenic peptide and Val, Leu, Ile or Ala in position P9 [48, 49]. P1, P3 and P7 residues also contribute to the stability of the peptide-MHC interaction [50, 51]. Identification of allele-specific peptide binding motifs allowed the design of peptides that bind to MHC with high affinity and helped to predict MHC-binding epitopes within a known protein sequence. Increasing the peptide binding affinity to MHC molecule by substituting anchoring amino acids represents one of the strategies to improve the immune reaction against tumor antigens [52, 53].

1.2.2.3 Cross-presentation

Although MHC class I is mainly loaded with the peptides, derived from cytosolic proteins, $CD8^+$ T-cell responses are also induced by the exogenous pathogens. These pathogens enter the cell via endocytic pathway and thus theoretically should be restricted to the MHC class II antigen presentation pathway. The process allowing exogenous antigens to be presented in the context of MHC class I is called cross-presentation.

The ability to cross-present exogenous antigens *in vivo* was demonstrated mainly for DC and macrophages. However, other endocytic cells, including B-cells and

keratinocytes were shown *in vitro* to cross-present exogenous antigens on MHC class I with low efficiency [54, 55].

The molecular mechanism of cross-presentation is not yet completely understood. One possible mechanism of cross-presentation is described by the exchange model. According to this model, MHC class I can be internalized from the cell surface following monoubiquitination [56], and subsequently included into multivesicular bodies. In this compartment, MHC class I is colocalized with MHC class II [57]. Cross-presentation then can occur by exchange of MHC class I associated peptides for the antigens newly generated by proteolysis in the endosomal compartment. The endosomal antigen exchange pathway is dependent on lysosomal degradation. This pathway was shown to contribute at least in part to the cross-presentation of some antigens [58, 59]. However, the antigens that are cross-presented on MHC class I according to this model are produced by different proteolytic systems and in different conditions than antigens that are processed by the classical MHC class I pathway. This may in some cases result in a different pool of antigens. Usually, non-hematopoietic cells process antigens only via the classical MHC class I pathway. Thus, the antigens produced by exchange will not contribute much to an efficient $CD8^+$ T-cell response against exogenous pathogens restricted to the endocytic pathway and targeting non-hematopoietic cells. Moreover, in other studies, cross-presentation in DC was shown not to be influenced by inhibitors of lysosomal proteolysis or by the inhibitor of lysosomal acidification chloroquin [54, 60-62]. Instead, cross-presentation was shown to be dependent on the classical MHC class I antigen presentation pathway. In these studies, cross-presentation was highly sensitive to proteasomal inhibitors [62, 63] and also strictly depended on TAP function, as it was completely abrogated in TAP-deficient cell-lines and mutant mice [63, 64] These data suggest that there is more than one mechanism involved in antigen cross-presentation.

Experimental evidence suggests that exogenous antigens can successfully reach the cytoplasm [61, 65, 66]. The molecular mechanism of this process is yet unclear. One protein proposed to be responsible for ‘retrotranslocation’ of exogenous antigens is Sec61. Sec61 cotranslationally inserts secretory and transmembrane proteins into the ER but also shuttles misfolded and unstable proteins back to cytosol for proteolysis by proteasomes (ER associated degradation) [67-69]. Sec61 was indeed found in

phagosomes [70] and associated with internalized exogenous antigens in the endosomal compartment [71]. Moreover, cross-presentation of exogenous antigens was inhibited upon downregulation of Sec61 levels by RNA interference [71]. Apart from Sec61, phagosomes were shown to contain other ER associated factors, that are necessary for MHC class I loading. These include TAP, MHC class I molecules, calnexin, calreticulin and tapasin, as well as an ER aminopeptidase [71-73]. One potential mechanism that could explain the phagosomal localization of the ER proteins is ER-mediated phagocytosis. Gagnon and colleagues reported that in mouse macrophage cell lines, the ER can fuse with early forming phagosomes, donating a portion of its membranes together with ER-resident proteins [70]. As a result, phagosomes contain all the proteins needed for MHC class I loading. Additionally, proteasomes were also shown to be associated with the cytoplasmic surface of the phagosomes [74]. Several studies suggested that the cross-presented peptides are generated in the cytosol, probably by phagosome associated proteasomes, and are immediately transferred back to be loaded on the MHC class I molecules inside the phagosomes [72-74]. Alternatively, phagosomes may fuse with the ER at some point, releasing soluble antigens into the ER, so that the MHC class I loading occurs inside the ER. Consistent with this model are the findings that some exogenous soluble proteins after their internalization can access the ER in a nondegraded form [75].

Cross-presentation is a very important mechanism that enables the immune system to react efficiently with both, $CD4^+$ and $CD8^+$ T-cell responses to infections or somatic mutations, without antigen presenting cells having to be directly infected or endogenously express the antigens.

1.2.3 DC maturation and function

DC maturation is a complex process, converting them from weakly immunostimulating cells specialized in antigen uptake into potent initiators of both humoral and cellular immune responses. Maturation is accompanied by downregulation of antigen uptake by phago- and endocytosis, increase of surface expression of antigen-presenting molecules and co-stimulatory molecules, production of pro-inflammatory cytokines and acquiring the ability to respond to lymph node-directing chemokines.

1.2.3.1 Maturation stimuli: The danger model and intracellular signaling associated with DC maturation

According to the danger model proposed by Matzinger [76], the immune system is activated by so called "danger signals". These are the signals derived from pathogens or injured cells, exposed to pathogens, toxins or mechanical damage. Danger signals can induce DC maturation resulting in the subsequent activation of innate and adaptive immune responses. Main classes of signals that induce DC maturation are reviewed below.

Toll-like receptor signaling

Toll-like receptors (TLR) are a big family of evolutionary conserved pattern recognition receptors (PRR) (reviewed in [77]). 11 mouse and 10 human members of this family have been identified so far. TLR share a cytoplasmic domain with IL-1 receptors, which is known as toll/IL-1R (TIR) domain. The extracellular domain of TLRs contains 24-29 tandem copies of leucine-rich repeats. TLRs recognize a broad range of molecular patterns, including lipopolysaccharides (TLR4) [78], lipoproteins, glycolipids and peptidoglycans (TLR2) [79-81], double-stranded RNA (TLR3) [82], single-stranded RNA (TLR7 and 8) [83] and CpG-containing DNA (TLR9) [84]. TLR1, TLR2 and TLR4 are located on the cell surface and are recruited to the phagosomes upon ligand binding. In contrast, TLR3, TLR7 and TLR9, which recognize RNA and DNA, are not expressed on the cell surface. Localization to the ER and recruitment to the endosomal/lysosomal compartment upon stimulation with CpG-containing DNA was demonstrated for TLR9 [85].

After ligand binding, TLRs dimerize and recruit the adaptor molecule MyD88 (myeloid differentiation primary response protein 88). MyD88, in turn, recruits IL-1R-associated kinase 4 (IRAK4). Upon binding of MyD88 to IRAK4, IRAK4 phosphorylates IRAK1, which then becomes active. IRAK1 additionally autophosphorylates residues in its N-terminus, thus enabling TNF-receptor associated factor 6 (TRAF6) to bind to this complex. The IRAK1-TRAF6 complex dissociates from the receptor and initiates a series of events that lead to TGF-β-activated kinase 1 (TAK1) activation. TAK1 subsequently activates IKK (IκB kinases). IKK phosphorylate IκB (inhibitor of NF-κB) thus targeting it to proteasomal degradation. IκB degradation results in the release of NF-κB and the consequent transcription of

NF-κB target genes. Furthermore, active TAK1 activates mitogen-activated protein kinases (MAPK) [77].

Apart from the MyD88-dependent pathway, an MyD88-independent pathway plays a role in the response to some TLRs, e.g. TLR3 and TLR4. The MyD88-independent pathway involves other adaptor molecules (TIR-domain-containing adaptor protein inducing IFN-β, TRIF, and TRIF-related adaptor molecule, TRAM). This pathway leads to activation of IFN-regulatory factor 3 (IRF3) [86] as well as NF-κB (although with a delayed kinetics as compared to the MyD88-dependent pathway) [87]. IRF3 and NF-κB activation results in IFN-β production, which in an autocrine manner activates the transcription of a number of IFN-inducible genes, including late IFNα/IFNβ production [88].

TLRs play an important role in the recognition of foreign pathogens, including bacteria, viruses and fungi. Myeloid DC express TLR1-6 and TLR8, whereas plasmacytoid DC express TLR7 and 9 [89]. TLR signaling leads to DC maturation. In response to TLR-ligands, DC upregulate the expression of surface co-stimulatory molecules (e.g. CD40, CD80 and CD86). This can occur via both, MyD88-dependent and MyD88-independent pathways [88, 90]. In contrast, TLR-mediated production of pro-inflammatory cytokines is controlled by the MyD88-dependent pathway, although the second pathway can enhance pro-inflammatory cytokine production [87, 91]. Thus, expression of co-stimulatory molecules and production of pro-inflammatory cytokines are differentially regulated during TLR signaling.

NOD-signaling

Another class of receptors, the nucleotide binding oligomerization domain (NOD) family of proteins, is involved in TLR-independent recognition of certain intracellular pathogens (reviewed in [92]). They are located in the cytoplasm and recognize bacterial peptidoglycan structures. The human NOD-family includes 20 proteins. One of the molecules that is expressed in DC and is involved in pathogen recognition is NOD2. NOD2 recognizes bacterial lipopolysaccharide (LPS) and peptidoglycan. Interaction of NOD2 with its ligands results in NF-κB activation as well as activation of a number of caspases. Recent analyses have indicated that the essential structure within peptidoglycan for the recognition by NOD2 is muramyldipeptide (MDP). MDP is present in peptidoglycans from almost all Gram-positive and Gram–negative

bacteria. Upon activation with MDP, DC secrete pro-inflammatory cytokines, including IL6 and IL12 [93]. Moreover, DC respond to MDP stimulation by upregulating surface expression of co-stimulatory molecules [93], indicating that NOD2 can activate DC in a manner similar to TLR.

Fc-receptor-signaling

Immune complexes containing antigens opsonised by immunoglobulins (Ig) were also shown to promote DC maturation [94-96]. This is mediated by the Fc-receptor (FcR) family. The role of FcRs in human DC was recently reviewed by Bajtay *et al* [97]. Human DC express FcRs recognizing IgG, IgA and IgE isotypes (FcγR, FcαR and FcεR, respectively). FcRs can be functionally divided into activating (i.e. FcγRI, FcγRIIA, FcγRIIIA) and inhibitory receptors (i.e. FcγRIIB). The activating FcRs contain an immunoreceptor tyrosine-based activation motif (ITAM) whereas the inhibitory FcRs contain an immunoreceptor tyrosine-based inhibition motif (ITIM). Crosslinking of the activating FcRs by immune complexes leads to tyrosine phosphorylation of ITAM. The signal cascade initiated by this event results in recruitment of phoshatidylinositol-3 kinase (PI3K) and phospholipase-Cγ (PLCγ) which trigger protein kinase C (PKC) activation and sustained calcium elevation [98]. Crosslinking of the activating FcγRIIA induces phagocytosis, upregulation of DC maturation markers and enhances efficient antigen cross-presentation to T-cells [94-96]. In contrast, the ITIM-containing FcγRIIB inhibits DC maturation, as suggested by studies of FcγRIIB-deficient mice that showed enhanced potential to generate T-cell responses [99]. This was further supported by *in vitro* experiments with the human DC by Dhodapkar *et al*. Blocking immune complex binding to FcγRIIB by monoclonal antibodies was sufficient to induce DC maturation and IL12 secretion in response to immune complexes normally present in plasma [100].

Cytokine-induced maturation

Pro-inflammatory cytokines, such as TNF-α [101, 102] and IL-1β [103], were demonstrated to induce maturation of DC. Other cytokines, including IL-6 [104], IFN-α [105] and prostaglandin E2 (PGE2) [104, 106] can synergize with TNF-α and IL-1β promoting DC-maturation *in vitro*.

T-cell dependent maturation: CD40/CD40L interaction

CD40 is a type I transmembrane glycoprotein cell surface receptor belonging to the tumor necrosis factor α (TNF-α) receptor family. Its ligand, CD40L (CD154), is a type II integral membrane protein expressed on activated T cells [107], activated B cells [108], and activated platelets [109]. Stimulation through CD40 results in a complex series of events within an APC. Engagement of CD40 by CD40L leads to multimerization of CD40 and subsequent recruitment of TNF receptor–associated factors (TRAFs) to the cytoplasmic tail [110]. TRAF binding results in formation of a signaling complex that includes multiple kinases, such as NF-κB inducing kinase (NIK), receptor interacting protein (RIP), PI3K, members of the mitogen-activated protein kinase (MAPK) family and possibly others. These kinases then initiate a downstream cascade of signaling events, resulting in the activation of the NF-κB and, possibly NF-AT and CREB transcription factors (reviewed in [111]). Crosslinking of CD40 in iDC results in upregulation of DC maturation markers [112, 113], production of pro-inflammatory cytokines (i.e. IL-12p70 [114] and TNF-α [113]) and chemokines [115] as well as in prolonged DC survival [116].

1.2.3.2 Maturation induced changes in DC phenotype and functions

Antigen presentation

MHC class II presentation is highly regulated in DC. Following activation, iDC upregulate MHC-II expression. In iDC, MHC class II is mainly detected in the endosomal compartment, whereas after stimulation, more MHC class II molecules are expressed on the cell surface. Two models were proposed to explain how MHC class II is regulated. The first model suggests that peptide generation and formation of peptide-MHC class II complexes is more efficient in mature as compared to immature DC. The other model proposes that increase of surface MHC class II expression in the mature DC state is achieved by a disruption of the peptide-MHC complex turnover. Peptide-MHC complexes are then retained in the plasma membrane (reviewed in [117]). Endosomal protein degradation and peptide-MHC complex formation was shown to be enhanced during DC maturation. Maturation is associated with increased activity of lysosomal hydrolases [118] due to increased acidification of lysosomes. Maturation is also accompanied by increased invariant chain degradation by cathepsin S due to downregulation of the cathepsin S inhibitor, cystatin C [119]. On the other

hand, iDC and mature DC differ in the rate of MHC class II turnover. In iDC, peptide-MHC class II complexes are only transiently expressed on the cell surface and then rapidly internalized. In contrast, in mature DC peptide-MHC class II complexes are retained on the plasma membrane [120]. Therefore, the available experimental evidence suggests that both mechanisms may contribute to the regulation of MHC class II.

Although peptide-MHC class I complex formation is more efficient in iDC, the surface expression levels of loaded MHC class I molecules is increased upon maturation. This results from increased synthesis and lower turnover of peptide-MHC complexes in mature as compared to immature DC [120, 121].

Cross-presentation was also shown to be regulated upon DC maturation [94, 122, 123]. The enhancement of cross-presentation could be a result of the increased synthesis of MHC class I [120, 121] and other components of peptide-processing machinery [123]. However, not all the signals that enhance synthesis of MHC class I result in increased cross-presentation [122], suggesting the existence of additional mechanism of regulation of cross-presentation [117].

Adhesion molecules and co-stimulatory molecules

During maturation, DC upregulate surface expression of a number of molecules. These include co-stimulatory molecules CD40, CD70, CD80, CD83 and CD86 and OX40L, most of which are involved in bidirectional signaling between DC and T-cells.

CD80 and CD86 are members of the B7 receptor family. They provide co-stimulation of T-lymphocytes via CD28 signaling. CD28 is constitutively expressed on T-cells. CD28 signaling influences the threshold for T-cell activation and significantly decreases the number of TCR engagements needed for effective T-cell activation [124]. Ligation of CD28 by its ligands, CD80 (B7-1) and CD86 (B7-2), synergizes TCR signaling and leads to increased production of IL-2, proliferation, and enhanced survival of T-cells (reviewed in [125]).

Co-stimulatory functions of CD83 were recently reported by Hirano *et al*. They showed that CD83 ligand (CD83L) expression is upregulated on $CD4^+$ and $CD8^+$ T-cells. This upregulation requires CD28 co-stimulation. Using artificial APC expressing CD80 and CD83 alone or in combination, Hirano N. and co-workers

demonstrated that CD83:CD83L signaling specifically supported priming and expansion of naïve $CD8^+$ T-cells. Moreover, engagement of CD83 enabled long-term survival of in vitro generated antigen-specific CTL-cultures by enhancing proliferation and inhibiting apoptosis [126].

Mature DC also express several adhesion molecules, including CD2, CD11a, CD54 (ICAM-1), CD58 (LFA-3) and several integrins. They are believed to play a role in attracting the T-cells and, together with other co-stimulatory molecules, are involved in formation of the immune synapse between T–cells and DC [127].

Cytokine production

Different maturation stimuli may induce cytokine secretion by DC. The cytokine profile can be different depending on the type of stimuli and the DC subtype. For example, high levels of type I IFN (IFNα/β) are mainly produced by plasmacytoid DC. Major pro-inflammatory cytokines that can be secreted by mature DC include IL-12p70 [114, 128], IL-6, IL-1β and TNF-α [113, 129, 130]. Human monocyte-derived DC are also capable of producing the anti-inflammatory cytokine IL-10 in response to maturation stimuli [131, 132]. The profile of DC-secreted cytokines plays an important role in defining the type of immune response that will be induced.

Chemokines, chemokine receptors and migration

In order to prime T-cell responses, DC have to reach secondary lymphoid organs. The main receptor that mediates recruitment of DC to the lymph nodes is CCR7. The CCR7 ligands CCL19 (MIP-3β) and CCL21 (6Ckine) are secreted by lymphatic endothelial cells and lymph node stromal cells [133-135]. Expression of CCR7 is upregulated by most maturation-inducing stimuli. Maturation is also accompanied by downregulation of chemokine receptors responsible for tissue retention of DC (i.e. CCR1, CCR5) [136].

The expression of CCR7 is necessary but not sufficient for migration. A variety of other factors were implicated to influence migration. This includes lipid mediators, prostaglandins, adhesion molecules and matrix metalloproteinases (as reviewed in [137]).

Mature DC also start to secrete a number of chemokines, such as TARC, MDC, IP-10 that recruit different T-cell subtypes [138]. In addition, mature DC can secrete RANTES, MIP-1α and MIP-1β that can attract monocytes [138].

1.2.4 Adaptive differentiation of DC

Human monocyte-derived dendritic cells (MoDC) respond to activation stimuli with an adaptive differentiation program that allows the development of diverse functional DC phenotypes. Depending on the type, strength and persistence of the activation stimulus [139] DC can acquire a pro-inflammatory, a migratory, or a mixed phenotype. For instance, DC activated in the presence of PGE2 become migratory DC: they are unable to secrete inflammatory IL-12p70, but can migrate towards lymph node-directing chemokines (i.e. CCR7 ligands). In contrast, CD40L-activated DC acquire a pro-inflammatory function: they secrete large amounts of cytokines, especially IL-12p70, IL6 and TNF, but are incapable of migrating toward CCR7 ligands [139-141]. DC activated with live pathogens (i.e. *E.coli*) can accomplish both functions – they secrete high levels of pro-inflammatory cytokines and migrate towards CCR7 ligands [139]. IL-12p70 production requires persistent stimulation whereas for migration, weak and non-persistent signals are sufficient. Furthermore, migration is inhibited by persistent CD40L signaling [139]. Migration and cytokine secretion are independently regulated by a network of extra- and intracellular factors and pathways [141]. IL-12p70 production is enhanced by IL-4, IL-1β [142], IFN-γ and IFN-α. [143, 144] and inhibited by IL10 [145], PGE2 [140, 146] or cyclic adenosine monophosphate (cAMP) [147, 148]. Both, ERK1/2 and p38 MAPK activity are needed for IL-12p70 secretion by human monocyte-derived DC, whereas PI3K inhibited IL-12p70 production [139, 141]. Migration of DC towards lymph nodes is enhanced by PGE2 and cAMP and requires p38 MAPK activity, whereas ERK1/2 inhibits the migratory capacity of DC [139]. Our group suggested that the intracellular players comprising the molecular network required for a specific function are organized as a functional unit or “module” (signal response module, SRM) [141]. Despite accumulating knowledge of factors influencing any given functional module, principles that govern the signalling network operation remain to be defined.

1.2.5 DC interactions with different immune cell types

1.2.5.1 DC-T-cell interactions

One of the main functions of DC is to induce T-cell responses. The outcome of T-cell-DC interaction depends on the functional state of DC (i.e. MHC class I and II expression level, expression of co-stimulatory molecules and secreted cytokines). DC are capable of priming naïve T-cells to proliferate and differentiate into effector and memory T-cells as well as polarizing immune reactions towards type 1 or type 2 responses. However, DC are also involved in the generation of T-cell tolerance either by inducing abortive proliferation and anergy of antigen-reactive T-cells or by activating regulatory T-cell subsets.

Priming of naïve T-cells: strength and persistence of the signal

The efficiency of T-cell priming depends on strength and persistence of the priming signal. Immature DC are unable to form stable conjugates with T-cells and thus are not efficient in priming T-cell responses [149, 150]. This is probably due to the low levels of MHC expression and the absence of co-stimulation in iDC. Immature DC form short, transient contacts with T-cells, which result in abortive proliferation and subsequent deletion of antigen-specific T-cells [149, 151]. Several studies on mouse and human T-cells demonstrated that prolonged APC-T-cell interaction in the presence of co-stimulation is necessary to achieve efficient priming of naïve T-cells [149, 151, 152]. *In vivo* imaging of lymphocytes trafficking in mouse lymph nodes showed that mature DC acquire the ability to form stable contacts with T-cells within 15-20 hours after activation [151]. These contacts are maintained for several hours resulting in generation of effector T-cells [151, 152]. Concentration of MHC-antigen complexes and co-stimulation contribute to the strength and persistence of the priming signal. Indeed, Bousso et al showed that the duration of DC-T-cell interaction in vivo correlated with the antigen density [152]. As mentioned before, co-stimulatory CD28 signal lowered the threshold for the number of TCR-MHC interactions needed for T-cell activation [124]. Other co-stimulatory molecules, including members of the tumor necrosis factor receptor (TNFR) family, such as CD27 and OX40, are also involved in both priming naïve T-cells and sustaining long-lived T-cell immunity (reviewed in [153]).

Polarization of immune responses: Th1 versus Th2

T-helper type 1 effector cells (Th1) secrete high levels of IFN-γ and IL-2 and mediate mainly cellular immune responses. T-helper type 2 effector cells (Th2) produce mainly IL4, IL5, IL10 and IL13, support humoral immune responses and downregulate Th1 responses. Depending on the subtype and the cytokine profile, DC can direct T-cells towards type 1 or type 2 responses.

One of the main type 1-polarizing cytokines is IL-12p70. Different types of pathogens and pathogen-derived products can induce DC to secrete high levels of IL-12p70. This includes *Leishmania* [154], some types of live bacteria [155-157] and live viruses [158] as well as their derivatives. Additionally, DC can secrete IL-12p70 in response to CD40L activation [142-144]. The production of IL-12p70 can be enhanced by various cytokines, e.g. IFN-α and IFN-γ [143, 144], IL-1β and IL-4 [142]. Other cytokines, produced by DC, such as IL-23, IL27, IL18 and type I IFN can synergize with IL-12 to promote Th1 responses (reviewed in [159]).

Th2 polarizing signals are less well defined than Th1-directing signals. Absence of IL-12 is a prerequisite for the induction of Th2 responses. Some products released by parasites, e.g. *Schistosoma mansoni* [160] are known to trigger TLR2 and promote a Th2 response. Additionally, IL-4, OX-40L and several chemokines (e.g. MCP-1) are proposed to play a role in Th2 polarization [159].

Inducing tolerance

DC are capable of inducing tolerance by at least two different mechanisms. Immature DC can induce abortive proliferation and subsequent depletion and/or anergy of antigen-recognizing T-cells [149, 151]. Additionally, both, iDC [161] and mature DC [162], were reported to activate regulatory T-cells in mouse and human systems. IL-10 was implicated as one of the main factors involved in the induction of regulatory T-cells. However, several other molecules, including inhibitory members of the B7 family (e.g. PD-L1, PD-L2, B7-H3 and B7-H4) and the immunoglobulin-like transcripts 3 and 4 (ILT3 and ILT4) may also be involved in this process (reviewed in [159]). DC can be modulated to become tolerogenic in culture by IL-10, TGF-β or corticosteroids [163]. Depletion of antigen-recognizing T-cells as well as activation of regulatory T-cell subsets by DC is an important mechanism of maintaining peripheral tolerance to self antigens.

1.3 DC-Based Cancer Immunotherapy

DC have the unique capacity to stimulate both humoral and cell-mediated immune responses. Therefore, they are attractive candidates for active vaccination strategies. The ability of DC-based vaccines to induce effective immune responses is currently being evaluated in mouse models as well as in human clinical studies. One of the main clinical applications of DC-based vaccinations is immunotherapy of cancer.

The two main sources of DC used in clinical applications are 1) proliferating $CD34^+$ precursors and 2) non-proliferating $CD14^+$monocytes. $CD34^+$ DC precursors can be separated from bone marrow or peripheral blood following stem cell mobilization by Flt3 or granulocyte-colony stimulating factor (G-CSF) administration. $CD34^+$ cells can then be differentiated into DC by culturing with different cytokine combinations, including TNF-α, granulocyte macrophage-colony stimulating factor (GM-CSF), Flt3 ligand and CD40L. $CD14^+$ monocytes can be obtained in large amounts from peripheral blood. Immature DC can be generated from $CD14^+$ monocytes by culturing for 5-7 days in the presence of GM-CSF and IL-4. Immature DC can further be induced to mature by different activation stimuli.

1.3.1 Antigen loading strategies

The most common strategy used for antigen loading of DC is pulsing them with synthetic peptides. These peptides represent epitopes derived from tumor-proteins. Synthetic peptides are easy to produce on large scale and the antigen-loading process can be well-controlled. Moreover, the immune response will be mainly epitope-specific and is easy to monitor. However, this approach has certain disadvantages. The application is restricted by the patient's HLA type: tumor-derived epitopes have to be defined for individual HLA alleles. Moreover, this approach has mainly focused on inducing CD8+ T-cells, ignoring that the CD4+ help is often needed for efficient anti-tumor response [164]. The need for CD4+ help, however, can be overcome depending on the type of DC maturation stimuli used [165-167].

Several clinical studies employed DC pulsed with tumor-derived peptides. These studies were mainly performed on patients with advanced metastatic melanoma [168-172], as a number of melanoma-associated antigens have been well characterized. Metastatic prostate, breast and ovarian cancers were also targeted by DC-peptide therapy [173, 174]. In these studies, expansion of antigen-specific cytotoxic $CD8^+$ T-

lymphocytes occurred in a significant fraction of patients. Clinical responses could be detected in approx. 30% of patients. Recently, Tuettenberg and coworkers demonstrated induction of long-lived tumor antigen-specific T-cells in stage II melanoma patients after vaccination with DC pulsed with MelanA/MART-1 peptide. 10 out of 13 patients after the vaccination remained disease-free with the follow-up period of 21-33 months [175].

Importantly, epitope-spreading after vaccination with a single peptide has been observed, demonstrating the generation of immune responses against tumor antigens different from those used for vaccination [173, 176, 177].

Purified or recombinant proteins are used as an alternative strategy for DC loading with tumor-specific antigens [178, 179]. This approach has the following advantages: 1) the need of characterization of the individual epitopes is circumvented; 2) no selection for the patient's HLA type is needed; 3) the protein contains epitopes for both classes of MHC and thus $CD4^+$ and $CD8^+$ can be stimulated simultaneously. The protein is most efficiently uptaken by immature DC. However, for efficient T-cell stimulation, fully mature DC are required. Therefore, the DC maturation stage has to be carefully controlled. Moreover, it is difficult to control efficiency of DC loading if the exact epitopes are not known. The same applies for the monitoring of immune responses after vaccination.

Other strategies of DC loading with a broad spectrum of tumor-derived antigens include entire tumor cell lysates [170, 180], apoptotic tumor cells or the fusion of DC with tumor cells [181, 182]. This allows to generate both, $CD4^+$ and $CD8^+$ T-cell responses simultaneously against a broad range of tumor associated antigens. This strategy may also reduce tumor escape from immune recognition by clonal selection of antigen-negative tumor cells, which occurs upon immunization with a limited number of epitopes. This strategy, however, is limited by the availability of sufficient tumor tissue. The potential risk of inducing autoimmunity against shared self-antigens should also be considered.

Another way of antigen loading is DC transfection with RNA (tumor antigen-specific or total tumor RNA) or DNA (e.g. recombinant viruses, encoding tumor antigens). This method, although efficient [183-186], is technically more demanding than the other strategies mentioned above.

1.3.2 Enhancing the efficiency of anti-tumor vaccines by manipulating DC *in vitro*

The efficiency of a DC-based vaccination depends on the maturation state and functional properties of the DC. The first recognition of the importance of DC maturation state came from several clinical studies that demonstrated that immature, but not mature DC can induce antigen-specific inhibition of effector T-cell function and induction of regulatory T-cells [161, 187, 188]. Mature DC, however, depending on the time of activation, may have already exhausted the production of cytokines, such as IL-12p70, at the time they reach lymph nodes, and therefore fail to activate an efficient immune response [189]. The type of maturation stimuli used for the activation of DC also matters for the outcome of vaccination. For example, Bullock and coworkers demonstrated that DC preactivated via CD40L and, to a less extent, via TLR4 or TLR9 ligands were capable of activating $CD8^+$ T-cells independently of $CD4^+$ help. They could correlate this effect with the surface expression of CD70 costimulatory molecule, which was not induced by other activation stimuli [165].

The dose and frequency of vaccination is another variable that effects the induction and maintenance of T-cell responses. Several mouse experiments show that weekly administration of peptide-pulsed DC led to diminished antigen-specific T-cell activity [190] either due to clonal exhaustion of expanded T-cells or due to rapid lysis of antigen-pulsed DC by $CD8^+$ T-cells [191]. Therefore, the frequency of vaccine administration has to be optimized to induce persistent antigen-specific responses in tumor patients.

Another important issue for using DC as adjuvants for vaccination is the route of delivery of DC. Several mouse and human studies reported dependence of vaccination efficiency on the route of delivery of DC. Intradermal or subcutaneous injections were found to be superior as compared to intravenous injection [190, 192]. The dependence of the vaccination efficiency on the route of DC delivery could reflect the ability of ex vivo activated DC to migrate towards lymph nodes. It was recently estimated in human and mouse experiments, that upon intradermal injection approximately 5% of DC reach the lymph nodes [193, 194]. Therefore, enhancing the migration ability of DC could also improve the vaccination efficiency.

DC-based vaccination is a rapidly developing field of immunotherapy. Early clinical trials and mouse models provided a proof of principle and established general safety of this approach. The capacity of DC to direct cancer-specific responses was demonstrated in a number of mouse and human trials. Intensive research is focused on improving the effectiveness of DC-based vaccines. The generation of successful anti-tumor vaccines requires a better understanding of the fundamental biology of DC as well as establishing and characterization of tumor-associated antigens that can be used for vaccinations.

1.4 Motivation and scope of the thesis

The first part of this work is focused on identification and characterization of new potential MHC class I antigens for active vaccination against hematological malignancies in context of stem cell transplantation.

Hematological malignancies are among the leading forms of cancer. Autologous and allogeneic stem cell transplantation (SCT) is often used as a therapy mainly for patients with hematological malignancies who relapsed after standard chemotherapy, or even as a primary therapy, in the case of multiple myeloma. However, additional therapeutic strategies are required to improve clinical outcome of conventional therapeutic approaches. One possibility to increase the therapeutic effect is provided by immunotherapy after SCT.

The most serious complication after allogeneic SCT is graft versus host disease (GvHD). Although GvHD is an important cause of mortality after allogeneic SCT, patients developing GvHD achieve higher rates of disease remission, than patients without GvHD [195]. This suggests that GvHD is accompanied by immune reaction against malignant cells (graft vs leukemia/lymphoma, GvL). T-lymphocytes play an important role in both GvHD and GvL after allogeneic SCT [195]. Although little is known about antigens that are recognized by T-cells, GvHD and GvL effects presumably involve polymorphism of normal cellular proteins. These proteins are present on both normal and malignant stem cells but are not shared between recipient and donor. Peptides derived from polymorphic regions of these proteins can be presented in the context of HLA and are named minor histocompatibility antigens. Minor histocompatibility antigens (mHA) specifically expressed on recipients' cells can be recognized by donor T-cells. In the case of complete MHC match between donor and recipient, GvHD is considered to be the consequence of immune responses against mHA, specifically expressed on recipients' cells. These mHA have a wide tissue distribution, including skin, liver, and gastrointestinal tissues. In contrast, antigens that are expressed only by blood cells could induce an exclusive graft vs hematopoietic (GvH) reaction [196] that can include putative GvL effect. As the hematopoietic system of recipient is replaced by donor stem cells, this immune reaction will not induce a severe clinical pathology. Therefore, mHA that are

expressed exclusively by hematopoietic cells represent attractive candidates for vaccination after SCT.

In unrelated allogeneic SCT, the majority of HLA-A, -B, -C and HLA-DR-matched recipient-donor pairs show mismatches in HLA-DP [197]. Single allele mismatches in HLA-DP do not appear to influence the risk of acute GvHD, whereas patients mismatched for two HLA-DP alleles have a significantly increased risk of GvHD [198], suggesting that immune responses against HLA-DP could be involved in GvHD development. Indeed, a $CD4^+$ response against HLA-DP was previously demonstrated in a patient [199, 200]. However, the major role in GvHD and GvL is attributed to $CD8^+$ T-cells. Therefore, in the context of HLA-DP-mismatched allogeneic SCT, the induction of donor $CD8^+$ T-cells specifically recognizing MHC class I antigens, derived from polymorphic region of the recipients' HLA-DP variant can represent a promising therapeutical approach.

A large subset of hematological cancers is represented by B-cell malignancies, including non-Hodgkin's lymphomas and multiple myelomas. B-cell malignancies appear to be suitable candidate targets for immunotherapy approaches, as a number of surface markers are well characterized. These include markers that are tumor-specific or shared by B–cell malignancies and normal B-cells.

Immunotherapy against B-cell malignancies may include monoclonal antibodies as well as T-cell therapy. Most studies on T-cell mediated therapy have targeted idiotypic sequences of immunoglobulin molecule. B-cell malignancies are usually derived from a single expanded B-cell clone, which expresses an immunoglobulin (Ig) with a unique idiotype (variable regions of Ig). Therefore, the idiotype can be regarded as a tumor-specific antigen. However, idiotype vaccine has to be produced individually for each patient. The alternative approach is to vaccinate against the isotype (i.e. heavy chain constant regions) of the Ig expressed on malignant cells. Ig-isotypes are not unique for the individual B-cell malignancy but are shared by many patients. An immune response against immunoglobulin isotypes may lead to elimination of malignant cells, as well as target a subset of B-cells and their progenitors. Thus, antigens, derived from immunoglobulin constant regions represent potential targets for vaccination against B-cell malignancies.

The aim of the first part of this study was to identify and characterize antigens derived from HLA-DP alleles and from Ig constant regions as potential targets for vaccination after SCT.

The generation of successful anti-tumor DC-based vaccines requires better understanding of the fundamental biology of DC. Therefore, the second part of this work is focused on the intracellular signaling pathways that regulate different functions of DC, in particular, glycogen synthase kinase 3 (GSK-3) pathway.

GSK-3 is a multifunctional enzyme now recognized as a key regulator of numerous signaling pathways. Broad regulatory influence on cellular function: cellular structure, growth, motility, apoptosis. Due to its involvement in numerous signaling pathways, some of which are known to influence DC differentiation, GSK-3 is a candidate regulator of DC function.

GSK-3 is linked to a diverse array of neurologic diseases, such as bipolar mood disease [201, 202], schizophrenia [203] and Alzheimer‘s disease [204], but also to non-neurologic diseases such as diabetes mellitus [205] and cancer [206-208]. Interestingly, most of these diseases are associated with immune deviations. Particularly, patients suffering from bipolar mood disorders and schizophrenia, who were reported to have an increased baseline GSK-3 activity [203], also have increased serum levels of pro-inflammatory cytokines such as IL-1β [209], IL-6 [210, 211] and IL12p70 [212]. Chronic treatment with the GSK-3 inhibitor lithium normalizes these deviations together with its therapeutic psychotropic effect. Furthermore, lithium has successfully been employed as a treatment in animal models of autoimmune diseases [213]. Recently, Martin et al. have demonstrated that GSK-3 inhibitors enhance IL-10 secretion and inhibit IL-12p40, IL-6 and TNF-α secretion of human monocytes [214]. Furthermore, mice challenged with endotoxin could be rescued from death by septic shock using GSK-3 inhibitors [214].

Based on these observations, the purpose of the second part of this work was to investigate the role of GSK-3 in DC differentiation and function.

2 Materials and methods

2.1 Materials

2.1.1 Antibodies

Primary antibodies for flow cytometry:

Anti human CD3-FITC	BD-Pharmingen, Heidelberg, Germany
Anti human CD14-FITC	BD-Pharmingen
Anti human CD19-PE	BD-Pharmingen
Anti human CD80-FITC	BD-Pharmingen
Anti human CD83-PE	BD-Pharmingen
Anti human CD86-PE	BD-Pharmingen
Anti human HLA-A2.1 (BB7.2), purified	BD-Pharmingen
Anti human HLA-ABC-FITC	BD-Pharmingen
Anti human HLA-DR-PE	BD-Pharmingen
Anti human IgD-PE	BD-Pharmingen
Mouse isotype control IgG2a-FITC	BD-Pharmingen
Mouse isotype control IgG1-PE	BD-Pharmingen
Mouse isotype control IgG2a-PE	BD-Pharmingen

Secondary antibodies for flow cytometry:

Goat anti mouse IgG, PE-labeled	BD-Pharmingen

Antibodies for enzyme-linked immunospot:

Anti human IFN-γ (clone), purified	BD-Pharmingen
Anti human IFN-γ (clone), biotinylated	BD-Pharmingen

Primary antibodies for Western Blot:

phospho-Akt (Ser473)	Cell Signaling, Frankfurt am Main, Germany

phospho-GSK3α/β (Ser21/9)	Cell Signaling
phospho-GSK3α/β (Tyr279/216)	BioSource GmbH, Solingen, Germany
β-catenin	Dianova GmbH, Hamburg, Germany
p38	Santa Cruz, Heidelberg, Germany
ERK-1/2	Santa Cruz
β-actin	MP Biomedicals, Illkirch, France

Secondary antibodies for Western Blot:

HRP-conjugated antibodies to rabbit IgG	Santa Cruz

2.1.2 ELISA sets, cytokines, chemokines and other materials

Cytokine ELISA-sets (OptEIA brand) for IFN-γ, IL-6, IL-12p40, IL-12p70, TNF-α and IL-10 were purchased from BD Pharmingen. IL-23 ELISA kit was purchased from Hölzel Diagnostika, Cologne, Germany.

The following cytokines were used: recombinant human (rh) GM-CSF (50 ng/ml, Berlex, Seattle, WA), rhTNF-α (10 ng/ml), rhIL-4 (50 U/ml) and IFN-α2a (2000 IU/ml) (all from PromoCell, Heidelberg, Germany). PGE_2 (used at 1 μM final concentration) was purchased from MP Biomedicals, Costa Mesa, CA, USA. Lipopolysaccharide (LPS from *E.coli* 055:B5) was purchased from Calbiochem, Merck Biosciences GmBH, Darmstadt, Germany. CCL21 (6Ckine) was purchased from PromoCell and used in migration assays at 40 ng/ml. IL-2 (Proleukin®) was purchased from Pharmacy, University Clinic Heidelberg, Germany. IL-7 was purchased from PeproTech EC Ltd., London, UK.

2.1.3 Protein kinase inhibitors

The following GSK-inhibitors were used: LiCl (Calbiochem), SB216763 and SB415286 (Sigma-Aldrich, Taufkirchen, Germany), 1-azakenpaullone (Calbiochem). The membrane permeable synthetic cAMP analogue and activator of protein kinase A (PKA) (Sp-5,6-DCl-cBIMPS) was purchased from Biolog, Bremen, Germany. The PI3K inhibitor Wortmannin was purchased from Sigma-Aldrich.

2.1.4 Media

RPMI 1640	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Iscove's MDM	Sigma-Aldrich Chemie GmbH
DMEM	Sigma-Aldrich Chemie GmbH

2.1.5 Cell lines

T2 (174 x CEM.T2)	human HLA-$A2^+$, MHC class II negative T-B lymphoblast hybrid was cultured in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich Chemie GmbH), with 60 mg/L penicillin G, 12.6 mg/L streptomycin, 2 mM L-glutamine.
BHK-CD40L	Baby Hamster Kidney (BHK) cells transfected with CD40L were cultured in DMEM containing 1 mM sodium pyruvate, 10% heat-inactivated FCS, with 60 mg/L penicillin G, 12.6 mg/L streptomycin, 500µg/ml G418, 2 mM L-glutamine.
BHK-mock	Baby Hamster Kidney (BHK) cell line transfected with an empty plasmid vector (pACF) was cultured similar to BHK-CD40L cells.
IM9, KMS-12-BM and LP-1	human multiple myeloma cell lines were cultured in RPMI supplemented with 10% heat- inactivated FCS, with 60 mg/L penicillin G, 12.6 mg/L streptomycin, 2 mM L-glutamine.

T2 cell line (ATCC number CRL-1992™) was purchased from American Type Culture Collection (LGC Promochem GmbH, Wesel, Germany). BHK-CD40L and BHK-mock cell lines were a gift of Dr. E. Leo, University of Heidelberg. Human multiple myeloma cell lines (IM9, KMS-12-BM and LP-1) were kindly provided by Dr. M. Hundemer, University Clinic, Heidelberg.

2.1.6 Bacteria

E. coli (XL-1) were cultured in LB-Broth* in a bacteria shaker (New Brunswick Scientific Co., Edison, USA) at 37 °C until an OD_{600} of 0.7. *E. coli* were then washed three times, resuspended in 60 mM CaCl2, 10 mM PIPES pH 7.0, 15% glycerol and frozen in 50 µl aliquots. Directly prior to activation, one *E. coli* aliquot was thawed, washed in double distilled water (dd H_2O) and resuspended in 1 ml H_2O. 20 µl of this solution was added to 1 ml of DC cultures, this concentration is referred to as 1:1.

* LB-Broth medium	10 g NaCl
	10 g tryptone
	5 g yeast extract
	The mixture was dissolved in dd H_2O to a final volume of 1 liter and autoclaved.

2.1.7 Synthetic peptides

Purified peptides were obtained as lyophilized powder from Dr. R. Pipkorn (Peptide synthesis lab, DKFZ, Heidelberg). Peptides were reconstituted in sterile DMSO to a concentration of 10mg/ml and then further diluted with PBS to the concentration of 1 mg/ml. Reconstituted peptides were stored in 50-100 µl aliquots at -80°C.

2.2 Methods

2.2.1 Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coat preparations or directly from fresh blood of healthy donors from the Red Cross Blood Bank (Heidelberg, Germany). The blood was diluted 1:1 with sterile PBS and carefully layered on 15 ml of Biocoll solution (contains Ficoll®400, Biochrom AG, Berlin, Germany) in 50 ml Falcon tubes. The blood was then centrifuged for 20 min, at 1100 g at room temperature. Resulting interphase was transferred to fresh 50 ml tube and washed twice with sterile PBS. PBMC were then counted and either used immediately for preparation of $CD14^+$ monocytes, B-cells or CTL lines, or frozen in freezing medium containing 45% FCS, 45% hetastarch (Sigma-Aldrich Chemie GmbH) and 10% DMSO (Merck Biosciences, Darmstadt, Germany).

2.2.2 $CD14^+$ monocytes and monocyte-derived DC (MoDC)

$CD14^+$ monocytes were affinity purified from PBMC of healthy donors using the MACS CD14 isolation kit (Miltenyi Biotech, Germany) according to manufacturer's instructions. $CD14^+$ monocytes were either activated immediately or cultured at 0.5-1×10^6 cells/ml in standard culture medium (RPMI with 10% heat-inactivated FCS, with 60 mg/L penicillin G, 12.6 mg/L streptomycin, 2 mM L-glutamine) supplemented with GM-CSF (50 ng/ml), and IL-4 (50 U/ml) in 24-well plates. By day 5-7, MoDC represented more than 95% of cultured cells. On day 5-7, cells were washed and readjusted to $2\text{-}3\times10^5$ DC per ml. The following maturation-inducing factors were added: a mixture of pro-inflammatory cytokines (rhGM-CSF (50 ng/ml), rhTNF-α (10 ng/ml), IFN-α2a (2000 IU/ml) and PGE2 (1 μM)), BHK-CD40L (DC to BHK-CD40L ratio of 20:1), *E. coli* at high (1:1) and low (1:1000) concentrations or LPS (1 μg/ml). All cytokines and DC stimuli used in the present study have previously been tested in dose-titration analyses, and the concentrations used in the figures represent those found to be optimal. Dendritic cells and supernatants were harvested 24-36 hours after adding the maturation stimuli and analyzed for migration capacity, expression of surface markers and secretion of cytokines. Alternatively, DC were harvested after 6 hours of activation, washed twice with sterile PBS, pulsed with 10μg/ml of synthetic peptide in serum-free RPMI medium (1 hour, 37°C) and used as stimulator cells for peptide-specific CTL lines.

2.2.3 CD40-activated B-cells

B-cell lines were generated from the PBMC of healthy donors based on the method published by Schultze (modified from [215]). B-cells were stimulated via CD40 using BHK cell line transfected with the human CD40L (BHK-CD40L). Expression of CD40L by BHK cells was confirmed by flow cytometry using an anti-CD40L mAb. BHK cells do not express human MHC class I and II and therefore do not activate $CD4^+$ and $CD8^+$ T-cells. For B-cell cultures, BHK-CD40L cells were irradiated (96Gy). PBMC (2×10^6 cells/ml) were cocultured in 6-well plates with BHK-CD40L (2×10^4 cells/ml) in presence of IL-4 (20U/ml) and 1×10^{-6} M cyclosporin A (CsA) in Iscove's MDM supplemented with 10% pooled human serum, 1×ITS (Insulin-Transferrin-Selenium supplement, Gibco®, Invitrogen GmbH, Karlsruhe, Germany), 15μg/ml gentamycin at 37°C in a humidified 5% CO_2 incubator. The medium was

changed and the fresh irradiated BHK-CD40L cells were added every 3-4 days. The purity of B-cell population was assessed by flow cytometry using anti-CD19 and anti-CD3 mAB starting from week three of culture.

2.2.4 Peptide-specific CTL lines

Peptide-specific CTL lines were generated from PBMC of healthy HLA-A2.1$^+$ donors. CD8$^+$ T-cells were purified from PBMC of healthy donors using MACS CD8 isolation kit (Miltenyi Biotech, Germany) on day 0. Stimulator cells (γ-irradiated (100Gy) T2 cells or autologous, preactivated DC, as specified in text) were washed twice with PBS and preincubated with 10μg/ml of synthetic peptide in RPMI (serum free) for 1 hour at 37°C. CD8$^+$ T-cells at the concentration of 1×10^6 cells/ml were cocultured with 2×10^5 stimulator cells/ml in T-cell medium (Iscove's MDM containing 10% pooled heat-inactivated human serum, with 60 mg/L penicillin, 12.6 mg/L streptomycin, 2 mM L-glutamine, 0.55 mM arginine, 0.24 mM asparagine) in 2 ml cultures in 24-well plates at 37°C, 10% CO_2. On day 3, IL-2 and IL-7 were added to CTL cultures to the final concentration of 20 U/ml IL-2 and 10 ng/ml IL-7. Starting from day 7, the CTL cultures were harvested weekly, washed twice with PBS, adjusted to the concentration of 1×10^6 cells/ml and restimulated with peptide-pulsed stimulator cells (prepared as described above) in the presence of 20 U/ml IL-2 and 10 ng/ml IL-7. Fresh T-cell medium with cytokines was added every 3-4 days.

Alternatively, PBMC at the concentration of 2×10^6 cells/ml were cocultured with peptide-pulsed stimulator cells (2×10^5 autologous DC/well + 1×10^5 irradiated T2 cells/well). The cultures were treated as described above, except for purification of CD8$^+$ T-cells from the cultures on day 7 using MACS CD8 isolation kit. CD8$^+$ T-cells were then readjusted to the concentration of 1×10^6 cells/ml, rested overnight in T-cell medium and further restimulated weekly with peptide-pulsed, preactivated autologous DC (2×10^5 DC per well).

2.2.5 CTL cloning

Peptide-specific CTL lines were cloned by limiting dilution in 96-well round-bottom plates. Each well received 1×10^4 γ-irradiated (30 Gy) autologous PBMC pulsed with corresponding peptide. T cells from previously generated peptide-specific T cell lines were added in 200 μl T-cell medium supplemented with 50 U/ml IL-2 and 10 ng/ml

IL-7. Three independent dilutions were made containing 10, 3, and 1 responder T cell from peptide-specific T-cell lines per well. Every 3-4 days half of the medium was removed and substituted with fresh T cell medium containing 50 U/ml IL-2 and 10 ng/ml IL-7. At days 7 and 14 of culture, T-cells were restimulated with 1×10^4 irradiated, peptide-pulsed autologous PBMC. After 3 weeks, wells exhibiting T-cell growth were identified under the microscope, and transferred into new 96-well plates for testing peptide-specificity and further cultivation. To test peptide-specificity, 1×10^4 irradiated (100 Gy) T2 cells pulsed with corresponding peptide were added to each well in 150 µl T-cell medium without cytokines. The supernatants were harvested 16 hours later and 200 µl fresh T-cell medium with 50 U/ml IL-2 and 10 ng/ml IL-7 was added to each well. The supernatants were then analyzed for IFN-γ in ELISA. The clones were further cultivated for additional 10 days; every 3-4 days half of the medium was substituted with fresh T-cell medium with IL-2 and IL-7. After 10 days, the clones that secreted IFN-γ in the first test were transferred into new plates, each divided between two wells. As in the first test, one well per clone was restimulated with T2 cells pulsed with the corresponding peptide and the second well was restimulated with T2 cells without the peptide as a negative control. The supernatants were harvested 16 hours later and tested in IFN-γ ELISA.

2.2.6 T2 binding assay

T2 cells were washed three times with PBS and resuspended in serum-free RPMI medium at the concentration of 1.5×10^6 cells/ml. T2 cells were then incubated overnight in 50 µl of serum-free RPMI in presence or absence of 50 µg/ml peptide in U-bottom 96-well plate at 37°C, 5%CO_2. The peptide binding to HLA-A2 was assessed by flow cytometry using HLA-A2.1 specific mAB (clone BB7.2) that recognizes stabilized HLA-A2.1 complexes. The fluorescence index (FI) was calculated from the mean fluorescence intensities (MFI) according to the following formula:

$$FI = \frac{MFI(T2 + peptide) - MFI(T2)}{MFI(T2)}$$

2.2.7 Flow cytometry

For phenotypic analysis, the cells were resuspended in PBS+2% heat-inactivated human serum and labeled with primary antibodies. The majority of the primary antibodies were directly fluorochrome-labeled except for anti-HLA-A2.1. For anti-HLA-A2.1, after labeling with the primary antibody the cells were washed 3 times with PBS and incubated with the secondary antibody. The antibody labeling was performed for 5 minutes at room temperature, except for anti-CCR7 and anti-HLA-A2.1 antibody (30 minutes at 4°C). Cells were washed three times with PBS buffer and either resuspended in PBS and analyzed immediately or fixed in PBS+4% formaldehyde. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest software (Becton Dickinson).

2.2.8 Phagocytosis assay

Effector cells were washed twice with PBS and incubated with *Saccharomyces cerevisiae* at a concentration of 10^6 cells/ml at 37°C in culture medium. After 1 hour, cells were washed and stained with Hematoxylin/Eosin. Yeast particles phagocytosed to more than 50 % were counted under the inverted microscope (Leica DMIL, Leica Mikrosysteme Vertrieb GmbH, Bensheim, Germany) in a total of 100 cells. Percent phagocytosis (number of cells containing yeast per 100 cells) and phagocytic index (mean number of yeast particles per phagocytic cells) were calculated.

2.2.9 Migration of dendritic cells

MoDC matured with the indicated stimuli for 48 hours were harvested, washed and tested for migration toward the chemokine CCL21 (6Ckine, a ligand for CCR7) in a transwell migration assay. Lower chambers of transwell plates (5.0 µm pore size, Costar, Corning, NY) were filled with 350 µl RPMI containing 10% FCS with or without CCL21 (40 ng/ml). $1\text{-}2\times10^4$ DC were added in 50 µl RPMI/10%FCS into the upper chamber. After 3-4 hours of incubation (37°C, 5% CO_2) the upper chambers were removed. Cells in the lower chambers were harvested, and counted using a hemocytometer. For each protocol, migration towards CCL21 and towards medium without chemokine was performed in duplicate wells.

2.2.10 Cytokine ELISA

Cytokine secretion by stimulated MoDC was measured by ELISA. IFN-γ, IL-6, IL-12p40, IL-12p70, TNF-α and IL-10 ELISA were performed according to the manufacturer's instructions. Briefly, the Maxisorp plates (Nunc, Wiesbaden, Germany) were coated with 50 µl/well Catch Antibody solution in Coating Buffer* overnight at 4°C. After overnight incubation, the antibody solution was removed and the plates were blocked with 300 µl PBS containing 10% FCS for 1 hour. The plates were next washed once with PBS containing 0.05% Tween-20 (PBS-T). The supernatants (50 µl/well) were incubated on ELISA plates for 2 hours at room temperature. The titration of the standard cytokine solution was included in each plate. Each condition was performed in duplicate wells. After 2 hours of incubation, the plates were washed 5 times with PBS-T and incubated with 50 µl/well detection solution (Detection Antibody + Streptavidin-HRP reagent) for additional 1 hour at room temperature. The plates were next washed 6 times with PBS containing 0.05% Tween-20 and 100 µl substrate solution was added to each well. The HRP-substrate was tetramethylbenzidine (TMB) + hydrogen peroxide (BD Pharmingen); the color reaction was terminated by adding 50 µL sulphuric acid (2 N) to each well. Plates were read in a Sunrise microplate ELISA reader (Tecan, Salzburg, Austria) using Magellan software (Tecan).

* Coating Buffer: 0.1 M Sodium Carbonate, pH 9.5

2.2.11 The enzyme-linked immunospot (ELISPOT) assay

ELISPOT assays were performed in 96-well PVDF membrane plates (MultiScreen®, Millipore, Schwalbach, Germany). ELISPOT plates were pre-wetted with 80% ethanol for 10 minutes. The plates were washed twice with sterile PBS and coated overnight at 4°C with 50 µl/well of the capture anti-human IFN-γ mAB solution (20 µg/ml). After overnight incubation, the antibody solution was removed and the plates were washed once with sterile PBS. In order to prevent unspecific protein binding, the plates were incubated with RPMI medium supplemented with 10% FCS for 2 hours at room temperature. The blocking solution was then removed and the plates were washed once with sterile RPMI. CTL were cocultured overnight (37°C, 5%CO_2) with target peptide-pulsed T2 cells in ELISPOT plates in 200 µl of Iscove's MDM

supplemented with 10% pooled heat-inactivated human serum at the concentration of 5×10^4 CTL and 10^4 T2 cells per well. Each condition was performed in duplicate wells. After overnight incubation, the cell suspension was removed and the wells were washed 6 times with 200 µl/well of PBS containing 0.05% Tween-20. The plates were further incubated with 100 µl of biotinylated detection anti-human IFN-γ mAB solution in PBS containing 10% FCS (the final antibody concentration is 0.5 µg/ml) for 2 hours at room temperature. The wells were then washed 6 times with PBS-T and 100 µl/well of Avidin-HRP solution (25 µg/ml in PBS with 10% FCS) was added for 1 hour at room temperature. The wells were washed 4 times with 200 µl PBS+0.05% Tween-20, then 2 times with PBS. 100 µl of Substrate Solution* was added to each well for 5-15min. As soon as the spots developed, the reaction was stopped by washing the plates under the tap water. The plates were air dried at room temperature in the dark. The spots were enumerated under the stereomicroscope (Nikon SMZ800, Nikon GmbH, Düsseldorf, Germany) using Lucia Measurement Version 4.8 software (Nikon GmbH).

* Substrate Solution: 10mg/ml stock solution of 3-amino-9-ethyl-carbazole (AEC) in N,N-dimethylformamide was prepared. 333.3 µl of AEC stock solution was mixed with 10 ml of 0.1 M Acetate Solution (pH 5.0) and filtered through 0.45 µm membrane filter. 5 µl of 30% H_2O_2 were added immediately before use.

2.2.12 Western Blot analysis

MoDC activated for 2 to 24 hours with the indicated stimuli were harvested, washed, resuspended at a density of $5x10^6$ cells/ml in Western Sample Buffer* and snap frozen. Prior to analysis, lysates were thawed, heated for 3 minutes to 96°C and homogenized with a sonicator. 10 µl extract (corresponding to 5×10^4 cells) per lane was loaded onto 10% SDS–polyacrylamide gel and proteins were separated by electrophoresis using the BioRad Minigel system (Bio-Rad Laboratories GmbH, Munich, Germany). The proteins were transferred to Hybond-P PVDF membrane (Amersham Biosciences Europe GmbH, Freiburg, Germany) by electroblotting for 1 hour using BioRad Trans-Blot apparatus. The membrane was incubated with Blocking Buffer** for 2 hours at room temperature. Primary antibodies were then added in

Blocking Buffer + 0.1% Tween 20 for overnight incubation at 4°C or for 2 hours at room temperature. After washing 4 times for 5 minutes in Blocking Buffer + 0.1% Tween 20, secondary antibodies were applied in Blocking Buffer for 2 hours at room temperature. Membranes were washed once with Blocking Buffer + 0.1% Tween for 15 minutes and additional 3 times for 5 minutes. Membranes were developed with ECL Western blot system (Santa Cruz). For quantitative Western blot analysis, the ECL-signal was quantified using the Lumi-Imager F1 and the LumiAnalyst Version 3.1 for Windows Software (Roche Diagnostics GmbH, Germany).

* Western Sample Buffer:	100 mM Tris-HCl, pH 6.8
	4% SDS
	0.2% Bromophenol-Blue
	20% Glycerol
	200 mM DTT
** Blocking Buffer:	PBS + 5% nonfat milk powder

2.2.13 Real-Time RT-PCR quantification

This assay was performed by Dr. Th. Giese, University clinic, Heidelberg and Dr. M. Conzelmann, DKFZ, Heidelberg.

2 x10^6 cells were collected in 400µl lysis buffer from the MagnaPure mRNA Isolation Kit I (RAS, Mannheim, Germany) supplemented with 1% (w/v) DTT and mRNA was isolated with the MagnaPure-LC device using the mRNA-I standard protocol. The elution volume was set to 50µl. An aliquot of 8.2 µl RNA was reverse transcribed using AMV-RT and oligo- (dT) as primer (First Strand cDNA synthesis kit, RAS) according to the manufactures protocol in a thermocycler. After termination of the cDNA synthesis, the reaction mix was diluted to a final volume of 500 µl and stored at –20˚C until PCR analysis.

Primer sets specific for the sequences of IL-12p35, IL-12p40, IL-6, IL-10 and TNF-α optimized for the LightCycler (RAS) were developed and provided by SEARCH-LC GmbH, Heidelberg (www.search-lc.com). The PCR was performed with the LightCycler FastStart DNA Sybr GreenI kit (RAS) according to the protocol provided

in the kits. To control for specificity of the amplification products, a melting curve analysis was performed. Only specific products were observed. The copy number was calculated from a standard curve, obtained by plotting known input concentrations of four different plasmids at log dilutions to the PCR-cycle number at which the detected fluorescence intensity reaches a fixed value. This approach dramatically reduced variations due to handling errors over several logarithmic dilution steps.

To correct for differences in the content of mRNA, the calculated copy numbers were normalizes according to the average expression of two housekeeping genes, Cyclophilin B and β-actin. Values were thus given as input adjusted copy number per µl of cDNA.

2.2.14 Statistical analysis

The Students t-test was performed for significance analysis using Microsoft Excel program.

3 RESULTS

3.1 Searching for the new vaccination antigens

3.1.1 HLA-DP-derived antigens

In unrelated allogeneic stem cell transplantation, HLA-DP is mismatched between donor and recipient in more than 70% of transplanted patients [198]. In the context of HLA-DP-mismatched allogeneic SCT, polymorphic antigens derived from different HLA-DP alleles represent potential targets for immunotherapy. MHC class II expression, including HLA-DP, is restricted mainly to hematopoietic cells and their progeny, but the expression was also found on most malignant blood cells. The immune reaction against recipient HLA-DP allele variant may therefore eliminate the recipients' hematopoietic system together with the malignant cells. Thus, we identified and characterized polymorphic antigens derived from different HLA-DP alleles as potential targets for vaccination in context of allogeneic SCT.

3.1.2 HLA-DP sequence analysis and MHC class I binding epitope prediction

The sequences for human HLA-DP are available from Immunogenetics database (IMGT/HLA, http://www.ebi.ac.uk/imgt/hla/index.html). The database contains sequences for all officially recognized HLA alleles [216]. There are more than 20 alleles for HLA-DPA1 gene and around 430 alleles for HLA-DPB1. We analyzed these sequences using two different MHC-binding prediction tools: BIMAS (http://bimas.cit.nih.gov/molbio/hla_bind/, [217]) and SYFPEITHI (http://www.syfpeithi.de/, [218]). These tools employ two different computational algorithms for MHC-binding prediction based on the published information on the allele-specific binding motifs. They allow to locate the peptides within the sequence containing peptide-binding motifs for HLA class I alleles. This study focused on the HLA-A*0201 allele of MHC class I, due to its abundance in the human Caucasian population (almost 50% are HLA-A*0201^{+} [219]). The 9-mer and 10-mer peptides derived from polymorphic regions of the HLA-DP molecules with peptide-binding motifs for HLA-A*0201 were identified. These peptides are derived from 3 polymorphic regions within HLA-DPA1 and 8 polymorphic regions within HLA-DPB1. The results of the prediction analysis are summarized in Table 1.

Table 1 Identification of peptides derived from the polymorphic regions of the HLA-DR molecules. The protein sequences of all known HLA-DPA1 and HLA-DPB1 alleles were analyzed using BIMAS and SYFPEITHI MHC-binding prediction tools. The peptides derived from polymorphic regions of the HLA-DP molecules with peptide-binding motifs for HLA-A*0201 were identified. Highlighted in yellow are 20 peptides with the best prediction scores that were synthesized for further analysis. The polymorphic amino residues are shown in bold.

HLA-DP	start position	sequence	BIMAS score	SYFPEITHI score	HLA-DP alleles expressing the peptide variant
HLA-DPA1	9	FMFEFD**E**DE**M**	122.685	20	*01031, *01032, *0105, *0107, *0203, *0301, *0302
		FMFEFD**D**DE**M**	122.685	18	*0104, *0108, *0401
		FMFEFD**E**DE**Q**	0.368	13	*0106, *020**
	13	FD**E**DE**M**FYV	8.555	11	*01031, *01032, *0105, *0107, *0203, *0301, *0302
		FD**D**DE**M**FYV	53.468	12	*0104, *0108, *0401
		FD**E**DE**Q**FYV	3.720	11	*0106, *020**
	51/52	AI**L**NnNLN**TL**	24.997	25	All but *03 and *04
		AI**S**NnNLN**TL**	6.756	27	*03
		AI**L**NNNLN**IA**	2.384	21	*04
		I**L**NNNLN**TL**	83.527	30	All but *03 and *04
		I**S**NNNLN**TL**	0.545	20	*03
		I**L**NNNLN**IA**	7.964	22	*04
HLA-DPB1	164	QQGDVY**I**CQV	66.498	15	*01011
		QQGDVY**T**CQV	28.912	11	*02012, *04
	81	YEL**DEAV**TL	28.986	19	*010**, *030**, *0501-1401, *1601, *1701, *1901-2201,*25-27, *29-31, *35-38, *44, *45, *50, *52, *54-58, *63, *65, *67-70, *76, *78-79, *84-85, *87-91
		YEL**GGPM**TL	7.070	20	*02, *04, *23,*24, *31, *32, *39, *41, *46-49, *51, *59-60, *66, *71-73, *75, *77, *80-83, *86
		YEL**VGPM**TL	7.070	18	*15, *18, *28, *34, *40, *53, *62, *74
	28	YIYNR**EE**Y**A**	27.099	15	All but…
		YIYNR**EE**F**V**	379.384	21	*0201*, *0301*, *0402, *06-10, *14, *16-20, *23, *25, *29, *30, *32, *35, *37, *41, *45-47, *50, *52, *54, *55, *57, *59-61, *67-71, *73, *75, *77-80, *82-84, *86-88, *9101
		YIYNR**EE**L**V**	68.979	21	*0202, *0501, *21-22, *34, *36, *38, *44, *48, *58, *62-63
		YIYNR**Q**E**YA**	27.099	15	*7401

HLA-DP	start position	sequence	BIMAS score	SYFPEITHI score	HLA-DP alleles expressing the peptide variant
		YIYNR**EE**F**A**	27.099	15	*0401, *2401, *2801, *3301, *5101, *5601, *6601, *7201, *7601, *8101, *9001
	33/34	E**LV**RFDSDV	19.822	18	*0202, *0501, *21-22, *34, *36, *38, *44, *48, *58, *62-63
		QEYARFDSDV	16.445	11	*11, *15, *74
	64	DI**L**EE**K**RA**V** DI**L**EE**E**RA**V** DI**L**EE**E**RA**D** D**F**LEE**E**RA**V** D**F**LEE**K**RA**V** D**N**LEE**K**RA**V**	0.100	14	*03, *06, *11, *14-15, *20, *25, *28-29, *44-45, *50, *52, *56-57, *59, *61, *67, *69-70, *72-74, *76, *87, *91
		D**L**LEE**K**RA**V** D**L**LEE**R**RA**V** D**L**LEE**E**RA**V**	15.827	24	*31, *34
		DLLEE**K**RA**L**	4.861	24	*02, *04, *05, *06, *11, *15, *16, *17, *18, *20, *21, *22, *23, *24, *27, *28, *,46, *47, *48, *49, *51, *53, *55, *58, *59, *60, *62, *63, *66, *69, *71, *72, *74, *77, *80, *81, *82, *83, *85, *86
	75	R**M**CRHNYEL	15.428	21	*01, *08, *09, *10, *14, *25, *26, *29, *35, *37, *44, *45, *50, *54, *56, *57, *65, *67, *68, *70, *73, *75, *76, *78, *79, *84
		R**V**CRHNYEL	1.900	17	*01, *15, *18, *50, *03, *06, *11, *13, *20, *21, *25, *26, *27, *29, *36, *37, *44, *52, *56, *61, *69, *74, *78, *79, *85
	7	Y**VY**QG**R**QEC, Y**VY**QL**R**QEC	6.399	11	*02, *04, *05, *16, *19, *22, *23, *24, *28, *31, *32, *33, *34, *39, *40, *41, *46, *47, *48, *49, *51, *53, *59, *60, *62, *63, *65, *68, *71, *72, *73, *77, *80, *81, *82, *83, *84
		Y**L**FQG**R**QEC	84.555	17	*03, *06, *11, *13, *20, *21, *25, *26, *27, *29, * 36, *37, *44, *52, *56, *61, *69, *74, *78, *79, *85
		Y**V**H**Q**L**R**QEC	2.000	10	*09, *10, *14, *17, *30, *35, *45, *54, *55, *58, *66, *67, *76, *86
		Y**V**D**Q**L**R**QEC	0.800	9	*70
	9	**Y**Q**G**R**Q**ECYA, **F**Q**G**R**Q**ECYA	12.744	6	*01, *02, *04, *05, *08, *15, *16, *18-19, *21-23, *27, *31-34, *38-41, *46-51, *53, *57, *59-60, *62-65, *68, *71-73, *75, *80-84, *89-90
		YQ**L**R**Q**ECYA	47.151	8	*03, *06, *11-13, *20-21, *25-27, *29, *36-37, *44, *52, *56, *61, *69-70, *74, *78-79, *85, *87-88,
		HQ**L**R**Q**ECYA	0.349	6	*09-10, *14, *17, *30, *35, *45, *54-55, *58, *66-67, *76, *86, *91
		DQ**L**R**Q**ECYA	0.769	5	*70

We selected and synthesized 20 peptides derived from the polymorphic regions of the HLA-DP molecules with the highest probability of binding to HLA-A*0201 according to both prediction tools. (Table 1 and 2)

3.1.3 HLA-A*0201 binding affinity of HLA-DP-derived peptides

The binding affinity of the peptides to HLA-A2 molecules, as predicted by theoretical analysis, was validated by the T2 cell binding assay. T2 is an HLA-A2 positive cell line that lacks the transporter associated with antigen presentation (TAP) [220, 221]. This results in an impaired ability to transport peptides that are endogenously produced from intracellular proteins into the ER. Therefore, only the peptides derived from the C-terminus of the transmembrane and soluble proteins of the secretory pathway have access to MHC class I molecules in the ER [46]. Most of these peptides have low affinity for MHC class I and thus form unstable complexes resulting in low surface MHC class I expression levels [42]. Addition of exogenous HLA-A2 binding peptides to the TAP deficient T2 cells stabilizes the peptide/MHC complexes and increases the expression level of the HLA-A2 on the cell surface. This allows the

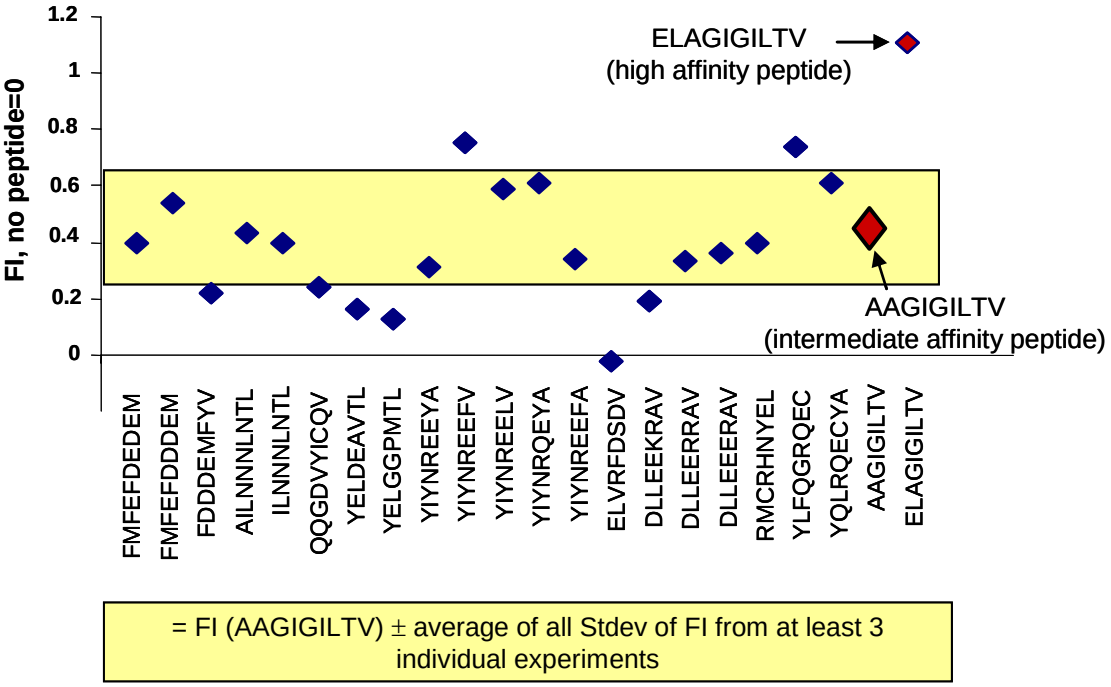


Figure 1 Binding of HLA-DP-derived peptides to HLA-A2. Relative binding affinity of synthetic HLA-DP-derived peptides was assessed in T2 binding assay. Two Melan-A derived HLA-A*0201-restricted peptides were used as positive controls: intermediate affinity AAGIGILTV peptide and high affinity ELAGIGILTV peptide. Mean fluorescence index (FI) values of at least three independent experiments are shown.

determination of the relative affinity of peptides to the MHC-I molecule. Two Melan-A derived HLA-A*0201-restricted peptides that are known to be immunogenic were used as positive controls: the naturally occurring AAGIGILTV peptide with intermediate affinity for HLA-A2 and its synthetic analog (10-mer peptide ELAGIGILTV), modified to bind to HLA-A*0201 with higher affinity. Among the 20 synthetic HLA-DP-derived peptides, 14 peptides showed intermediate affinity binding (comparable with intermediate affinity peptide AAGIGILTV or higher), 5 peptides showed low affinity binding and 1 did not show any detectable binding to HLA-A*0201 (Figure 1 and Table 2). These results demonstrate the reliability of the theoretical prediction of MHC class I peptide binding by BIMAS and SYFPEITHI tools.

3.1.4 In vitro generation of antigen-specific CTL lines: immunogenicity and cross-reactivity of HLA-DP-derived peptides

In order to investigate whether HLA-DP-derived peptides could stimulate $CD8^+$ responses, we next attempted to generate $CD8^+$ T-cell lines against HLA-DP-derived peptides from PBMC of healthy HLA-A2 positive donors. $CD8^+$ cells were separated from PBMC and were then activated with irradiated TAP-deficient T2 cells pulsed with synthetic peptides. The Melan-A-derived peptide ELAGIGILTV, which is known to be highly immunogenic [52], was used as a positive control. CTL cultures were further restimulated every week with peptide-pulsed, irradiated T2 cells.

We checked the peptide specificity of the CTL-lines by means of IFN-γ production in response to peptide-pulsed T2 cells in ELISPOT assays and/or ELISAs starting from week 4 of culture. Specific T-cell responses were observed against 9 out of 12 tested peptides (#4007-#4011, #4013-#4015, #4016, Figure 2).

Peptides #4007-#4011 and #4013-#4015 represent two groups of polymorphic peptides. Peptides #4007-#4011 differ in positions P6, P8 and P9 and peptides #4013-#4014 in positions P2, P4 and P9. P2, P8 and P9 are known to be anchor positions for HLA-A*0201 and influence the binding affinity of the peptide to MHC molecules (see Chapter 1.2.2.2). However, different amino acids in P6 and P4 theoretically could alter the recognition of MHC-bound peptide by the TCR. To verify this possibility we checked whether the CTL-lines generated against one of the peptide cross-recognized all other polymorphic variants within one polymorphic group with the same

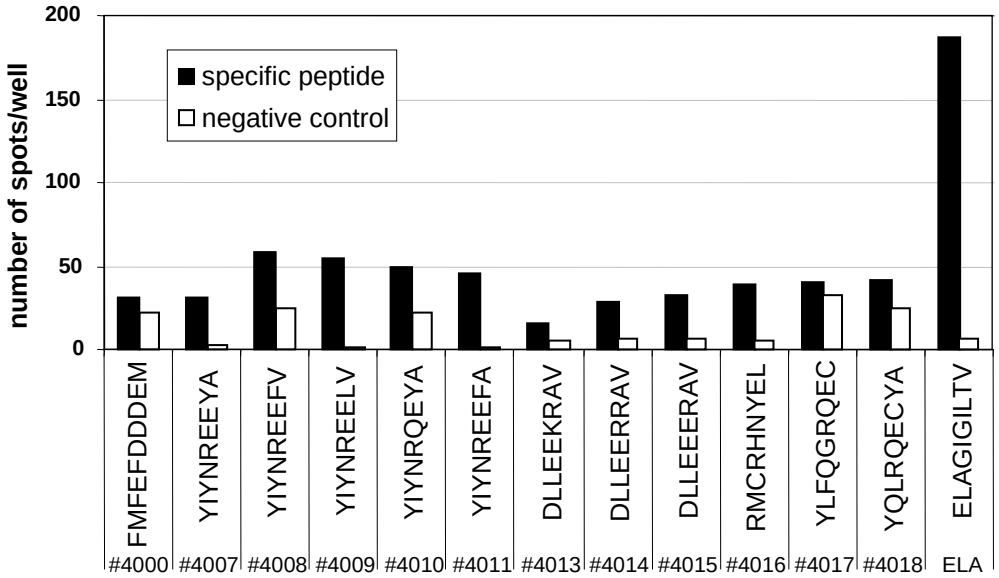


Figure 2 Immunogenicity of HLA-DP-derived peptides. $CD8^+$ T-cell lines against twelve HLA-DP-derived peptides (#4000, #4007-4011, #4013-4018) and Melan-A derived ELA peptide were generated from PBMC of healthy HLA-A2 positive donors. $CD8^+$ cells were purified from PBMC and were weekly stimulated with irradiated peptide-pulsed T2 cells. Peptide-specificity of CTL cultures was assessed by IFN-γ release in response to peptide-pulsed target cells in ELISPOT assay. T2 cells pulsed with the corresponding peptide or with an unrelated peptide (negative control) were used as targets. The results of the ELISPOT assay of 4 week old CTL cultures from one out of 6 donors are shown. Results are mean values from an experiment done in duplicates.

efficiency. As shown in Figure 3, CTL cultures generated against the peptide #4013, #4014 and #4015 recognized all peptides within the polymorphic group with equal efficiency, and the same was true for the second polymorphic group of peptides (#4007-4011). These results show that the polymorphic synthetic peptides within each group are cross-reactive.

For the aim of vaccination against the recipients' HLA-DP allele after HLA-DP mismatched allogenic SCT, it is essential that the CTL against recipients' HLA-DP do not recognize the donor HLA-DP variant. Differential recognition of polymorphic peptides could result from 1) differential recognition of polymorphic peptides by CTL or 2) different natural processing of various HLA-DP variants and natural presentation of HLA-DP-derived peptides on MHC class I molecules. As shown in Figure 3, the polymorphic peptides were cross-recognized by CTL cultures generated against the other peptides within the group. We therefore next investigated whether the polymorphic peptides are differentially processed and presented.

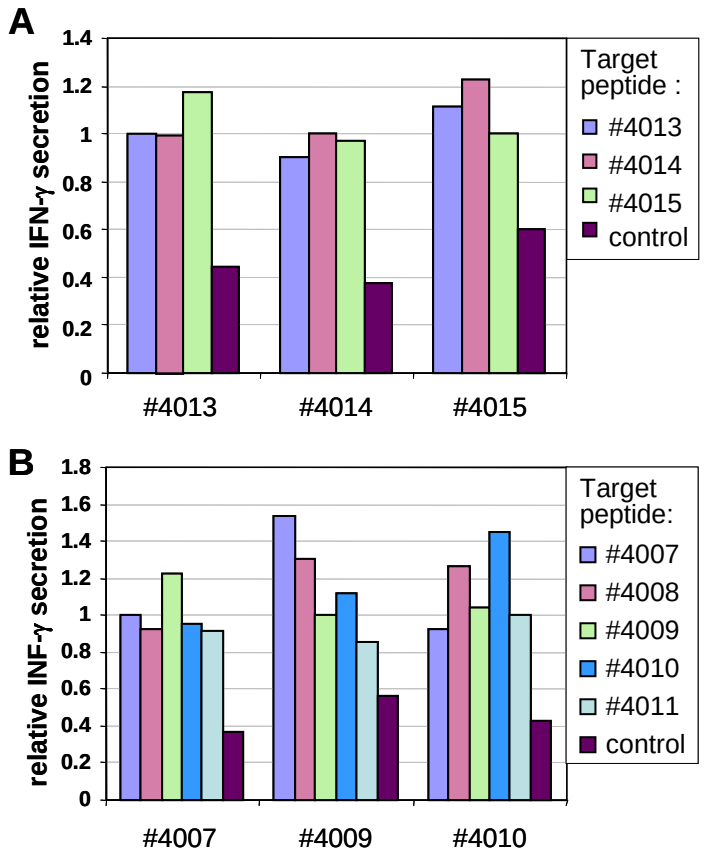


Figure 3 Cross-reactivity of HLA-DP-derived peptides within the polymorphic peptide group.
CTL-lines generated against the peptides **(A)** #4013, #4014, #4015 and **(B)** #4007, #4009, #4011 belonging to two polymorphic groups of HLA-DP-derived peptides (#4007-4011 and #4013-4015) cross-recognized all other polymorphic variants within the group with the same efficiency. The results of one representative IFN-γ ELISA are presented. T2 cells pulsed with every peptide within the polymorphic group or with an unrelated peptide (control) were used as target cells. Results are normalized to the IFN-γ amount secreted by CTL-cultures in response to T2 cells pulsed with the specific peptide.

The main sources of MHC class I-binding antigenic peptides are cellular proteins which are degraded as part of the normal protein turnover (see Chapter 1.2.2.2). This degradation involves the ubiquitin-proteasome system as well as several downstream aminopeptidases. The protein degradation system though enabling generation of a broad pool of MHC class I epitopes, still has a certain specificity for the sequence of the substrate which is not yet totally understood. Not all the peptides theoretically predicted to bind MHC class I will be produced naturally by the cell's proteolytic system. We therefore investigated, whether the HLA-DP-derived peptides used in this study are naturally presented. This could be checked using allogeneic HLA-A*0201^{+} B cells or EBV-transformed B cell lines, expressing various HLA-DP-alleles. We generated B-cell lines from different donors typed for HLA-DP alleles. These B-cell lines were allogeneic, therefore, recognition of these cell lines by CTL cultures could involve antigens different from the HLA-DP alleles relevant for this study. Thus, allogeneic activity of CTL cultures had to be excluded in order to determine the specific CTL recognition of the HLA-DP-derived peptide variant. To avoid non-HLA-

DP-related allogeneic recognition of target cells, peptide-specific CTL had to be cloned.

In order to obtain monoclonal CTL, peptide-specific CTL cultures were further cloned in 96-well plates using peptide-pulsed, irradiated autologous PBMC as stimulator cells. After 3 weeks, the generated T-cell clones were checked for peptide specificity by measuring IFN-γ in ELISA assays, using peptide-pulsed T2 cells as targets. However, no peptide-specific clones could be detected (124 clones tested, data not shown). Most of the tested clones recognized T2 cells which were not pulsed with any peptide, suggesting that the T2 cells, which were used to restimulate the CTL-cultures induced nonspecific responses themselves. Thus, the protocol of peptide-specific CTL lines generation had to be optimized to reduce nonspecific CTL responses against T2-cells.

3.1.5 Optimization of the in vitro generation of peptide-specific CTL cultures from PBMC of healthy donors

a) Using autologous DC as APC

As peptide-pulsed T2-cells primed a strong non-peptide-specific CTL response, we next attempted to generate peptide-specific T-cell-lines using autologous DC as APC. It was previously published by Kaiser and colleagues [222] that DC most efficiently induced specific $CD8^+$ responses when used in maturing state, 6 hours after activation. Therefore, DC were activated for 6 hours with low concentration of E.coli or a mixture of GM-CSF, IFN-α, TNF-α and PGE2 to induce maturation. Following these stimuli, DC were pulsed with the peptides and co-cultured with $CD8^+$ T-cells. The CTL cultures were further restimulated weekly with similarly prepared autologous DC. This method allowed the generation of highly-specific CTL-lines against the control ELAGIGILTV peptide. However, although stimulation with autologous DC reduced unspecific reactivity against T2 cells, this method failed to induce any HLA-DP-derived peptide-specific CD8 response (n=5, 8 out of 20 HLA-DP-derived peptides were tested, data not shown). This suggests that the reduction of nonspecific reactivity of CTL cultures stimulated with autologous DC coincided with a loss of peptide-specific reactivity.

b) Using autologous DC and T2 as APC

The efficiency of naïve T-cell activation and expansion in response to MHC-associated antigens depends on the strength and persistence of the signal, delivered by the antigen-presenting cell. Variables modifying T-cell response induction involve 1) the affinity between the TCR and the MHC–peptide complex, 2) the stability of the MHC-peptide complex itself and 3) the co-receptors and co-stimulatory molecules associated with T-cell activation [223]. Thus, high affinity peptides provide stronger stimuli and may not need additional costimulation, whereas low-affinity peptides require additional help to be able to induce effective T-cell responses.

In contrast to TAP-deficient T2 cells, dendritic cells present a large variety of internally processed peptides. Due to the competition of peptides for the binding to MHC class I in the ER, most of the peptides presented on the surface of TAP-competent cells will have a high affinity to the specific MHC allele [224]. Therefore, exogenous low-affinity peptides may have a limited chance of being presented on MHC class I for an extended period of time [224, 225]. TAP-deficient T2 cells can provide prolonged presentation of low-affinity peptides, required for efficient activation of naïve T-cells, but they lack costimulatory molecules, such as CD80 and CD86. In contrast, dendritic cells express high levels of CD80/CD86, but may rapidly loose peptide expression. Comparing two control peptides ELAGIGILTV (high affinity) and AAGIGILTV (intermediate affinity), we investigated whether the combination of autologous DC and T2 cells is able to induce stronger specific $CD8^+$ T-cell responses against moderate-affinity peptides than DC alone.

Therefore, the protocol for priming peptide-specific CTL-responses was changed. Unsorted PBMC were activated on day 0 with peptide-pulsed, preactivated, autologous DC either alone or in combination with T2 cells. After one week, $CD8^+$ cells were purified from these cultures in order to remove persisting T2 cell and to avoid non-specific CTL-responses. Cultures were further restimulated every week with peptide-pulsed, preactivated, autologous DC. Peptide-specific reactivity was determined in IFN-γ ELISPOT assays starting from week 4. As shown in Figure 4, both, DC alone and DC in combination with T2 cells induced similar responses against high affinity ELAGIGILTV peptide. In contrast, CTL-responses against intermediate affinity AAGIGILTV peptide were significantly stronger induced when primed with DC in combination with T2 cells. As most of the synthetic peptides had

an intermediate affinity to HLA-A2 similar to the AAGIGILTV peptide, this latter method was further applied for the generation of peptide-specific CTL-lines.

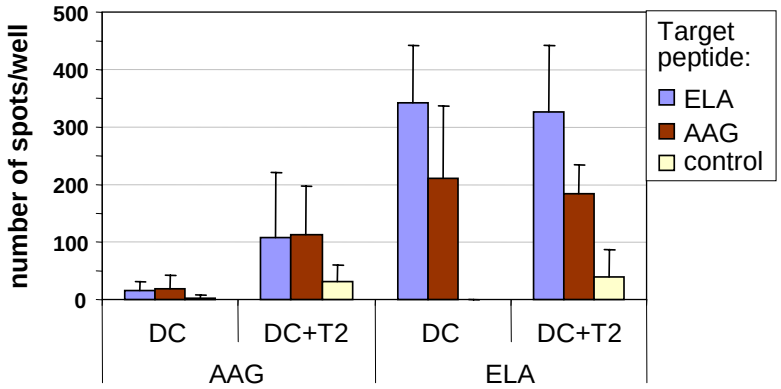


Figure 4 Optimization of the method of *in vitro* generation of peptide-specific CTL cultures. CTL lines were generated against high affinity peptide ELAGIGILTV (ELA) and intermediate affinity AAGIGILTV (AAG) by priming peptide-specific response with peptide-pulsed autologous DC either alone or in combination with irradiated T2 cells. The number of the cells producing IFN-γ in response to peptide-pulsed T2 cells in week 4 CTL cultures was assessed in IFN-γ ELISPOT assay. Results are the mean values + Stdev from the experiments done in duplicates with CTL cultures generated in 2 independent experiments from PBMC of 6 different healthy donors.

3.1.6 Generation of CTL lines against HLA-DP derived peptides with an optimized method

HLA-DP-derived peptide-specific CTL cultures were generated from PBMC of 6 healthy donors using the optimized protocol as described above. CTL-lines were stimulated either with the single peptide or with a mixture of peptides. CTL-cultures from 1 out of 6 donors responded weakly against the peptides #4007-#4010, #4013-#4015 and #4017-#4018 after 4 weeks of restimulations (Figure 5). These cultures were further restimulated for additional 2 weeks with DC pulsed with the individual peptides and then cloned. However, no peptide-specific clones could be detected (254 clones were checked, data not shown).

Thus, although some of the HLA-DP-derived peptides were immunogenic (results summarized in Table2), it was not possible to generate peptide-specific clones in our experimental conditions. The final characterization of the natural presentation of the HLA-DP-derived peptides was therefore not possible. This failure to induce peptide-

specific CTL-clones may reflect low frequencies of T-cells recognizing HLA-DP-derived peptides in PBMC of normal donors.

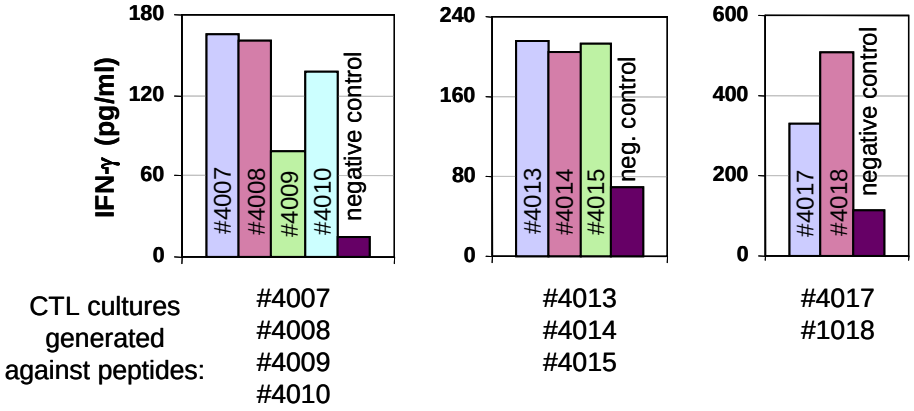


Figure 5 Immunogenicity of HLA-DP-derived peptides. $CD8^+$ T-cell lines against HLA-DP-derived peptides and Melan-A derived ELA peptide were generated from PBMC of healthy HLA-A2 positive donors with an optimized protocol. IFN-γ release by CTL cultures in response to peptide-pulsed T2 cells was measured in ELISA. Shown are the results of IFN-γ release by CTL generated against 3 peptide mixtures (#4007-4010, #4013-4015 and #4017-4018) from one out of 6 healthy donors.

Table 2 Immunological characteristics of the HLA-DP-derived synthetic peptides: HLA-A2 binding, immunogenicity and crossreactivity. HLA-DP-derived synthetic peptides were analyzed for HLA-A2 binding in T2 binding assay. Relative HLA-A2 binding is shown as mean fluorescence index (FI) ± standard deviation (Stdev) of three independent experiments. Immunogenicity is given as a number of positive CTL cultures/number of different donors tested. The ability of CTL-lines generated against one of the peptide to cross-recognize other polymorphic variants within one polymorphic group was tested (n.d. = not done).

Peptide name	sequence	BIMAS score	SYFPEITHI score	HLA-A2 binding (FI±STDIV)	Immuno-genicity	Cross-reactivity
#3999	FMFEFDEDEM	122.685	20	0.4±0.086	0/8	n.d
#4000	FMFEFDDDEM	122.685	18	0.54±0.138	0/10	
#4001	FDDDEMFYV	53.468	12	0.22±0.155	0/8	n.d
#4002	AILNNNLNTL	24.997	25	0.43±0.341	0/7	n.d
#4003	ILNNNLNTL	83.527	30	0.4±0.4	0/7	
#4004	QQGDVYICQV	66.498	15	0.24±0.449	0/6	n.d
#4005	YELDEAVTL	28.986	19	0.16±0.145	0/9	n.d
#4006	YELGGPMTL	7.070	20	0.13±0.196	0/9	
#4007	YIYNREEYA	27.099	15	0.31±0.039	3/12	Cross-reactive
#4008	YIYNREEFV	379.384	21	0.75±0.25	3/15	
#4009	YIYNREELV	68.979	21	0.59±0.526	3/14	
#4010	YIYNRQEYA	27.099	15	0.61±0.299	3/14	
#4011	YIYNREEFA	27.099	15	0.34±0.241	2/11	
#4012	ELVRFDSDV	19.822	18	-0.02±0.049	0/6	n.d
#4013	DLLEEKRAV	15.827	24	0.19±0.22	4/14	Cross-reactive
#4014	DLLEERRAV	15.827	24	0.33±0.235	4/14	
#4015	DLLEEERAV	15.827	24	0.36±0.263	4/15	
#4016	RMCRHNYEL	15.428	21	0.4±0.191	1/9	n.d
#4017	YLFQGRQEC	84.555	17	0.74±0.12	2/12	n.d
#4018	YQLRQECYA	47.151	8	0.61±0.197	2/12	

3.1.7 IgA and IgD constant region-derived antigens

Hematological malignancies of B-cell origin usually express one or several immunoglobulin isotypes. Antigens, derived from immunoglobulin constant regions represent potential candidates for vaccination against B-cell malignancies. This part of the study focuses on IgA and IgD isotypes. IgA and IgD are expressed on a subset of non-Hodgkin lymphomas and multiple myelomas. We identified MHC class I antigens, derived from IgA and IgD constant regions and characterized them as potential candidates for a vaccination therapy against B-cell malignancies after SCT.

3.1.8 IgA and IgD derived peptides

Similar to the analysis of HLA-DP alleles, the sequences of the IgD and IgA constant regions were screened for the occurrence of specific peptides containing HLA-A*0201 peptide-binding motifs with BIMAS and SYFPEITHI prediction tools. The 6 top-scored peptides for each isotype were synthesized and taken into further analysis (Table 3). These peptide sequences were specific for IgA or IgD isotypes, and were not present in any other Ig isotype (in the IgG isotypes or the IgE or in the IgD/IgA respectively).

Table 3 Identification of peptides derived from the constant regions of the IgA and IgD molecules. The protein sequences of IgA and IgD constant regions were analyzed for the HLA-A2 binding motifs using BIMAS and SYFPEITHI MHC-binding prediction tools. The peptides with the best prediction scores that were synthesized for further analysis are listed.

Peptide name	Isotype	Start position	sequence	BIMAS score	SYFPEITHI score
#4844	IgA	137	LLLGSEANL	134.369	28
#4845	IgA	331	RLAGKPTHV	69.552	25
#4846	IgA	239	ELALNELVTL	4.0	27
#4847	IgA	144	NLTCTLTGL	49.134	24
#4848	IgA	232	LLPPPSEEL	17.795	27
#4849	IgA	128	SLHRPALEDL	24.075	26
#4850	IgD	239	SLWNAGTSV	577.282	25
#4851	IgD	235	TLPRSLWNA	85.698	18
#4852	IgD	171	YLLTPAVQDL	836.253	28
#4853	IgD	306	LMWLEDQREV	141.755	23

Peptide name	Isotype	Start position	sequence	BIMAS score	SYFPEITHI score
#4854	IgD	365	LLNASRSLEV	118.238	26
#4855	IgD	255	SLPPQRLMAL	49.134	29

3.1.9 CTL lines and cloning

To assess the immunogenicity of the IgA and IgD-derived peptides, peptide-specific CTL-lines were generated using an optimized CTL-generation protocol. The cultures were first stimulated with the mixture of 3 peptides for 4 weeks and then checked in IFN-γ ELISAs for the specific responses against each individual peptide. Positive cultures were then further restimulated for additional two weeks with the individual peptide. As shown in Figure 6A, only a weak specific response against a number of peptides could be detected after 4 weeks of restimulations. After additional 2 weeks of restimulation with individual peptides, positive CTL cultures against IgD-derived peptide #4854 could be generated in 4 out of 10 donors tested (Figure 6B).

The best responder culture was then used for cloning. Out of 22 clones generated, 21 recognized specifically peptide #4854 (Figure 6C).

3.1.10 Natural presentation of the LLNASRSLEV (#4854) epitope

As the IgD-derived peptide #4854 was capable of inducing $CD8^+$ response, natural presentation of this epitope had next to be determined.

In order to investigate if the MHC class I antigen processing pathway is capable of generating the LLNASRSLEV peptide (#4854) from the IgD protein, we checked peptide-specific CTL cultures and clones for their ability to recognize normal and malignant IgD-expressing cells.

IgD is expressed in healthy individuals on a subset of B-cells. B-cell lines were generated from PBMC of the healthy donors as described in *Materials and Methods*. After 4 weeks these cultures contained 60-80% of $CD19^+$ B-cells and 2-4% of $CD19^-$ $CD3^-$ non-B non-T cells. IgD was expressed on 60-70% of the B-cell population. The cells were sorted by FACS into $CD3^-IgD^+$ and $CD3^-IgD^-$ B-cells (Figure 7). These two populations of B cells were further used as target cells. Peptide #4854-specific CTL cultures and CTL-clones were used as effector cells in order to assess if the LLNASRSLEV epitope is naturally presented.

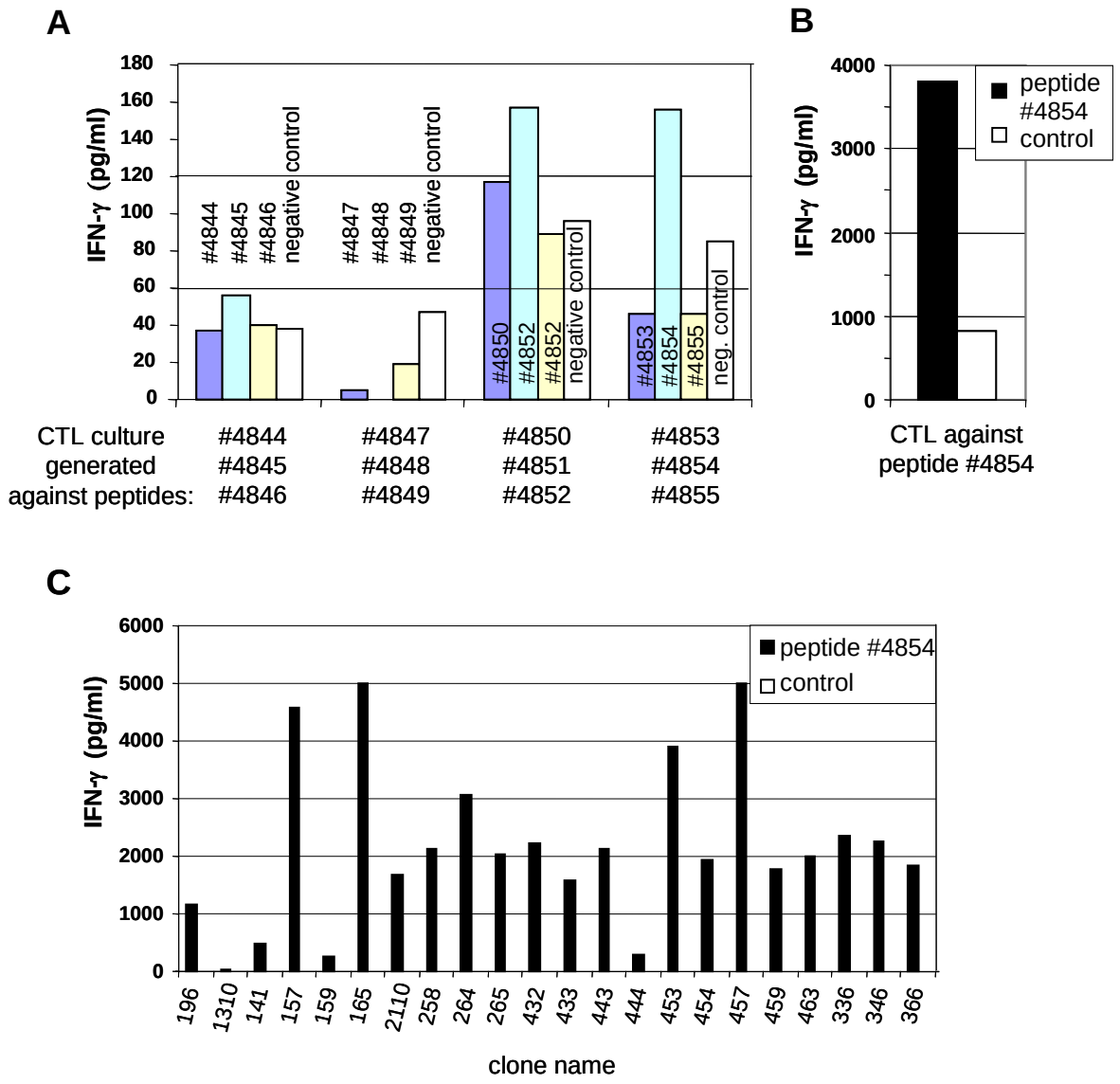


Figure 6 **IgD and IgA derived peptides immunogenicity and CTL clones.** CTL cultures were stimulated with the mixture of 3 peptides. After 4 weeks IFN-γ release in response to T2 cells pulsed with individual peptides and unrelated peptide as a negative control was tested in ELISA. **(A)** The IFN-γ ELISA results for week 4 CTL cultures of one out of 10 donors are shown. Positive cultures were further restimulated for additional two weeks with autologous DC pulsed with the individual peptide. **(B)** The best responder CTL culture against IgD-derived peptide #4854 after 6 week of culturing is shown. This culture was then used for cloning. **(C)** The peptide-specificity of CTL clones was screened by IFN-γ release in response to peptide-pulsed T2 cells. The representative ELISA results are shown.

It has been reported that IFN-γ changes the MHC class I peptide presentation profile [35-37]. IFN-γ promotes formation of immunoproteasomes that have altered cleavage site preferences as well as different cleavage rate as compared to conventional proteasomes [33, 34]. Thus, a proportion of IgD^+ B-cells was exposed to IFN-γ overnight before being used as target cells in order to include the possibility that peptide #4854 is better presented under this condition.

As shown in Figure 7B, peptide-specific CTL culture and clones recognized T2 cells pulsed with peptide #4854 and moderately recognized IgD^- peptide-pulsed B-cells, indicating that CTL cultures and clones can recognize the peptide, presented on B-cells. However, no IFN-γ production could be detected neither by the CTL clones nor by the CTL culture in response to IgD^+ B cells (Figure 7B). These results suggest that LLNASRSLEV epitope is not naturally presented by IgD^+ B-cells.

In order to further confirm that the LLNASRSLEV epitope is not naturally presented, we examined if tumor cell lines expressing IgD and HLA-A2 are recognized by peptide #4854-specific CTL culture.

Several multiple myeloma cell lines (KMS-12-BM, LP-1 and IM9) were tested for the expression of HLA-A2 allele and IgD protein by flow cytometry analysis. All cell lines tested expressed intracellular IgD (Figure 8A). No surface IgD expression could be detected. The IgD^+ $HLA\text{-}A2^+$ IM9 cell line was taken as a target cell line for testing peptide #4854 presentation and the IgD^+ $HLA\text{-}A2^-$ LP-1 cell line was used as a negative control.

As shown in Figure 8B, the peptide #4854-specific CTL culture was able to recognize only the $HLA\text{-}A2^+$ IM9 cell line if exogenously pulsed with peptide #4854, but not the IM9 cell line alone. The $HLA\text{-}A2^-$ LP-1 cell line was not recognized, even if pulsed with peptide #4854. These data indicate that although IM9 cell line is capable of presenting LLNASRSLEV peptide in context of HLA-A2, this epitope is not occurring naturally in malignant cells.

Taken together, we could not detect naturally presented LLNASRSLEV peptide on the surface of normal IgD^+ B-cell, nor on a malignant IgD^+ multiple myeloma cell line. This epitope is therefore not a suitable target for vaccinations.

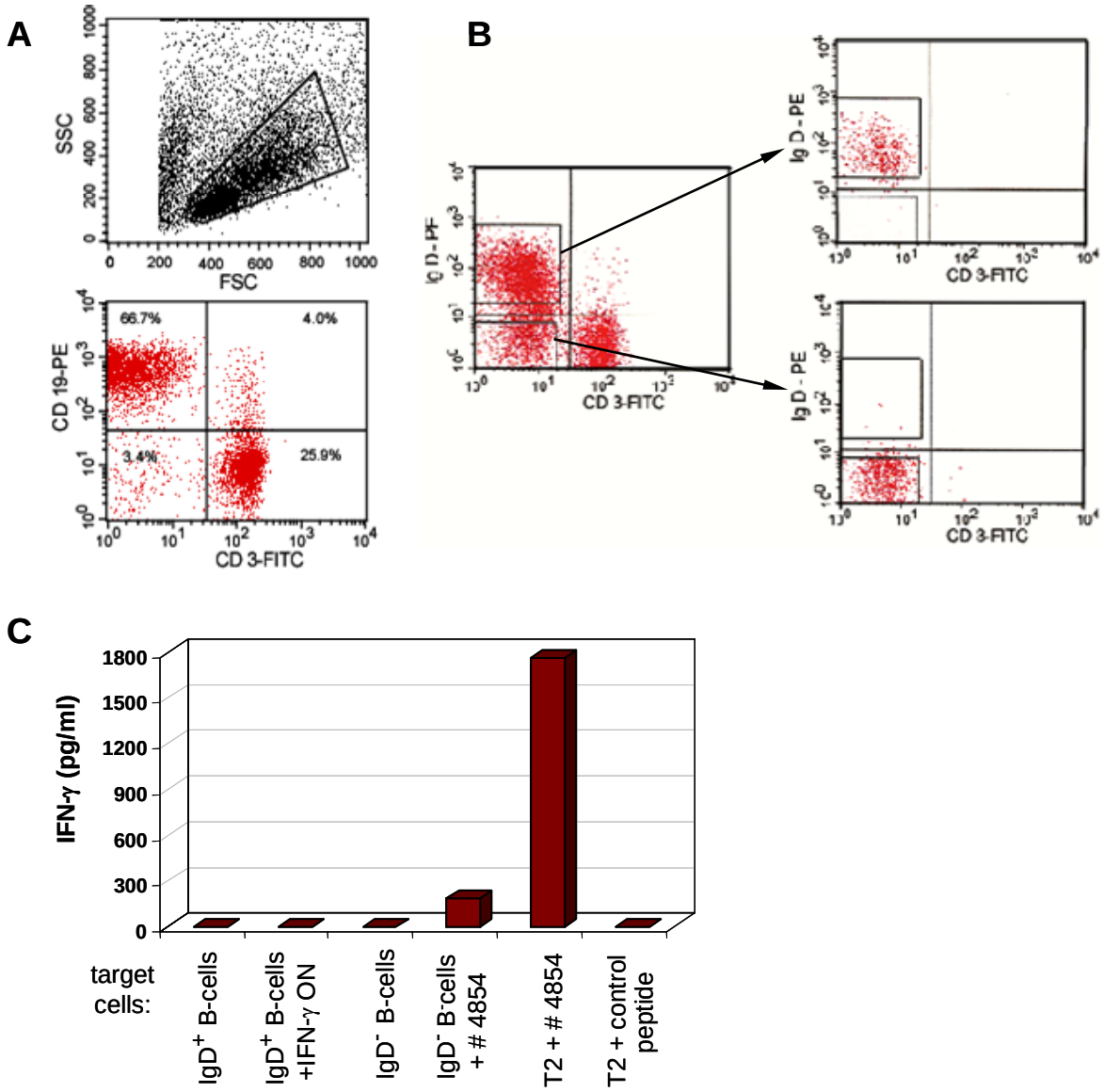


Figure 7 Natural presentation of the LLNASRSLEV (#4854) epitope: recognition of IgD$^+$ and IgD$^-$ B cells. B-cell lines were generated from PBMC of the healthy donors by culturing for 4-6 weeks in presence of CD40L-tranfected BHK cells and IL-4. Starting from week 4 the cultures were checked for CD19 and CD3 expression by FACS analysis to assess the percentage of B-cells and T-cells in the culture. **(A)** One representative B-cell culture after 4 weeks of cultivation is shown. **(B)** The cells were then sorted by FACS into CD3$^-$IgD$^+$ and CD3$^-$IgD$^-$ B-cells. The FACS analysis of the B-cell populations before and after the sorting is presented. These two populations of B cells were rested overnight and further used as target cells for IgD-derived peptide #4854-specific CTL cultures and clones to assess the natural presentation of the peptide. Peptide #4854-specific CTL culture or CTL clones were coincubated with IgD$^+$ B-cells, IgD$^+$ B-cells exposed overnight to IFN-γ (IgD$^+$ B-cells + IFN-γ ON) or IgD$^-$ B-cells. T2 cells pulsed with peptide #4854 and with an unrelated peptide were used as positive and negative controls correspondingly. IgD$^-$ B-cells exogenously pulsed with peptide #4854 were used to control the ability of CTL clones to recognize the peptide presented on B-cells. Supernatants after 16 hours were checked for IFN-γ production in ELISA **(C)**.

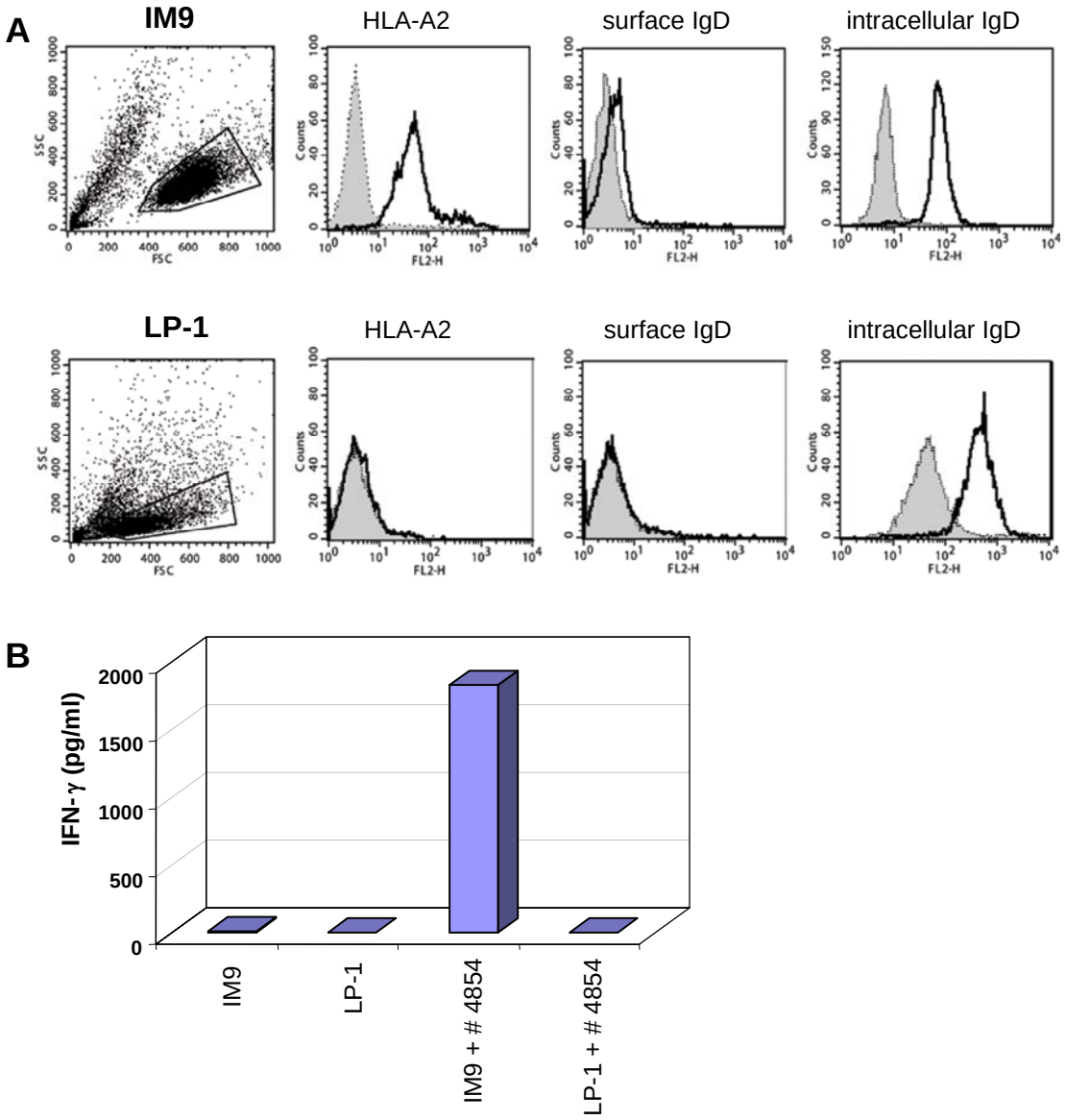


Figure 8 Natural presentation of the LLNASRSLEV (#4854) epitope: Recognition of IgD$^+$ multiple myeloma cell lines. (A) FACS analysis of IgD and HLA-A2 by LP-1 and IM9 multiple myeloma cell lines is shown (IgD and HLA-A2 specific antibodies: open histogram, solid line; antibody isotype control: filled histogram, dotted line). **(B)** CTL culture specifically recognizing peptide #4854 was coincubated with IgD$^+$ HLA-A2$^+$ IM9 cell line alone or pulsed with peptide #4854 and the peptide #4854-pulsed IgD$^+$ HLA-A2$^-$ LP-1 cell line as a negative control. Supernatants after 16 hours were checked for IFN-γ production in ELISA.

3.2 The role of GSK-3 in differentiation and activation of pro-inflammatory DC

GSK-3 is a multifunctional enzyme critical for cellular differentiation, apoptosis, self renewal and motility. More than 40 proteins including 17 transcription factors have been reported to be substrates of GSK-3 (reviewed in [226]). Two isoforms of GSK-3 (GSK-3α and GSK-3β) have been described which are highly homologous in their kinase domain, but little is known about isoform-specific functions. GSK-3 is regulated by multiple mechanisms including phosphorylation of GSK-3 itself and its target proteins, intracellular localization and formation of protein complexes, such as β-catenin/GSK-3 in the canonical wnt-pathway [226].

Here we investigate whether GSK-3 plays a role in regulating differentiation of DC from $CD14^+$ monocytes and DC functions.

3.2.1 GSK-3 promotes DC differentiation of human monocytes

Differentiation of human DC from CD14+ monocytes requires 4-7 days of culture in GM-CSF and IL-4. Addition of the GSK-3 inhibitors LiCl, SB415286 or SB216763 on day 0 inhibited the development of DC so that monocytes differentiated toward macrophage-like cells (Figure 9). Cell yields in day-5 cultures were lower in the presence of GSK-3 inhibitors (62±9% in presence of SB415286 compared with cultures in the absence of the inhibitor (100%) (n=6)). In contrast to DC produced in cultures without GSK-3 inhibitors, these cells showed an elongated macrophage-like morphology and strongly adhered to culture dishes (Figure 9A). They also displayed strong phagocytic activity when exposed to *Saccharomyces cerevisiae* (Figure 9B). Following activation with *E. coli*, they expressed similar levels of MHC class I and II as did DC. However they differed in expression of DC-specific and monocyte/macrophage specific markers. In contrast to DC they showed no CD83 and only low CD80 and CD86 expression but strongly expressed CD14 (Figure 9C). Furthermore, upon *E. coli* activation, these macrophage-like cells secreted similar levels TNF-α, and higher levels of IL-6 and IL-10, but significantly lower levels of IL-12p40 and no IL-12p70 as compared with conventional MoDC (Figure 9D). These data suggest that GSK-3 activity is essential for differentiation of monocytes into

MoDC and in its absence monocytes will instead differentiate into macrophage-like cells.

3.2.2 GSK-3 is constitutively active in immature DC and inhibits spontaneous maturation

The effects of GSK-3 inhibitors on immature DC and during activation of DC were next investigated.

GSK-3 is negatively regulated by phosphorylation of an N-terminal serine (Ser-21 for GSK-α and Ser-9 for GSK-β). In contrast, GSK-3 activity is facilitated upon phosphorylation of Tyr-279 in GSK-α and Tyr-216 in GSK-β [227, 228]. This allows monitoring the activity of GSK-3 by comparing the level of Ser-21/9 and Tyr279/216 phosphorylation. GSK-3 activity can be additionally controlled by the cellular levels of GSK-3 substrate β-catenin. Active GSK-3 associates with β-catenin within a multiprotein complex. When phosphorylated by GSK-3, β-catenin becomes a target for proteasomal degradation [229]. Inactivation of GSK-3 releases β-catenin from the complex and thus allows its' accumulation in the cell.

Figure 9 GSK-3 inhibits macrophage differentiation of human monocytes. $CD14^+$ monocytes were cultured for 5 days in the presence of GM-CSF and IL-4. The GSK-3 inhibitors LiCl (10mM), SB415286 (10μM) and SB216763 (10μM) were added on day 0. After 5 days of culture, cells were either tested in phagocytosis assay or were activated with intact *E. coli* (1:1000, see Methods) for an additional 36h and examined for phenotypic maturation and cytokine secretion. **(A)** Photomicrographs of DC culture on day 5 in the absence (upper panel) or presence (lower panel) of the GSK-3 inhibitor (SB415286). One representative of 6 cultures is shown. Similar results were observed in the presence of LiCl and SB216763. **(B)** Phagocytosis of day 5 cultures with and without GSK-3 inhibitors. Left panel: Phagocytic Index (Number of yeast particles per cell), right panel: Proportion of cells containing yeast particles. (Mean values ± Stdev, n=4, * $p<0.05$; ** $p<0.01$). **(C)** Flow cytometric analysis of DC surface maturation marker expression following activation with *E. coli* for 36h. One representative of 4 experiments is shown. **(D)** Cytokine secretion of *E. coli* activated cells. Supernatants were harvested 36 hours following activation. Absolute cytokine levels of E.coli-activated MoDC (set 1): IL-12p70: 5.4±2.5 ng/ml; IL-12p40: 22.2±7.9 ng/ml; IL-6: 28.1±20 ng/ml; IL-10: 2.3±2.3 ng/ml; TNF-α: 10.6±9.5 ng/ml; (Mean values ± stdev, n=7-10, * $p<0.05$; ** $p<0.01$).

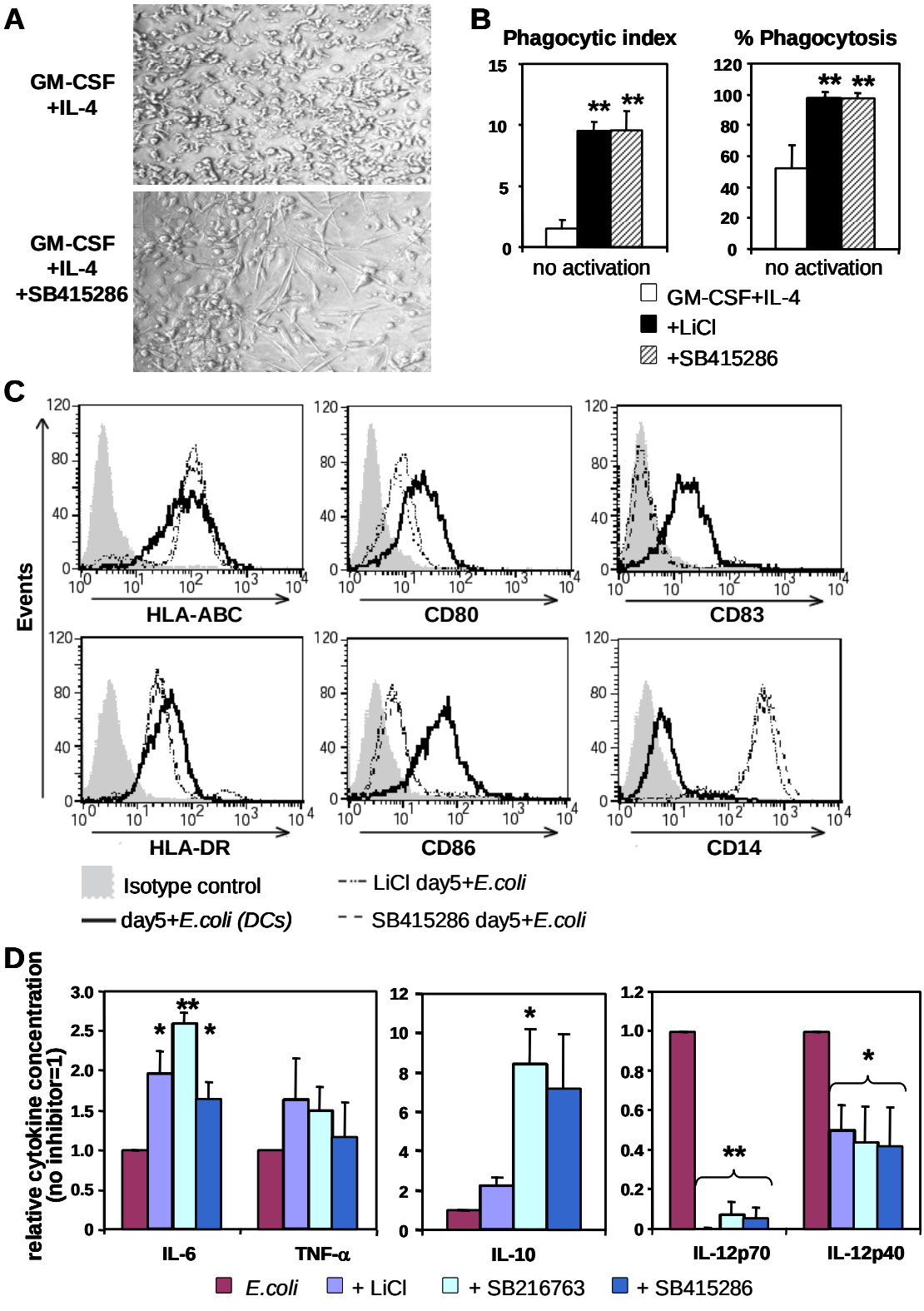
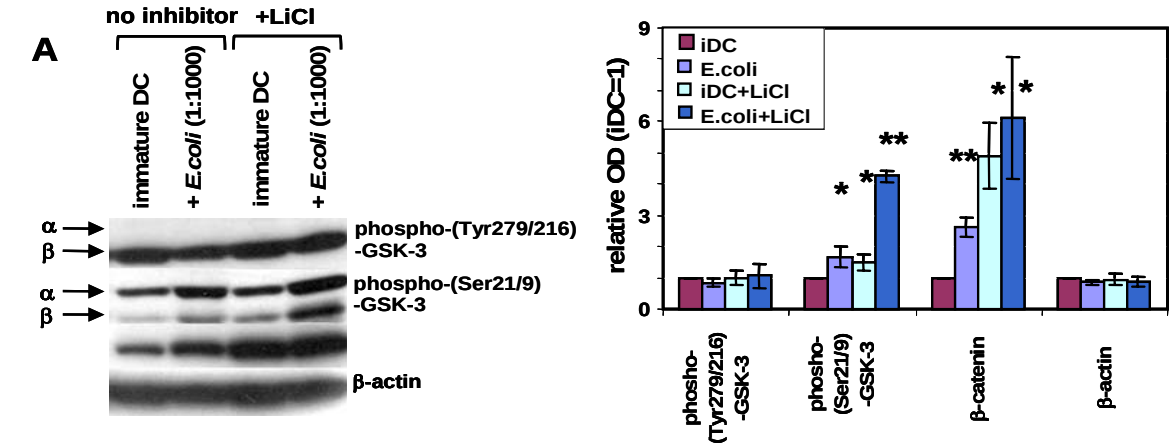
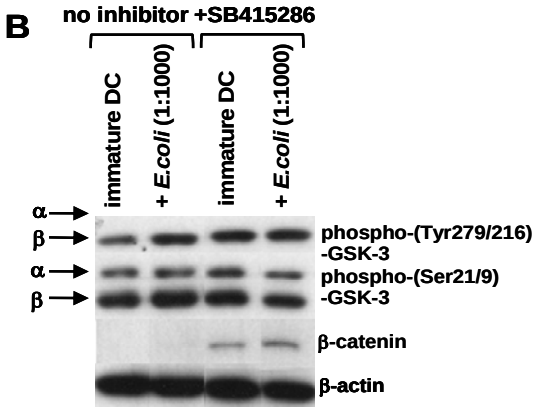
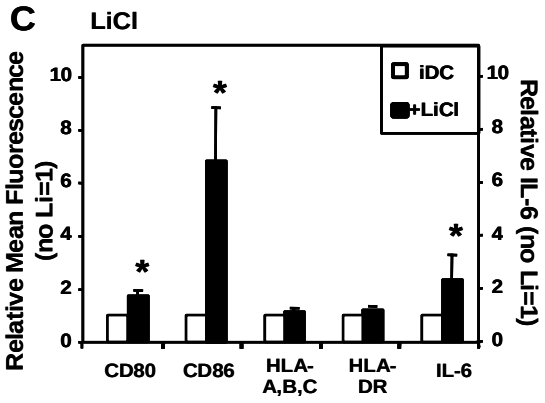


Figure 9 GSK-3 inhibits macrophage differentiation of human monocytes.







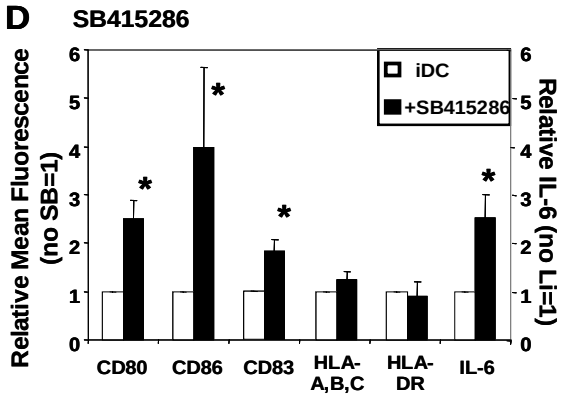


Figure 10 GSK-3 is constitutively active in and inhibits spontaneous maturation of immature DC. Immature MoDC were washed on day 5-7 of culture and resuspended in culture medium at a concentration of 2-3x10^5 cells/ml. Cells were either continued in GM-CSF and IL-4 (iDC), or activated with E. coli (1:1000). LiCl (10 mM) or SB415286 (10µM) were added 20 min prior to activation or together with GM-CSF and IL-4. **(A)** Western blot analysis of DC lysates harvested 2 hours following activation and/or addition of LiCl. β-actin was used to control for protein loading. One representative picture and quantification of 4 experiments is shown. The quantification results were normalization to the iDC values, set as 1. Shown are the normalized means+Stdev of 4 experiments, * $p<0.05$; ** $p<0.01$. **(B)** One representative western blot analysis of DC lysates harvested 2 hours following activation and/or addition of SB415286 is shown. **(C)** Flow cytometry analysis and IL-6 ELISAs of the supernatants were performed 36 hours after exposure to LiCl. Mean fluorescence values of CD80, CD86, HLA-A,B,C and HLA-DR and IL-6 levels in the supernatants are shown for DC relative to DC cultured in the absence of LiCl (Mean values ± Stdev, n=4 (FACS), n=14 (IL-6), * $p<0.05$ as compared to DC not exposed to LiCl). **(D)** Mean fluorescence values of CD80, CD83, CD86 and IL-6 levels in the supernatants are shown for DC exposed to SB415286 relative to DC cultured in the absence of inhibitor (Mean values ± Stdev, n=4, * $p<0.05$ as compared to DC not exposed to SB415286)

GSK-3 is constitutively active in immature DC as shown by high Tyr279/216-phosphorylation and low Ser9/21-phosphorylation of GSK-3 (Figure 10A). Lithium inhibited GSK-3 activity in immature DC as demonstrated by increased Ser9/21-phosphorylation, whereas tyrosine phosphorylation was not altered. The reduction of GSK-3 activity by lithium was reflected by increased level of GSK-3 substrate β-catenin (Figure 10A). In contrast, SB415286 did not increase serine phosphorylation of GSK-3 but enhanced β-catenin expression (n=4, Figure 10B).

Inhibition of GSK-3 activity in immature DC resulted in increased expression of DC maturation markers CD80, CD83 and CD86, and higher levels of IL-6 secreted within 24-36 hours (Figure 10C and 10D). Absolute levels of IL-6 secretion without lithium were 177±77 pg/ml; in the presence of GSK-3 inhibitors, lithium and SB415286, these were increased to 298±131 pg/ml and 384±131 pg/ml correspondingly. The increase in IL-6 production was not due to the differences in cell yields, as GSK-3 inhibitors did not alter viability or cell yields of DC during the 36-48 hours activation period (n=5). These results demonstrate that GSK-3 is constitutively active in immature MoDC and inhibits their spontaneous maturation.

3.2.3 GSK-3 is inhibited during DC activation

GSK-3 is negatively regulated by several upstream pathways, including PI3K/Akt-1 pathway [226]. It was recently demonstrated that Akt-1 is activated (Ser473-phosphorylated) during pro-inflammatory stimulation of DC [141]. Akt-1 has been shown to phosphorylate GSK-3 at the inhibitory Ser9/21-moiety [230, 231]. Consistent with these observations, the Ser473 phosphorylation of -Akt-1 correlates with Ser9/21 phosphorylation of GSK-3 (Figure 11A and 11B). *E. coli* activated DC that showed highest levels of phospho-Akt-1 also showed highest levels of phospho-Ser9/21-GSK-3. PI3K inhibitor, wortmannin inhibited Akt-1 phosphorylation, and this resulted in reduced Ser9/21-phosphorylation of GSK-3 (Figure 11 A and 11B). Consistent with the observed higher levels of Ser9/21-phosphorylation of GSK-3, the level of β-catenin also increased during DC activation (Figure 11C).

These results suggest a net inhibition of GSK-3 activity following pro-inflammatory DC activation.

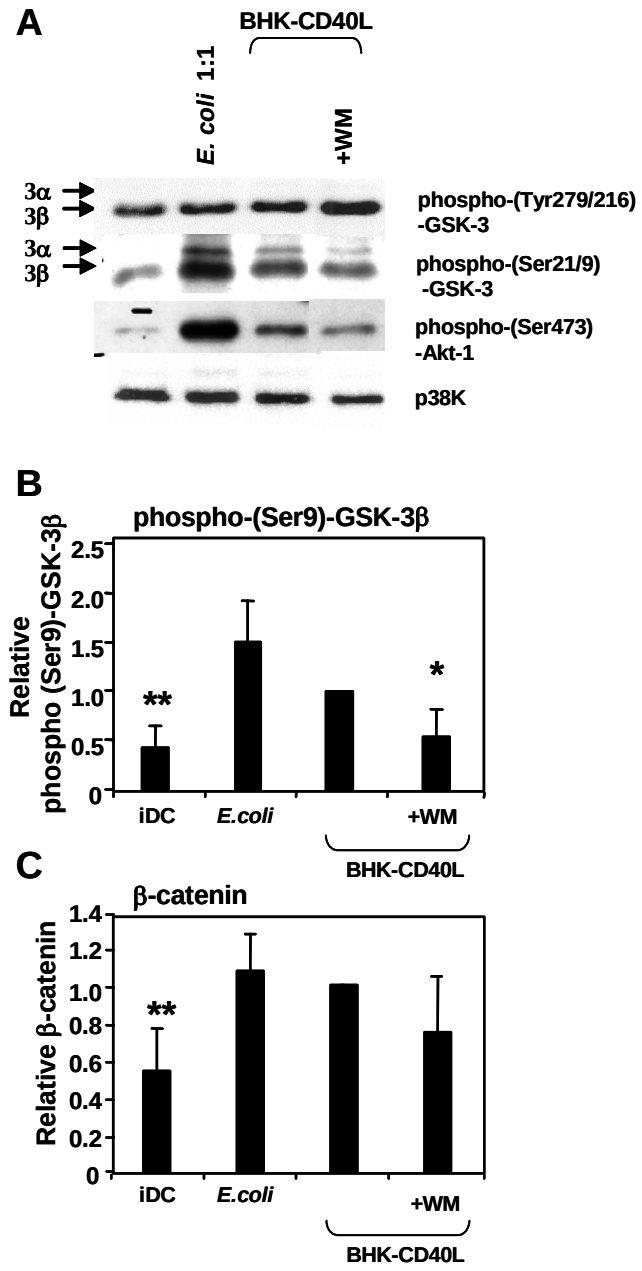


Figure 11 GSK-3 is inhibited during DC activation. MoDC were prepared as in Figure 10. Cells were either continued in GM-CSF and IL-4 (iDC), or activated with *E. coli* (1:1) or a BHK-CD40L (in ratio of DC to BHK cells 20:1) in presence or absence of wortmannin (100ng/ml). **(A)** Ser21/9 and Tyr279/216 phosphorylation of GSK-3 and Ser473 phosphorylation of Akt-1 2 hours following activation were analysed by Western Blot. p38K acts as protein loading control. One representative of 4 experiments is shown. **(B)** Strength of Ser9-phosphorylation of GSK-3β and **(C)** intracellular β-catenin expression was assessed by quantitative immunoblotting. Means ± Stdev of 4 experients are shown.

3.2.4 GSK-3 inhibitors reduce pro-inflammatory cytokine secretion by monocytes and DC activated with *E.coli*

Martin and coworkers have recently demonstrated that GSK-3 inhibitors enhance IL-10 secretion and inhibit IL-12p40, IL-6 and TNF-α secretion of human monocytes [214]. We therefore next compared the effects of GSK-3 inhibitors on cytokine production of activated monocytes and activated DC.

Monocytes stimulated with intact *E. coli* (Figure 12D) or LPS (n=6, data not shown) did not secrete IL-12p70. In contrast, human DC produced high levels of IL-12p70 (3400±1200 pg/ml), and this was nearly completely blocked by lithium and by

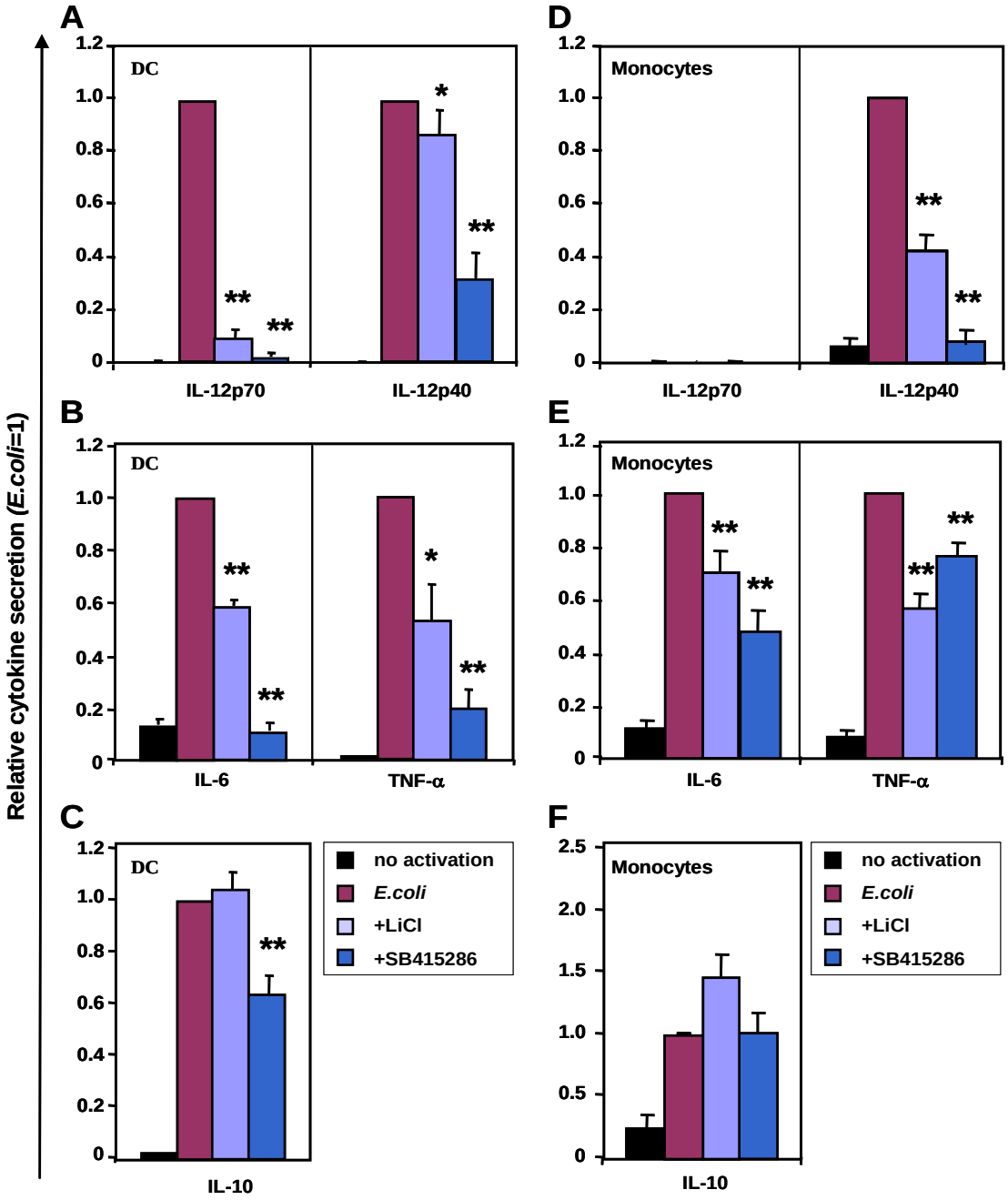


Figure 12 GSK-3 is necessary for IL-12p70 secretion of DC and enhances IL-12p40, IL-6 and TNF-α, but not IL-10 secretion by monocytes and DC activated with E. coli. Monocytes (right panels, **D-E**) were resuspended in culture medium at a concentration of $1x10^6$/ml and directly exposed to *E. coli* in the presence or absence of LiCl (10mM) or SB415286 (10µM). MoDC (left panels, **A-C**) were either continued in GM-CSF and IL-4 (no activation), or activated similar to monocytes. Supernatants were harvested after 36 hours. Mean values ± SEM, n=8-18 (monocytes) and n=6 (DC), * $p<0.05$; **$p<0.01$ as compared to activation without a GSK-3 inhibitor. Absolute cytokine levels in the absence of inhibitors (set 1) of *E. coli*-activated MoDC were: IL12p70: 2.1±1 ng/ml; IL12p40: 54.2±10.5 ng/ml; IL6: 109.7±25 ng/ml; IL10: 7.2±0.8 ng/ml; TNF-α: 16.1±3.8 ng/ml. Absolute cytokine levels of *E. coli*-activated monocytes (set 1): IL12p70: 0±0 ng/ml; IL12p40: 0.77±0.68 ng/ml; IL6: 58.2±24.1 ng/ml; IL10: 2.2±0.7 ng/ml; TNF-α: 3.2±1.2 ng/ml.

SB415286 (Figure 12A). IL-12p40 secretion was partially inhibited in both, monocytes and DC (Figure 12A and D). Furthermore, IL-6 and TNF-α were also partially inhibited to similar degrees in monocytes and DC by the two GSK-3 inhibitors (Figure 12B and E). We could not detect any significant effect of lithium on IL-10 secretion by monocytes and DC (Figure 12C and F). IL-10 secretion was inhibited to a minor degree by SB415286 in DC only. As GSK-3 inhibitors have been reported to enhance IL-10 secretion [214], we confirmed our observations using positively and negatively selected $CD14^+$ monocytes activated with LPS and *E. coli* at high (1:1) and low (1:1000) concentrations (see Methods). However, we did not observe significantly enhanced IL-10 secretion in the presence of lithium or

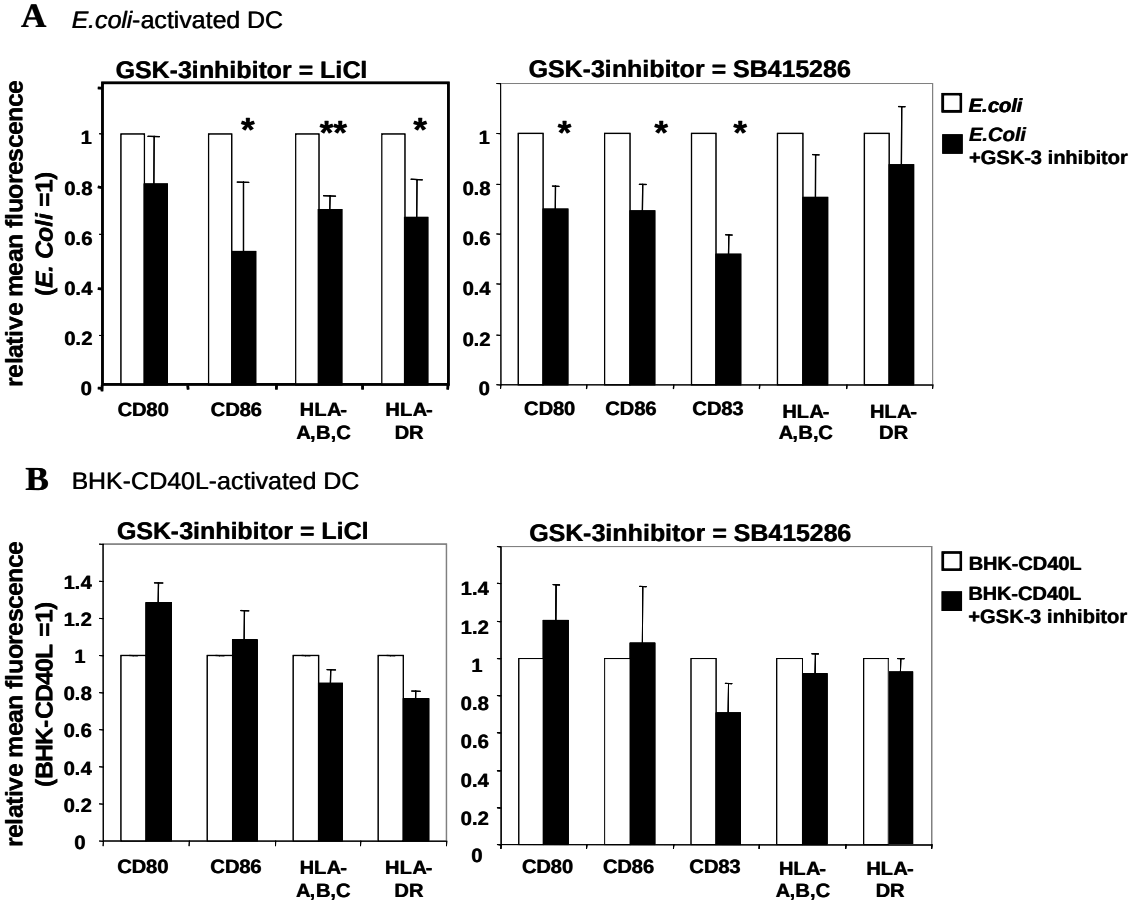


Figure 13 Influence of LiCl and SB415286 on phenotypic maturation of DC. MoDC were activated with *E.coli* **(A)** or BHK-CD40L **(B and C)** in the presence or absence of LiCl (10 mM) or SB415286 (10 µM). Flow cytometry analyses were performed 36-48 hours after activation. Relative mean fluorescence values of CD80, CD83, CD86, MHC class I and class II are shown. Mean values are related to DC activated in the absence of inhibitor (mean values ± Stdev, n=4). * $p<0.05$; **$p<0.01$ as compared to activation without a GSK-3 inhibitor.

SB415286 under any of these experimental conditions (n=4-16, data not shown).

Flow cytometry analysis of DC activated with *E.coli* in the presence or absence of LiCl or SB415286 (Figure 13A) showed that the inhibitors reduced, but did not abrogate the expression of the mature antigen-presenting DC phenotype.

The capacity of lithium and SB415286 to inhibit GSK-3 in activated DC was confirmed by Western blot analysis. Addition of lithium enhanced both, β-catenin expression and GSK-3 Ser(9/21)-phosphorylation, within 2 hours following activation of DC with low concentrations of *E. coli* (Figure 10A).

3.2.5 GSK-3 inhibitors reduce pro-inflammatory cytokine secretion of CD40L-activated DC

Activation of DC using a baby hamster kidney (BHK) cell line transfected with CD40L mediates a strong pro-inflammatory signal resulting in the secretion of high levels of cytokines. Similar to their effects on *E. coli* activated DC, LiCl, SB415286 and 1-Azakenpaullone blocked IL-12p70 secretion and reduced IL-12p40, IL-6 and TNF-α secretion of CD40L-activated DC (Figure 14A and data not shown). IL-10 was not significantly influenced (Figure 14A).

Flow cytometry analyses of DC activated with BHK-CD40L in the presence or absence of LiCl or SB415286 showed a reduced up-regulation of CD83, but no influence on CD80, CD86, MHC class I and class II (Figure 13B).

3.2.6 GSK-3 inhibitors specifically inhibit *E.coli*- induced migration, but not CD40L/cAMP-induced migration

DC activated with BHK-CD40L migrate poorly toward CCR7 ligands [141], whereas upon *E. coli* activation DC acquire significant migratory capacity. Addition of CD40L together with Sp-5,6-DCl-cBIMPS (cBIMPS, a membrane-permeable cAMP analogue and activator of PKA) induced migratory capacity.

We next investigated the influence of GSK-3 inhibitors on CD40L- and *E. coli*-induced migration. LiCl and SB415286 did not influence migration of MoDC induced by CD40L in presence of cAMP or without cAMP (Figure 14B). However, migration induced by *E. coli* was reduced to about 60% by all GSK-3 inhibitors (LiCl and SB415286, Figure 14B, and 1-Azakenpaullone, data not shown). This difference between CD40L-activated and *E. coli* activated DC was further supported by flow

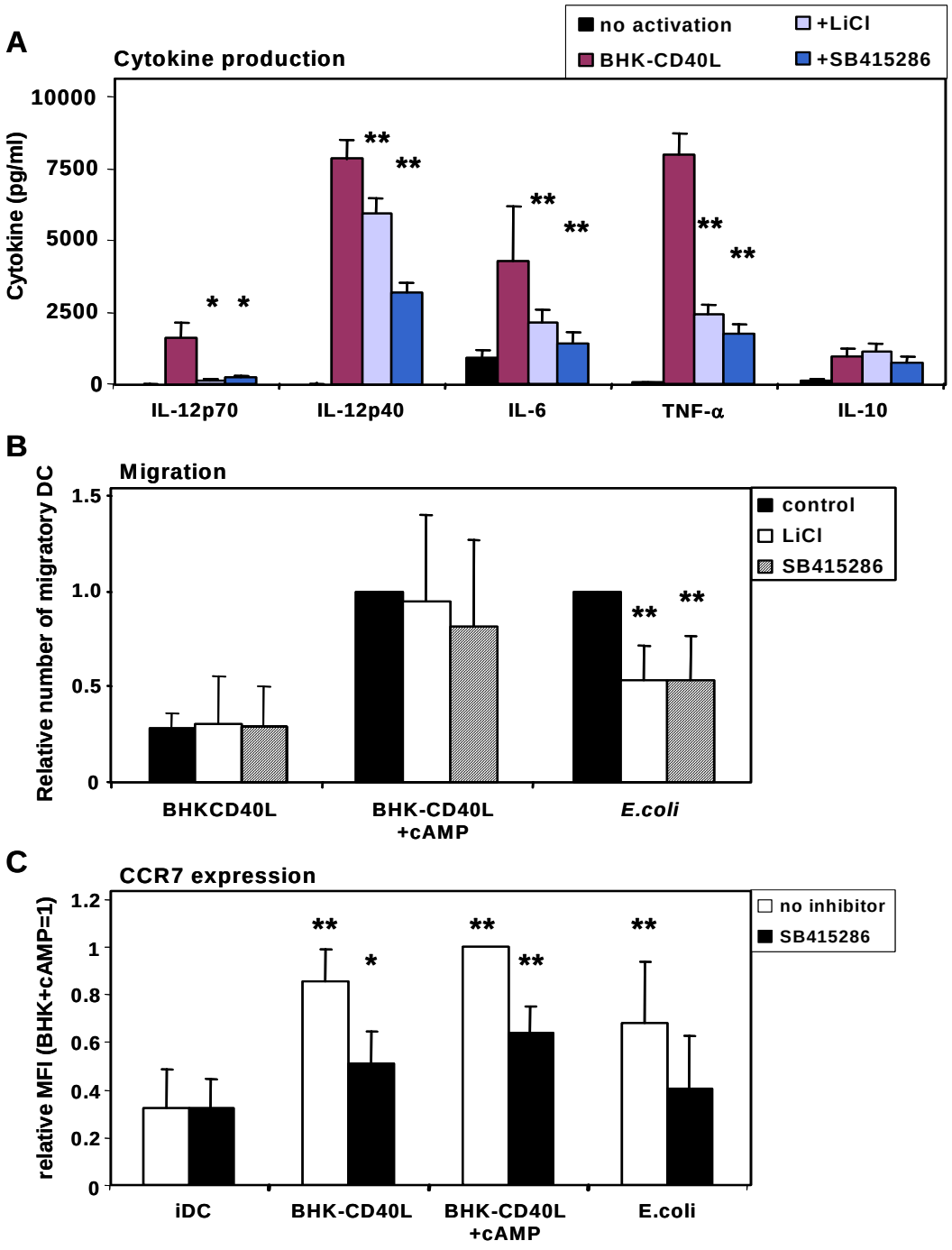


Figure 14 GSK-3 enhances pro-inflammatory cytokine secretion by CD40L-activated DC and reduces migration of E. coli activated DC. MoDC were either continued in GM-CSF and IL-4 (no activation), or activated with a BHK-CD40L in the presence or absence of LiCl (10mM) or SB415286 (10µM). **(A)** Cytokine levels in culture supernatants 36 hours following activation. Shown are Means ± SEM of 6 experiments, (*p<0.05, **p<0.01 as compared to activation with the BHK-CD40L). **(B)** Migration of DC activated with BHK-CD40L cells in the presence or absence of cAMP (cAMP-analogon Sp-5,6-DCl-cBIMPS, 50µM) or with *E. coli* was tested in transwell assays. Migration toward CCL21 (40 nM) relative to BHK-CD40L+cAMP is shown. Number of separate donors: BHK-CD40L+cAMP n=6; *E. coli* n=15, **p<0.01 as compared to activation without inhibitor. **(C)** CCR7 expression was analyzed by FACS 36hours after the activation. Shown are mean fluorescence values related to BHK-CD40L+cAMP ± Stdev. n=6).

cytometry analysis of CC chemokine receptor 7 (CCR7) surface expression. SB415286 significantly reduced CCR7 expression in all protocols of DC activation (Figure 14C). However, this reduction was most pronounced for *E. coli* activated DC. Here, surface CCR7 expression in the presence of the inhibitor did not differ from expression by immature DC.

This suggests that depending on the activation stimulus, GSK-3 influences migration of MoDC.

3.2.7 Reverse effects of PI3K and GSK-3 on IL-12 and IL-10 secretion

As mentioned previously, the PI3K/Akt pathway is activated during pro-inflammatory DC stimulation, thus inhibiting GSK-3. It was shown that inhibition of PI3K by wortmannin increases IL-12p70 secretion by activated DC [141]. Therefore, we investigated whether wortmannin effects on cytokine secretion could be reversed by inhibiting GSK-3 activity.

Lithium has been shown to inhibit GSK-3 both, directly by replacing Mg^{2+} [232] and indirectly by enhancing PI3K/Akt-1 activity that results in increased Ser9/21-phosphorylation levels of GSK-3 ([233] and Figure 10A). Thus, some effects of lithium may be explained by a modulation of PI3K activity. Therefore, we investigated interactions of wortmannin and SB415286, a PI3K-independent GSK-3 inhibitor which does not increase GSK-3 Ser9/21-phosphorylation (Figure 10B).

Figure 15 demonstrates that the opposing effects of wortmannin and SB415286 were significant for IL-12p70. SB415286 inhibited the wortmannin-enhanced IL-12p70 secretion of MoDC activated with *E.coli* (Figure 15) and BHK-CD40L (n=4, data not shown). SB415286 significantly decreased wortmannin-mediated IL-12p40 secretion only when DC were activated with low concentrations of intact *E. coli* but not with high *E.coli* concentrations or with BHK-CD40L. No effect on wortmannin-enhanced secretion of IL-6 and TNF-α could be detected (Figure 15). Similar results were obtained using the second GSK-3 inhibitor, 1-Azakenpaullone (n=4, data not shown). This suggests that other, indirect effects may explain the activity of GSK-3 inhibitors or wortmannin on the secretion of these cytokines. Alternatively, higher levels of GSK-3 inhibitors may be required to block the secretion of IL-6 and TNF-α, if GSK-3 activity is enhanced by wortmannin. Indeed, SB415286 at a concentration of 25 μM and 1-Azakenpaullone at a concentration of 5 μM significantly reduced secretion of

all cytokines (induced by wortmannin in combination with BHK-CD40L), however, viability was also reduced to about 70% (n=4, data not shown) suggesting that these concentrations were approaching cell toxic levels.

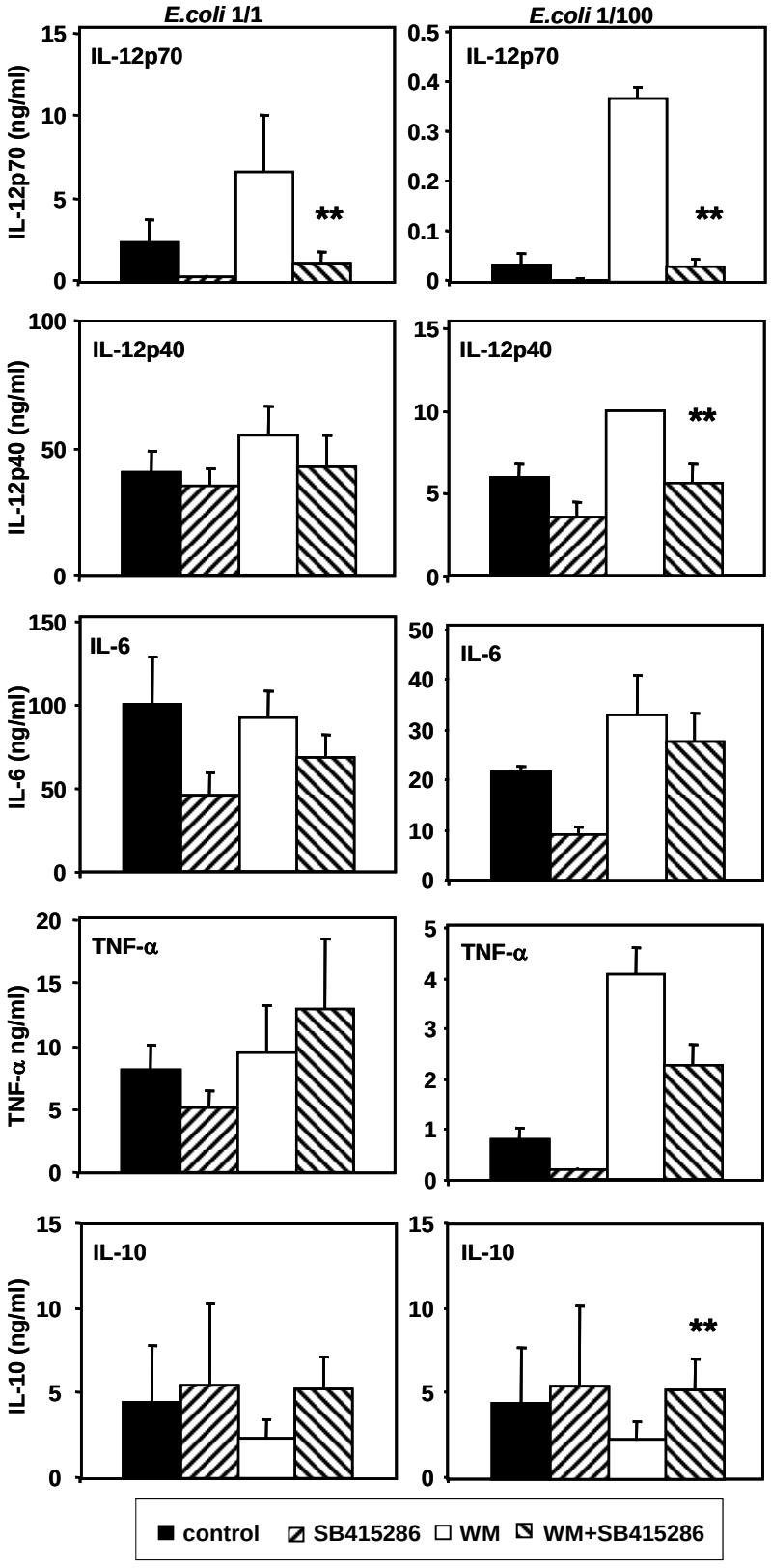


Figure 15 Reverse effects of PI3K and GSK-3 on IL-12 and IL-10 secretion. Immature MoDC were prepared and activated as in Figure 10. SB415286 (10µM) and wortmannin (WM, 100 ng/ml) were added 20 minutes prior to activation; *E. coli* were used in two concentrations (1:1 and 1:100; see Methods). Supernatants were harvested 36 hours following activation. Means ± SEM of 8 experiments (*E.coli* 1:1) and 4 experiments (*E. coli* 1:100) are shown, *p<0.05; **p<0.01 (comparison of WM vs WM+SB415286 only).

Interestingly, significant opposing effects of wortmannin and SB415286 were observed for IL-10 secretion induced by low concentrations of *E. coli* (Figure 15). Here however, wortmannin inhibited and SB415286 enhanced IL-10 secretion, which suggests that *E. coli*- induced IL-10 secretion is partially inhibited by GSK-3. Similarly, BHK-CD40L-induced IL-10 secretion was enhanced by the GSK-3 inhibitors in the presence of wortmannin (n=4, data not shown).

Our results substantiate that the enhancing effects of wortmannin on IL-12p70 secretion depend on GSK-3; and that the phosphorylation of the Ser9/21 moiety of GSK-3 by Akt-1 inhibits IL-12p70 secretion. Furthermore, this data support observations of Martin et al. [214] of GSK-3 being a partial inhibitor of IL-10 secretion.

3.2.8 GSK-3 specifically enhances IL-12p35 mRNA levels and inhibits IL-10 mRNA levels

Using real-time PCR quantification, we investigated whether GSK-3 regulates cytokine secretion at the mRNA level. CD40L-induced IL-12p35 mRNA was significantly inhibited by SB415286 (Figure 16) as early as 3 hours following activation. In contrast, IL-12p40 mRNA was reduced in MoDC only after 24 hours of activation. IL-10 mRNA was enhanced by SB415286 in all three MoDC cultures after 12 and 24 hours, however, due to the donor to donor variability (1.6 to 3.8 fold enhancement), statistical significance was not reached with three experiments. IL-6 mRNA was inhibited after 3 and 6 hours but enhanced after 12 hours following activation. TNF-α mRNA was not significantly changed by the GSK-3 inhibitor (Figure 16).

These results suggest that GSK-3 regulates IL-12p70 secretion on the level of IL-12p35 mRNA. In contrast, less direct and less consistent effects were observed for the other three pro-inflammatory cytokines, supporting the hypothesis that indirect effects of GSK-3 may modulate the secretion of IL-6, IL-12p40 and TNF-α.

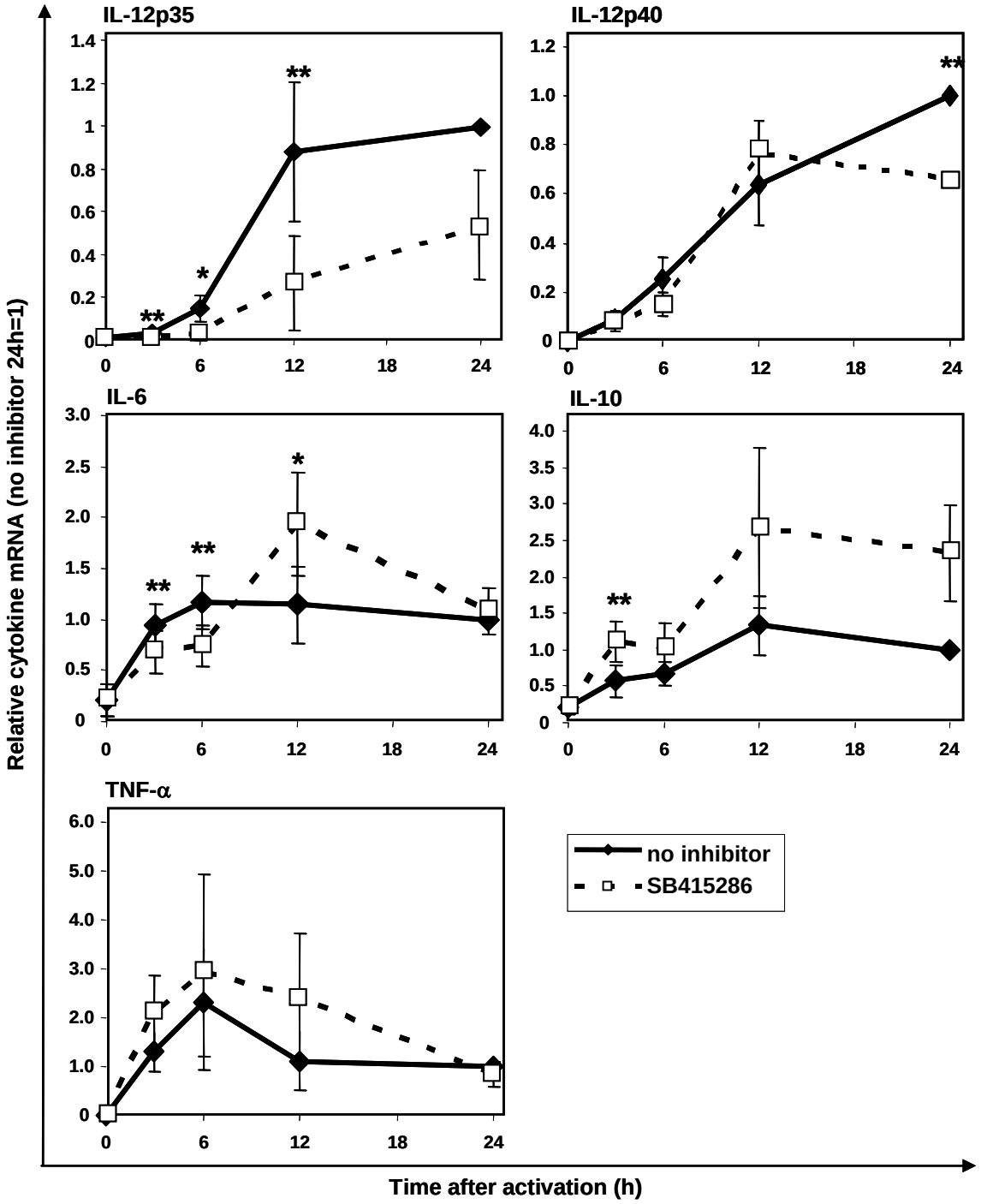


Figure 16 GSK-3 specifically enhances IL-12p35 mRNA levels and inhibits IL-10 mRNA levels. Real-time PCR quantification of mRNA in MoDC stimulated with BHK-CD40L cell line in the presence or absence of the GSK-3 inhibitor SB415286. Specificity of the mRNA signal was controlled by adding BHK-CD40L cells to the unstimulated MoDC (0 hours) and by analysing the influence of mock-transfected BHK cells (no CD40L) (n=1, not shown). Shown are the means ± Stdev of 3 independent experiments normalized to the 24 hour mRNA levels without inhibitor. * $p<0.05$; ** $p<0.01$ (comparison with and without SB415286).

4 Discussion

4.1 Identification of new potential vaccination antigens

Immunotherapy provides an opportunity to enhance the effects of conventional therapy in cancer patients. This approach aims to induce effective immune responses against the tumor cells in patients. These responses are directed against tumor-associated antigens. Identification and characterization of TAA that can be used for vaccination in clinics is an important step of anti-tumor vaccine development.

TAA can be identified by several strategies including: 1) molecular dissection of tumor-reactive T cells isolated from patients [234]; 2) elution of peptides naturally presented on the MHC of tumor cells [235]; 3) predicting epitopes from a particular protein that are likely to bind to common MHC alleles using bioinformatic programs [217, 218]. The latter approach was employed for identification of HLA-A2-binding antigens in this study. Predicted peptides can be synthesized, confirmed experimentally to bind to MHC, and used to generate peptide-specific CTL. This approach allows to focus the research only on specific proteins with desirable expression profiles. Moreover, synthetic peptides are easy to produce and the induced immune responses are relatively easy to monitor. A major disadvantage of this approach, however, is that processing and presentation of the predicted peptides on the MHC of the tumor cells can not be guaranteed.

In this work, MHC class I antigens derived from HLA-DP alleles and from immunoglobulin A and D constant regions were identified and evaluated as potential targets for vaccination of patients with hematological malignancies after stem cell transplantation (SCT).

BIMAS and SYFPEITHI MHC-binding prediction tools were used to identify potential HLA-A*0201-binding epitopes derived from polymorphic regions of HLA-DP alleles and from constant regions of IgA and IgD. This study focused on the HLA-A*0201 allele of MHC class I, due to its abundance in the human Caucasian population [219]. The theoretical prediction of MHC class I peptide binding was reliable as the experimentally measured HLA-A2 binding affinity of 14 out of 20 synthetic HLA-DP-derived peptides was comparable to that of the control

intermediate affinity AAGIGILTV peptide or higher; and only 1 HLA-DP-derived synthetic peptide did not show any detectable binding to HLA-A2 (Figure 1).

4.1.1 Induction of $CD8^+$ T-cell responses against intermediate affinity peptides

Generating peptide-specific $CD8^+$ T-cell lines and CTL clones was a prerequisite for the immunological characterization of the peptides in this study. The peptides analyzed in this study were binding to HLA-A2 with intermediate to low affinity. Moreover, similar to most of the tumor associated antigens, they represent non-mutated self antigens and thus might be tolerated by the immune system. Therefore, the optimization of the method of generating specific CTL lines against moderate affinity self peptides represented the main challenge.

The method initially used for generation of CTL lines employed T2 cells pulsed with peptides for priming and restimulation of antigen-specific $CD8^+$ T-cells. This cell line is deficient for TAP and therefore endogenously produced antigens do not reach the ER and are not presented on the cell surface. Due to the lack of competition of endogenous antigens for binding to MHC class I, TAP-deficient T2 cells can provide prolonged presentation of exogenously delivered low-affinity peptides [224]. This method allowed for generating specific CTL lines against HLA-DP-derived antigens. However, this method had a major disadvantage. Repetitive stimulation of CTL-cultures with T2 cells caused expansion of T-cells recognizing T2 cells independent of the exogenous antigen they were pulsed with. This T2 cell-directed response usually occurred after 4-5 restimulations and "T2-specific" T-cells rapidly overgrown peptide-specific responses thus impeding the generation of peptide-specific CTL clones. The target antigens of CTL responses against TAP-deficient T2 cells are not known, but could be induced by MHC class I associated peptides, entering the ER via TAP-independent pathways. The peptides derived from C-terminus of the transmembrane- and soluble proteins of the secretory pathway have access to MHC class I molecules in ER in the absence of TAP activity [46]. Moreover, it was recently reported that such peptides could serve as neoantigens inducing CTL-responses against TAP-deficient tumor variants [47].

In order to avoid induction of a strong non-peptide-specific CTL response against T2-cells, we next used autologous DC as APC for generating peptide-specific T-cell-

lines. DC have the unique capacity to prime T-cell responses due to expression of high levels of co-stimulatory molecules and secretion of T-cell activating cytokines. However, this method failed to induce any HLA-DP-derived peptide-specific CD8 response. The failure of DC to stimulate CTL cultures against intermediate and low affinity peptides could probably be explained by the inefficiency of presentation of these exogenous peptides by DC. As DC are TAP-competent, they present a large variety of internally processed peptides that compete for the binding to MHC class I in the ER. Most of the peptides presented on the surface of TAP-competent cells will have a high affinity to MHC [224]. Therefore, exogenous low-affinity peptides may have a limited chance of being presented on MHC class I for an extended period of time [224, 225].

The efficiency of T-cell responses against any antigen depends on the avidity of the interaction between the T-cell and the antigen presenting cell. This includes 1) the affinity of antigen binding to the MHC molecule, 2) affinity between the TCR and the MHC–peptide complex and 3) the co-receptors and co-stimulatory molecules associated with T-cell activation [223]. Whereas high affinity peptides provide stronger stimuli due to the increased stability of the MHC–peptide complex, low-affinity peptides might require additional help to be able to induce effective T-cell responses. We assumed that for the induction of efficient CTL responses against intermediate affinity peptides the combination of prolonged peptide presentation and efficient T-cell co-stimulation was necessary. TAP-deficient T2 cells can provide prolonged presentation of low-affinity peptides, required for efficient activation of naïve T-cells, but they lack costimulatory molecules. In contrast, dendritic cells express high levels of the costimulatory molecules CD80/CD86, but rapidly loose peptide expression [224]. If our assumption is true, the combination of autologous DC and T2 cells will be able to induce stronger specific $CD8^+$ T-cell responses against moderate-affinity peptides than DC alone.

This hypothesis was tested on the model of two Melan-A derived HLA-A*0201-restricted peptides of known immunogenicity that have intermediate and high affinity of binding to HLA-A*0201. The Melan-A derived AAGIGILTV peptide occurs naturally and has an intermediate affinity. Its synthetic analog (10-mer peptide ELAGIGILTV) was modified on the anchor positions to bind to HLA-A*0201 with higher affinity [52, 53]. The advantage of these model antigens is that the frequency

of peptide-specific CTL precursors is similar. Therefore, the efficiency of the CTL response induction will depend only on the different affinity of peptide binding to MHC.

CTL were primed by DC either alone or in combination with T2 cells. In order to avoid non-specific CTL-responses, T2 cell were removed from these cultures after 7 days and CTL were restimulated by DC. Consistent with our hypothesis, DC alone and DC in combination with T2 cells induced similar responses against the high affinity ELAGIGILTV peptide, whereas CTL-responses against the intermediate affinity AAGIGILTV peptide were significantly stronger when primed with DC in combination with T2 cells.

These results suggest that the induction of CTL responses against intermediate affinity peptides requires prolonged presentation on the cell surface. Such prolonged presentation is achieved by TAP-deficient T2 cells and not by TAP-competent DC, as priming CTL with DC did not suffice to induce an efficient response. However, non-specific responses were also induced by T2 cells. Induction of CTL responses with the combination of DC and T2 with subsequent removal of T2 cells from the cultures allowed generation of peptide-specific CTL responses against intermediate affinity peptides and reduced nonspecific reactivity of these cultures.

4.1.2 Characterization of HLA-DP-derived antigens

4.1.2.1 Immunogenicity of HLA-DP-derived peptides

Although it was previously demonstrated that HLA-DP can be a target antigen of $CD4^+$ T lymphocytes [199, 200], $CD8^+$ T-cell responses were not reported. We selected for this study 20 peptides derived from the polymorphic regions of the HLA-DP molecules according to their predicted capacity to bind HLA-A*0201. These peptides were synthesized and used to generate peptide-specific CTL lines from PBMC of healthy HLA-$A2^+$ donors. Here we demonstrate that $CD8^+$ CTL responses could be generated against 11 out of 20 HLA-DP-derived synthetic peptides. Peptide-specific CTL responses were, however, relatively rare. CTL responses against different HLA-DP peptides could be detected in 11 to 28% of tested healthy donors (Table 2).

4.1.2.2 Crossreactivity of HLA-DP-derived peptides

For the aim of vaccination against the recipients' HLA-DP allele after HLA-DP mismatched allogenic SCT (See Chapter 1.4), it is essential that the CTL against recipients' HLA-DP do not recognize the donor HLA-DP variant. There are two possible situations where such differential recognition of polymorphic peptides can take place: 1) the polymorphic peptide from the donor HLA-DP allele is presented on HLA-A2, but is not recognized by the CTL raised against the recipients' HLA-DP allelic variant or 2) polymorphic peptide from the donor HLA-DP allele is not presented on HLA-A2 due to different natural processing or to a loss of affinity to HLA-A2. The first possibility was tested for 2 polymorphic HLA-DP derived peptide groups (#4007-#4011 and #4013-#4015), for which peptide-specific CTL lines could be generated. We demonstrated that the polymorphic synthetic peptides within each group were cross-reactive. The next step in the characterization of polymorphic HLA-DP-derived peptides was testing their natural presentation.

4.1.2.3 Cloning of HLA-DP-derived peptides

As mentioned before, candidate epitopes that are theoretically predicted to bind to MHC (even if the MHC binding is conferred experimentally for synthetic peptides) will not necessarily be processed and presented on the MHC molecule on the cell surface. During processing of the full-length protein, the proteasome may generate peptides that are too short to fit in the groove of MHC, or it may cleave the protein at sites within the epitope. Sequence and size-preferences for peptide transfer into the ER by TAP can additionally contribute to restrictions of the cellular antigen repertoire [40, 41].

The natural presentation of HLA-DP-derived peptides could be tested using HLA-A*0201^{+} antigen presenting cells (e.g. B-cells or DC) expressing different HLA-DP alleles. However, potential allogeneic activity of peptide-specific CTL cultures had to be excluded in order to determine the specific CTL recognition of the HLA-DP-derived peptide variant. Therefore, in order to address the question of the natural presentation of HLA-DP-derived peptides, the generation of peptide-specific CTL clones was required. However, despite using our method of CTL response induction which was optimized for the model intermediate affinity AAGIGILTV peptide, CTL clones specific for HLA-DP-derived peptide could not be obtained in this study. The

possible difference between the Melan-A derived AAGIGILTV peptide and the HLA-DP-derived peptides used in this study is the frequency of the peptide-specific CTL precursors. It was previously shown that T cells specific for the AAGIGILTV have an exceptionally high precursor frequency in HLA-A*0201$^+$ individuals [236, 237]. It is the only self-antigen for which specific naïve T-cells could be directly detected in humans [236]. Therefore, the low frequency of the CTL precursors recognizing HLA-DP-derived peptides could explain why CTL clones specific for HLA-DP-derived peptides could not be generated.

4.1.2.4 Are HLA-DP derived antigens involved in GvHD development?

As mentioned in Chapter 1.4, patients mismatched for two HLA-DP alleles have a significantly increased risk of GvHD [198], suggesting that immune responses against HLA-DP could be involved in GvHD development. This study demonstrated that CD8$^+$ CTL responses could be generated against several HLA-DP-derived synthetic peptides. The characterization of the natural presentation of the HLA-DP-derived peptides, however, was not possible due to an inability to generate CTL clones specific for HLA-DP-derived peptides. It would therefore be important to investigate whether CTL recognizing these peptides have any impact on GvHD development in patients after HLA-DP mismatched allogeneic SCT. This can be tested by monitoring CTL responses against HLA-DP derived peptides *ex vivo* in patients who developed GvHD after allogeneic SCT. For this purpose, HLA-A*0201$^+$ donor-recipient pairs have to be identified that show particular mismatches in HLA-DP alleles. At the onset of GvHD, before steroid treatment is initiated (depleting CTL), T-lymphocytes have to be obtained and tested against antigens derived from recipients HLA-DP allele. These experiments are planned for the future.

4.1.3 Characterization of the antigens derived from immunoglobulin A and D constant regions

Antigens derived from immunoglobulin constant regions represent potential candidates for vaccination against B-cell malignancies. The potential advantage of vaccinating against immunoglobulin constant regions (isotypes) as compared to idiotypes (variable regions of Ig) is that isotypes are not unique for the individual B-cell malignancy but are shared by many patients. This study characterized antigens

derived from IgA and IgD isotypes, which are expressed on a subset of non-Hodgkins lymphomas and multiple myelomas.

4.1.3.1 Immunogenicity of IgD- derived LLNASRSLEV epitope

For this study we selected 6 peptides for each isotype (IgA and IgD) that were predicted to bind to HLA-A*0201 (Table 3.). These peptide sequences were specific for IgA or IgD isotypes, and were not present in any of the IgG isotypes, the IgE or the IgD/IgA respectively. IgA- and IgD- derived peptides were used to generate peptide-specific CTL lines and clones. We demonstrated that positive CTL responses against the IgD-derived LLNASRSLEV peptide could be induced in 40% of healthy HLA-$A2^+$ donors. This epitope could therefore be a promising target for immunotherapy against B-cell malignancies. Therefore, CTL clones specifically recognizing LLNASRSLEV epitope were generated for further characterization of the natural presentation of this peptide.

4.1.3.2 Immunogenic IgD- derived LLNASRSLEV epitope is not naturally presented on the cell surface

IgD is expressed on a subset of B-cells in healthy individuals and on some B-cell malignancies, e.g. multiple myeloma. If the IgD-derived LLNASRSLEV epitope is produced following proteasomal degradation of IgD, it can be transported into the ER and presented within the MHC class I complex. Therefore, the natural presentation of LLNASRSLEV epitope was examined by testing the ability of peptide-specific CTL to recognize IgD^+ B-cells and IgD^+ multiple myeloma cells. However, such a recognition could not be detected. This failure was not due to inefficient presentation of MHC-class I antigens by the target cells, as the same cells were recognized by CTL when pulsed with LLNASRSLEV peptide exogenously.

It has been reported that IFN-γ changes the MHC class I peptide presentation profile [35-37] via formation of immunoproteasomes. However, the LLNASRSLEV-specific CTL failed to recognize IgD^+ B-cells even preactivated with IFN-γ.

The data presented in this work demonstrate that IgD-derived LLNASRSLEV-epitope was not efficiently processed and presented on HLA-A2 on the surface of either normal or malignant cells. Therefore, although CTL responses against this epitope can

be efficiently induced using a synthetic peptide, it will not be presented on the surface of tumor cells.

This epitope was therefore not considered to be a suitable target for vaccinations.

4.2 GSK-3 regulates DC differentiation and pro-inflammatory DC function

DC are attractive candidates for active vaccination strategies due to their unique capacity to stimulate both humoral and cell-mediated immune responses. DC undergo a complex developmental program through three main differentiation stages – DC precursors, immature DC and mature DC. Immature DC sample the environment for the presence of "danger signals". As soon as iDC detect a danger signal, they undergo a complex maturation process. In the process of maturation, DC change their expression profile of surface markers resulting in an augmented capacity to activate naive and memory T-cell responses. Furthermore, DC may also secrete high levels of pro-inflammatory cytokines and can acquire the capacity to migrate towards lymph nodes. In order to use DC as an adjuvant for anti-tumor vaccinations, it is necessary to understand how DC development and function are regulated. We therefore investigated the intracellular signaling pathways that regulate different functions of DC. This study focused on the glycogen synthase kinase 3 (GSK-3) pathway.

GSK-3 is a multifunctional enzyme that is involved in a wide range of cellular processes, including cellular differentiation, apoptosis, self renewal and motility. More than 40 proteins including 17 transcription factors have been reported to be substrates of GSK-3 (reviewed in [226]). GSK-3 is negatively regulated by several upstream pathways, including the PI3K/Akt-1 pathway [226]. The PI3K/Akt-1 pathway was shown to inhibit pro-inflammatory cytokine production (including IL-12 [141, 238] and TNF-α [239]) of DC and monocytes, and enhance IL-10 production [240]. Furthermore, GSK-3 inhibitors were recently demonstrated to enhance IL-10 secretion and inhibit IL-12p40, IL-6 and TNF-α secretion of human monocytes [214]. Moreover, GSK-3 dysregulation is involved in several neurologic diseases such as bipolar mood disease [201, 202] and schizophrenia [203] that can be associated with an increased serum levels of pro-inflammatory cytokines (such as IL-6 and TNF-α and IL-12p70) [209-212]. These pro-inflammatory cytokines can be produced in large amounts by activated DC. Based on these observations, GSK-3 is a candidate regulator of DC function.

Using an *in vitro* model of DC differentiation from human $CD14^+$ monocytes, we investigated the involvement of GSK-3 in regulation of three main differentiation stages of DC – DC precursors (monocytes), immature DC and mature DC.

4.2.1 Specificity of GSK-3 inhibitors

In the present study, the involvement of GSK-3 was investigated by blocking GSK-3 activity with 4 different GSK-3 inhibitors: LiCl, SB216763, SB415286 and 1-azakenpaullone. Selectivity is a key issue when GSK-3 inhibitors are used to demonstrate the involvement of GSK-3 in a cellular process. We therefore used the inhibitors that inhibit GSK-3 by two different mechanisms. Lithium inhibits GSK-3 directly by outcompeting Mg^{2+} [232] and indirectly by activating the Akt-pathway [233]. Lithium therefore is not very specific for GSK-3. SB216763, SB415286 and 1-azakenpaullone compete with ATP in the ATP-binding site of the kinase. These inhibitors appear to be among the most specific GSK-3 inhibitors identified so far [241]. None of the inhibitors used in this study distinguish between GSK-3α and GSK-3β isoforms.

As the effects reported in this study were confirmed using several GSK-3 inhibitors that differ in chemical structure and mechanism of inhibition, they are most likely to result from the reduction of GSK-3 activity and not by side effects.

4.2.2 GSK-3 modulates monocyte differentiation: DC versus macrophages

Monocytes represent 5-10% of peripheral blood leukocytes. There are two major outcomes of monocyte differentiation: they can develop into macrophages or can become DC. Functionally, macrophages exhibit high phagocytic activity and rapidly degrade engulfed material [242], but fail to prime T-cell response *in vivo* [243, 244]. In contrast, although DC exhibit lower phagocytic activity and lower proteolytic activity than macrophages [242], they effectively process and present antigens on MHC class I and class II in context of appropriate costimulation [118] for effective priming of T-cell response. Moreover, macrophages and DC differ in migratory properties. Macrophages are mainly retained in the tissues, whereas DC can acquire the capacity to migrate towards lymph nodes in order to interact with T-cells and prime immune responses [137]. Therefore, macrophages are thought to be more

important for the local clearance of bacteria and dead cells, whereas DC have a major role in initiating adaptive T-cell responses.

It was recently demonstrated that GSK-3 is constitutively active in human $CD14^+$ monocytes [214]. Using an *in vitro* model of differentiation of human monocytes into DC in presence of GM-CSF and IL-4, we demonstrated that GSK-3 is crucially involved in this process. The present study shows that during the early stages of cell culture in GM-CSF and IL-4, GSK-3 inhibitors shift the differentiation of monocytes from developing into DC toward the macrophage lineage. The observed effect of GSK-3 inhibitors might be explained in several ways. In the presence of GM-CSF and IL-4, GSK activity can either promote differentiation of monocytes into DC or inhibit differentiation of monocytes into macrophages. IL-4 is known to be crucial for differentiation of monocytes into DC. In the presence of GM-CSF and absence of IL-4, human monocytes differentiate into macrophages [245]. Therefore, the effects of GSK-3 inhibitors on differentiation of human monocytes in the presence of GM-CSF and IL-4 suggest that GSK-3 might be involved in the IL-4 signaling pathway. Signaling pathways activated by IL-4 receptor engagement involve PI3K/Akt and Ras/MAPK pathways (reviewed in [246]). Activation of these pathways is mediated by Janus kinase family (Jak1 and 3) and insulin receptor substrate (IRS) 1 and 2. Jak1 and 3 also recruit Stat-6, the transcription factor that is critical for the activation or enhanced expression of many IL-4-responsive genes. Activation of gene transcription by Stat-6 may require cooperative interactions with additional transcription factors, such as C/EBPβ [247, 248] and AP1 [249]. Interestingly, C/EBPβ [250] and AP1 [251] are substrates of GSK-3. C/EBPβ was shown to be positively regulated by phosphorylation at a consensus ERK/GSK3 site [250], whereas AP-1 is negatively regulated by GSK-3 activity [251]. This suggests a possible mechanism how inhibition of GSK-3 activity can change the transcription profile induced by IL-4 and thus change the differentiation program of human monocytes.

Another issue to be considered is heterogeneity of the human monocyte population. Potentially, there can exist two subsets of monocytes, one predisposed to differentiate into DC and the other prone to differentiation into macrophages. For example, two major subsets of human peripheral blood monocytes differing in CD14 and CD16 expression were described: the so-called ‘classical’ $CD14^+CD16^-$ monocytes, representing around 90% of monocytes in healthy individual, and the ‘non-classical’

$CD14^{low}CD16^{+}$ monocytes comprising the remaining fraction [252, 253]. Several studies have analyzed the differentiation of these monocyte subsets *in vitro*. Some of them demonstrated that $CD14^{low}CD16^{+}$ monocytes in the presence of vascular endothelial cells are more prone to become DC [254]. However, it was demonstrated that in presence of human epidermal environment $CD14^{+}CD16^{-}$ monocytes can differentiate into Langerhans cells [255]. Therefore, the relevance of CD16 expression for monocyte differentiation fate is not yet clarified. Nevertheless, the possibility of the presence of two monocyte subsets differing in predisposition to differentiate toward DC or macrophage lineage can not be excluded. If this is the case, GSK-3 activity could differentially influence the survival of these two monocytic subpopulations.

We have not investigated, whether there is a defined subpopulation of monocytes that differentiates into DC and whether there are specific subpopulations that die. In our model yields of DC comprise 20-40% of the initial monocyte population, whereas 60-80% of the monocytes are dying within 5-7 days of culturing. Yields of viable cells were lower in cultures containing GSK-3 inhibitors (62+/-9% compared to 100% without inhibitors). As the yields were comparatively low, we are not able to exclude the existence of a few separate populations of monocytes in the cultures. We can therefore not tell whether GSK-3 inhibitors changed viability of a certain monocyte subpopulation or if the inhibitors changed the developmental program of the same population. However, both monocyte-derived cell types after 5 days (DC and macrophage-like cells differentiated in the presence of GSK-3 inhibitors), represented homogeneous populations by phenotype (Figure 9C) rather than two distinct populations. This observation indirectly favours the hypothesis that GSK-3 inhibitors change the developmental program of the same monocyte population. In order to finally clarify this issue, single cell experiments would be needed. However, single cell experiments with non-proliferating DC precursors are not possible at the moment.

4.2.3 Spontaneous maturation of monocyte-derived DC is inhibited by GSK-3

As outlined above, GSK-3 is constitutively active in immature MoDC and inhibits their spontaneous maturation in culture, as demonstrated by increased CD80, CD83 and CD86 expression and IL-6 secretion in presence of GSK-3 inhibitors. The mechanism by which GSK-3 inhibits spontaneous maturation of monocyte-derived

DC is not clear. One possible candidate for maintaining DC in immature state in the absence of maturation stimuli is the GSK-3 substrate β-catenin. Active GSK-3 associates with cytoplasmic β-catenin and reduces its levels by targeting β-catenin for proteasomal degradation. There are three functionally distinct pools of β-catenin in the cell: 1) in adherent junctions, directly associated with the cytoplasmic part of E-cadherin; 2) in the cytoplasm 3) in the nucleus, associated with transcription [256]. The levels of β-catenin in these three pools are influenced by one another. For example, differences in E-cadherin expression affect the cytoplasmic β-catenin pool and an increase in cytoplasmic β-catenin levels (e.g. as a result of GSK-3 inhibition) induce its nuclear translocation and subsequent initiation of β-catenin target gene transcription [257]. Interestingly, E-cadherin was shown inhibit maturation of $CD34^+$-derived human Langerhans cells [258]. In this study, spontaneous maturation (in presence of the cytokines used for differentiation of $CD34^+$ cells into Langerhans cells, i.e. TGF-β1, GM-CSF, TNF-α, SCF and Flt3 ligand) was induced by disruption of immature cell clusters. This effect was due to disruption of E-cadherin signaling, as the addition of an E-cadherin-specific mAB blocked spontaneous maturation of Langerhans cells [258]. As E-cadherin associates with β-catenin in adherent junctions, it is possible that the spontaneous maturation of monocyte-derived DC caused by GSK-3 inhibitors is a result of the changed cellular levels of β-catenin. Further experiments are required to verify the role of β-catenin in maturation of MoDC. This should be investigated in the future by transfecting DC with β-catenin and β-catenin siRNA constructs.

4.2.4 Stimulus-specific influence of GSK-3 on migration and maturation phenotype

We demonstrate that GSK-3 inhibitors exhibit different effects on phenotypic maturation and migration of DC, depending on the activation stimuli. GSK-3 inhibitors reduce phenotypic maturation of *E. coli* activated DC (which includes CD83 expression), although they do not completely prevent phenotypic maturation (Figure 13A). For BHK-CD40L activated DC, GSK-3 inhibitors reduce CD83 expression only (Figure 13B). Likewise, the migration of *E. coli*-activated DC was inhibited by GSK-3 inhibitors, whereas migration of DC activated with BHK-CD40L in presence or absence of a cell permeable cAMP analogue was not significantly

altered. This was also reflected by the levels of CC chemokine receptor 7 (CCR7) expression. Although SB415286 reduced CCR7 expression in all activation protocols, this reduction was most pronounced in E. coli-activated DC where surface CCR7 expression levels did not differ any more from immature DC (Figure 14C). This may explain the differences in DC migration in the presence of SB415286 of BHK-CD40L-activated DC and *E. coli*-activated DC shown in Figure 14B.

The mechanisms underlying these differential effects were not investigated in this study. *E.coli* activation of DC is a result of simultaneous engagement of several TLR receptors, whereas CD40L activates DC via CD40 signaling. It is possible that *E.coli* activation induce a strictly GSK-3-dependent phenotypic maturation of DC. GSK-3 inhibitors therefore decrease expression of all surface maturation markers, including CCR7. In contrast, CD40 crosslinking may additionally activate GSK-3-independent pathways leading to DC maturation.

4.2.5 GSK-3 regulates cytokine secretion by activated DC and monocytes

4.2.5.1 GSK-3 regulates IL-12p70 secretion of activated DC

Upon activation of DC with intact *E.coli* or CD40L, IL-12p70 secretion was almost completely blocked by GSK-3 inhibitors. GSK-3 is inhibited by the upstream PI3K/Akt pathway. This is achieved by Ser21/9 phosphorylation of GSK-3 by Akt-1. As was demonstrated previously, IL-12p70 secretion was enhanced in presence of the PI3K inhibitor wortmannin ([141] and Figure 15). This coincided with reduced phosphorylation of the GSK-3-Ser21/9 moiety. The enhancing effect of wortmannin on IL-12p70 secretion could be opposed with the GSK-3 inhibitors SB415286 and 1-Azakenpaullone, suggesting that the wortmannin effect indeed targeted GSK-3. These observations further support the critical role of GSK-3 for IL-12p70 production.

IL-12p70 is a biologically active form of IL-12. It is a heterodimer, composed of IL-12p40 and IL-12p35 subunits. Human IL-12p40 expression is primarily regulated at the transcriptional level, whereas IL-12p35 expression has been reported to be regulated on multiple levels, including both, transcriptional and translational mechanisms, as well as post-translational modifications (summarized in [259]). GSK-3 could therefore influence IL-12-p70 production on several levels, including transcription, translation, post-translational modifications and cytokine secretion.

In this study, we investigated whether IL-12 p40 and p35 mRNA levels were affected by the GSK-3 inhibitor SB415286. We could demonstrate that SB415286 inhibited CD40L-induced IL-12p35 mRNA in MoDC. In contrast, IL-12p40 mRNA levels responded to GSK-3 inhibitors only after 24 hours following activation. These results suggest that GSK-3 regulates IL-12p70 production via specifically regulating IL-12p35 mRNA levels.

4.2.5.2 Other pro-inflammatory cytokines

However, this picture is less clear for the other cytokines investigated. Compared with IL-12p70, cytokines such as IL-12p40, IL-6 and TNF-α were only partially inhibited by lithium, SB415286 or 1-Azakenpaullone suggesting that GSK-3 dependent and independent mechanisms may be involved in their regulation. This is further underlined by the lack of efficacy of the GSK-3 inhibitors to block wortmannin-enhanced IL-6 and TNF-α secretion. Furthermore, RT-PCR quantification demonstrated that IL-6 mRNA was differentially influenced in the early and late periods following activation, whereas TNF-α mRNA was not significantly influenced by GSK-3. IL-12p40 mRNA levels, as mentioned before, responded to GSK-3 inhibitors only after 24 hours following activation.

On the other side, wortmannin opposed the effects of SB415286 on IL-6 and TNF-α. Although more than one interpretation is possible, this observation may suggest the existence of additional inhibitory effects of the PI3K/Akt-1 pathway independent of GSK-3. These effects may also involve post-translational processing and regulation of secretion of these cytokines.

4.2.5.3 IL-10

GSK-3 inhibitors have previously been reported to enhance IL-10 secretion by human monocytes [214]. We therefore studied IL-10 secretion by activated human $CD14^+$ monocytes and MoDC. We could not detect a significant effect of lithium and SB415286 on IL-10 production of $CD14^+$ monocytes activated with intact *E.coli*. As this contradicted the data published by Martin *et al.*, we confirmed our observations using positively and negatively selected $CD14^+$ monocytes activated with LPS and intact *E. coli* at high and low concentrations (n=4-16, data not shown). However, the differences in IL-10 secretion in the presence or absence of lithium and SB415286

were not significant under any of these experimental conditions. Similarly, IL-10 secretion by MoDC activated with *E.coli* was not affected by the addition of lithium whilst addition of SB415286 only weakly inhibited IL-10 secretion. GSK-3 inhibitors as well did not influence IL-10 in CD40L-activated DC.

The PI3K inhibitor wortmannin inhibited CD40L- and *E.coli*-induced IL-10 secretion in MoDC. These results are consistent with the data that PI3K/Akt pathway enhances IL-10 production by human monocytes [240]. Interestingly, SB415286 opposed the effect of wortmannin on IL-10 secretion, suggesting that IL-10 production is partially inhibited by GSK-3. This was also supported by the observed increased levels of IL-10 mRNA in the presence of the GSK-3 inhibitor SB415286 in CD40L-activated MoDC. This suggests that GSK-3 is a partial regulator of IL-10 production by DC. However, additional, GSK-3-independent mechanisms seem to influence IL-10 secretion by monocytes and DC.

4.2.6 GSK-3 acquires pro-inflammatory function in activated DC

This study demonstrates that GSK-3 activity is crucial for IL-12p70 production of activated MoDC. Interestingly, GSK-3 is inhibited upon activation, as demonstrated by an increase in Ser21/9 phosphorylation levels of GSK-3 upon activation with intact *E.coli*. How can the enzyme that is inhibited upon DC activation enhance IL-12p70 secretion? Most GSK-3 substrates require “priming”, i.e. phosphorylation by other kinases, before they will be further phosphorylated by GSK-3 [260]. A positively charged pocket of GSK-3, comprising Arg96, Arg180 and Lys205 residues, optimizes binding and orientation of primed targets increasing the efficiency of substrate phosphorylation by 100-1000-fold [261]. The inhibitory Ser21/9-phosphorylation creates a pseudosubstrate that binds intramolecularly to the positively charged pocket. This mechanism of inhibition is competitive so that primed targets, at high enough concentrations, out-compete the pseudosubstrate and become phosphorylated [229]. Competition between primed targets and the phospho-Ser21/9-moiety of GSK-3 during DC activation may therefore modulate levels of IL-12p70 production. Kinases priming GSK-3 targets may include the MAPK, such as p38K and ERK1/2, as they are both activated following DC activation, and a combination of inhibitors of either MAPK also blocks IL-12p70 secretion [141].

Our data suggest a scenario where the GSK-3, which has inhibitory function of in immature DC, in response to activation stimuli changes into an enzyme with a pro-inflammatory function (Figure 17). This occurs due to a change of intracellular context that is characterized by the emergence of primed GSK-3 targets, i.e. proteins phosphorylated by other kinases such as p38K and ERK1/2. Pro-inflammatory cytokines such as IL-12p70 are cachectins and have to be tightly regulated. This regulation is partially achieved by PI3K/Akt-1 pathway that is activated by all maturation inducing stimuli used in our system. Akt-1 phosphorylates the Serine residues 21/9 of GSK-3, thereby inhibiting the phosphorylation of the GSK-3-primed targets. This results in a down-regulation of IL-12p70 secretion, an effect counteracted by wortmannin. However, the phospho-Ser21/9-moiety of GSK-3 acts as a competitive inhibitor of the phospho-binding pocket. High concentrations of primed GSK-3 targets may therefore successfully compete for access to GSK-3 active center resulting in a controlled pro-inflammatory activity of this enzyme.

Thus, production of IL-12p70 appears to be a result of tight interplay of 1) the activity of the kinases priming GSK-3 targets, 2) the activity of Akt-1 which inhibits GSK-3 and 3) the outcome of competition of primed GSK-3 targets and the phospho-Ser21/9-moiety for access to the phospho-binding pocket of GSK-3 (Figure 17).

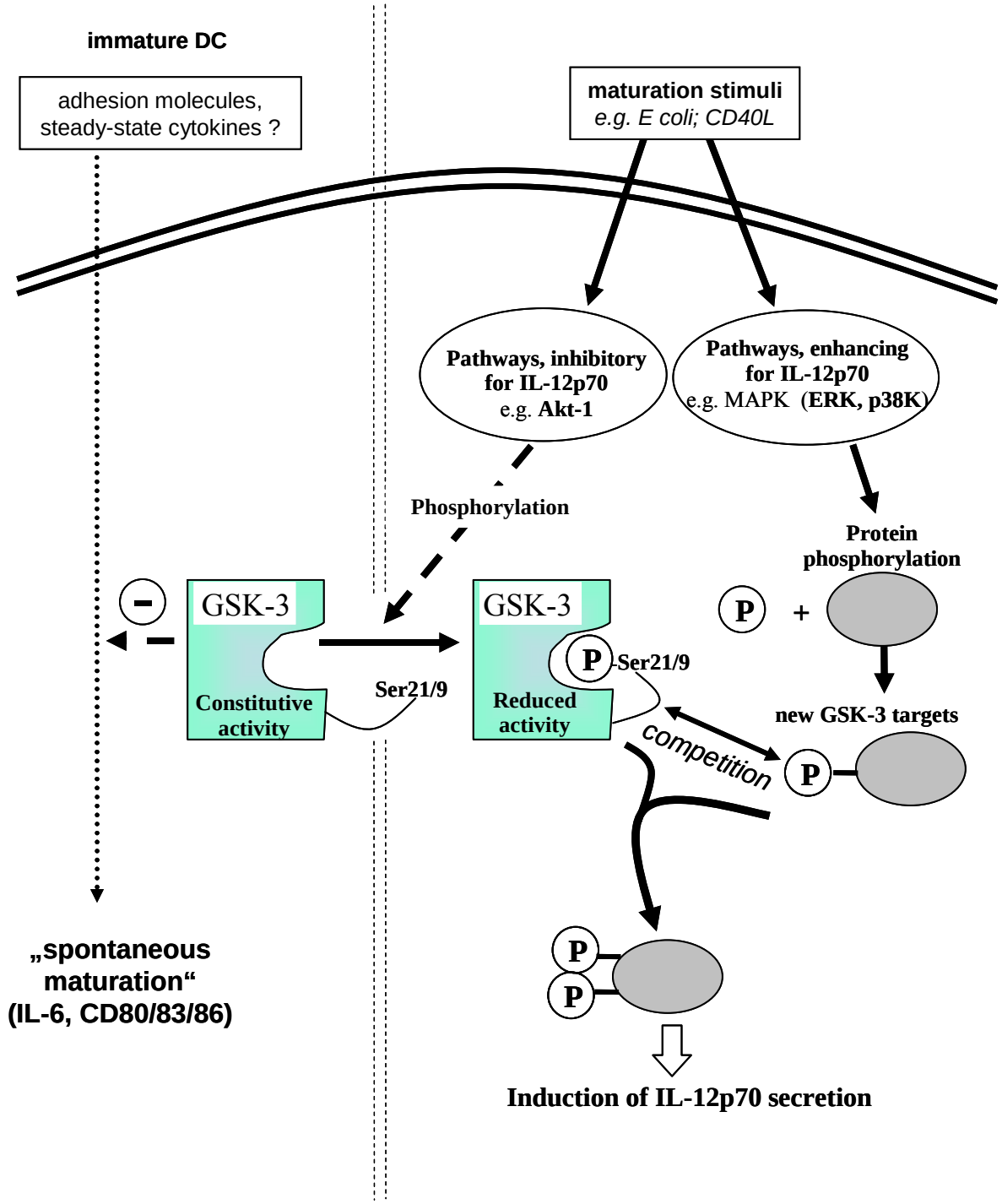


Figure 17 GSK-3 integrates activating and inhibitory pathways involved in IL-12p70 production. GSK-3 is constitutively active in immature DC and inhibits their spontaneous maturation. Maturation stimuli induce intracellular kinases that are enhancing (e.g. ERK1/2, p38K) or inhibitory (e.g. Akt-1) for IL-12p70 production. Enhanced kinase activity results in phosphorylation of proteins, which may thus become primed GSK-3 targets. Akt-1 inhibit GSK-3 by phosphorylating the Ser21/9-moiety, which bind the phospho-binding pocket of GSK-3 as an intramolecular pseudo-substrate. IL-12p70 production now depends on the outcome of competition of primed GSK-3 targets and the phospho-Ser21/9-moiety for access to the phospho-binding pocket of GSK-3.

Conclusions

In the first part of the presented work, MHC class I antigens derived from HLA-DP alleles and from immunoglobulin A and D constant regions were identified and evaluated as potential targets for vaccination of patients with hematological malignancies after stem cell transplantation (SCT).

We identified and characterized 20 peptides derived from polymorphic regions of HLA-DP and 12 peptides derived from constant regions of IgA and IgD (6 for each isotype). Immunogenicity, crossrecognition (when applicable) and natural presentation of selected antigens were tested.

The IgD-derived immunogenic LLNASRSLEV peptide was demonstrated to induce positive CTL responses in 40% of healthy HLA-$A2^+$ donors. This epitope, however, is not a suitable target for vaccinations due to the lack of efficient natural presentation on the surface of either normal or malignant cells.

$CD8^+$ CTL responses could be generated against 11 out of 20 HLA-DP-derived synthetic peptides. However, CTL clones specific for HLA-DP-derived peptides could not be generated and the final characterization of natural presentation of the HLA-DP-derived peptides was therefore not possible. This failure to generate peptide-specific CTL-clones may reflect low frequencies of T-cells recognizing HLA-DP-derived peptides in PBMC of normal donors. On the other hand, as CTL responses against some peptides were shown, further studies are required to investigate whether CTL recognizing these antigens have any impact on GvHD development in patients after HLA-DP mismatched allogeneic SCT. Therefore, CTL responses against HLA-DP derived peptides should be monitored in the future in patients who developed GvHD after allogeneic SCT.

In the second part of this work the role of GSK-3 pathway in differentiation of DC and regulation of DC functions was investigated.

Our results demonstrate that GSK-3 is a crucial enzyme involved in differentiation of DC from monocytes and in the maintenance of an immature phenotype of DC. Furthermore, in the context of DC activation, despite partial functional inhibition, GSK-3 acquires a novel, pro-inflammatory functional status mediating high levels of IL-12, IL-6 and TNF-α secretion, and partially inhibiting IL-10 production. Further

studies will have to confirm whether GSK-3 might become a target for therapeutic interventions in clinical immune imbalances, such as autoimmune diseases and transplantation settings. Modulation of this pathway during the course of vaccine-based immunotherapy may enhance DC activation in vivo and thus may enhance vaccine-mediated immune responses.

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List of publications:

1 Rodionova E, Conzelmann M, Maraskovsky E, Hess M, Kirsch M, Giese T, Ho AD, Zöller M, Dreger P, Luft T. GSK-3 mediates differentiation and activation of pro-inflammatory dendritic cells. Revised version submitted to Blood in August, 2006.

2 Luft T, Rodionova E, Maraskovsky E, Kirsch M, Hess M, Buchholtz C, Goerner M, Schnurr M, Skoda R, Ho AD (2006). Adaptive functional differentiation of dendritic cells: integrating the network of extra- and intracellular signals. Blood **107** (12):4763-4769.

List of presentations:

1 Rodionova E, Hess M, Kirsch M, Luft T. GSK-3 mediates differentiation and activation of pro-inflammatory dendritic cells. ENII-EFIS conference "Immunity and Autoimmunity". May 2006, Capo Caccia, Italy (poster presentation).

2 Rodionova E, Hess M, Kirsch M, Zöller M, Luft T. GSK-3 mediates differentiation and pro-inflammatory activation of dendritic cells. DKFZ PhD retreat. May 2006, Weil der Stadt (oral presentation).

3 Rodionova E, Maraskovsky E, Hess M, Kirsch M, Ho AD, Luft T. GSK-3 mediates differentiation and activation of pro-inflammatory dendritic cells. Cell Signaling World 2006, "Signal Transduction Pathways as therapeutic targets". January 2006, Luxembuorg (poster presentation).

4 Rodionova E, Maraskovsky E, Hess M, Kirsch M, Luft T. Adaptive functional differentiation of dendritic cells - integrating the network of extra- and intracellular signals. Cell Signaling World 2006, "Signal Transduction Pathways as therapeutic targets". January 2006, Luxembuorg (poster presentation).

5 Rodionova E, Hess M, Kirsch M, Zöller M, Luft T. GSK-3 mediates pro-inflammatory differentiation and activation of dendritic cells. DKFZ PhD poster competition. December 2005, DKFZ, Heidelberg (poster presentation).

6 Rodionova E, Hess M, Kirsch M, Luft T. Adaptive functional differentiation of dendritic cells - integrating the network of extra- and intracellular signals. ENII conference "Fundamentals of innate and adaptive immunity: basic advances and clinical potentials". May 2005, Ile des Embiez, France (poster presentation).

7 Rodionova E, Hess M, Zöller M, Luft T. Untersuchung neuer Peptid-Antigene für Vakzinestudien nach autologer und allogener Stammzell-Transplantation: a) polymorphe, HLA-DP-abgeleitete Peptide; b) IgA/D-Isotyp-peptide. Forschungssymposium des Tumorzentrums Heidelberg/Mannheim. November 2004, DKFZ, Heidelberg (poster presentation, poster prize).

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