

Induction of Cytotoxic CD8⁺ T Cells by a Soluble
HLA-derived Tumor-associated Peptide and Regulation of
CD8⁺ T Cell Activation by CTLA-4



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ABSTRACT

Part I. MHC peptides derived from tumor associated antigens (TAA) can serve as the basis for development of immunotherapeutics for treatment of human malignancies. Recently, we identified novel HLA-A*0201 (HLA-A2) restricted peptides recovered from soluble HLA (sHLA) molecules secreted by sHLA-transfected human tumor cell lines. Here, four candidate peptides eluted from sHLA-A2 were selected on the basis of their precursor proteins being TAA. Peptide p1028 (GLIEKNIEL), derived from DNA methyl transferase I (DNMT-1), which is over-expressed in various human tumors, showed the highest affinity to HLA-A2 and was relatively abundant in the sHLA/peptide complexes of all transfected breast, ovarian and prostate cancer cell lines. Peptide p1028 was immunogenic in vitro and p1028 effector CTL were capable of lysing tumor cells. p1028 CTL were peptide specific and HLA-A2 restricted. Since DNMT-1 is a widely expressed tumor-associated enzyme, the novel DNMT-1-derived and HLA-A2 restricted peptide GLIEKNIEL identified here, might find use as a therapeutic cancer vaccine.

However, since the success of cancer vaccines has been somewhat limited, it is necessary to improve the efficiency of these vaccines. This can be achieved by enhancing T cell activation e.g. through manipulation of inhibitory or stimulatory receptors. CD28 and cytotoxic T lymphocyte antigen-4 (CTLA-4) are critical costimulatory receptors on T cells. Thus, in the second part of this study, we investigated the inhibitory effects of CTLA-4 upregulation on CD8⁺ T cells.

Part II. CD28 and CTLA-4 are the critical costimulatory receptors that predominantly determine the outcome of T cell stimulation, with CD28 promoting positive costimulation and CTLA-4 inducing inhibitory signals. However, the exact mechanism of CTLA-4's inhibitory function remains elusive. Here, we investigated CD8⁺ T cell regulation by CTLA-4 and the influence of CTLA-4 upregulation on CD28 expression using CD8⁺CTLA-4⁺ T cell transfectants. Upregulation of CTLA-4 induced abrogation of IL-2 production as well as inhibition of the TcR proximal tyrosine kinases ZAP-70, p56^{lck} and p59^{lyn}, a phenotype of anergic T

cells. In addition, CTLA-4 upregulation promoted the internalization and proteolytic degradation of CD28, leading to decreased CD28 surface expression. These data suggest that apart from the established competition of CD28 and CTLA-4 for their ligands CD80 and CD86, inhibition of T cells by CTLA-4 might be explained by significant reduction of CD28 on the cell surface, which might impede T cell response to stimulation. Our data provide a previously unrecognized mechanism for T cell regulation by CTLA-4.

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LIST OF ABBREVIATIONS

mAb	monoclonal antibody
APC	antigen presenting cell
AP-1	activating protein-1
APS	ammonium persulfate
BSA	bovine serum albumine
CD	cluster of differentiation
cDNA	complementary DNA
CID	collision-induced dissociation
ConA	Concavalin A
cpm	counts per minute
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen-4
DAG	diacylglycerol
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNMT	DNA methyl transferase
EDTA	ethylene diamine tetraacetate
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ESI	electrospray ionization
EtOH	ethanol
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage-colony stimulating factor
GRB2	growth factor receptor bound protein 2
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLA	human leukocyte antigen
sHLA	soluble human leukocyte antigen
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
ICOS	inducible costimulatory molecule
IFN	interferon

IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
kD	kilo Dalton
LAT	linker of activation of T cells
LMP	low molecular weight protein
MAPK	mitogen-activated protein kinase
2-ME	2-mercaptoethanol
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
sMHC	soluble major histocompatibility complex
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NFAT	nuclear factor of activated T cells
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell

PCR	polymerase chain reaction
PD1	programmed cell death protein 1
PE	phycoerythrin
PI3K	phosphatidylinositol-3 kinase
PLC γ 1	phospholipase C γ 1
PP2A	protein phosphatase 2A
PTK	protein tyrosine kinase
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecylsulfate
SEB	staphylococcal enterotoxin B
SHP-2	Src homology 2-domain-containing tyrosine phosphatase-2
TAA	tumor associated antigen
TAE	tris-acetate-EDTA
TAP	transporter associated with antigen processing
TcR	T cell receptor

TEMED	N,N,N',N'-tetraethyl methyldiamine
TGF	transforming growth factor
TNF	tumor necrosis factor
TRP2	tyrosinase-related protein 2
TSA	tumor specific antigen
Tween 20	polyoxyethylene 20-sorbitan monolaurate
ZAP-70	ζ -associated protein of 70 kD

CHAPTER 1

GENERAL INTRODUCTION

1.1 Antigen Recognition by T Cells

The adaptive immune response is initiated by the recognition of antigens by lymphocytes. T lymphocytes play the central role in all immune responses to protein antigens. The principal functions of T lymphocytes are defense against intracellular microbes and activation of other immune cells such as B lymphocytes and macrophages. The basic mechanisms of T cell mediated immune responses are crucial to understand immunological reactions against tumors and transplants as well as diseases caused by abnormal immune responses. For example, T cell inhibition can be a desired mechanism in the case of immunologic rejection of a transplant, whereas T cell activation can be a goal in fighting tumors. The balance of T cell activation and inhibition is also necessary to control infections and, on the other hand, to prevent the onset of autoimmune diseases.

T cells recognize antigens in the form of peptides displayed on antigen presenting cells (APCs), as for example macrophages, B cells and dendritic cells. T cells are able to interact with these cells through the antigen receptor on T cells, the T cell receptor (TcR), which can only recognize antigens that are displayed on cells and not soluble antigens. These peptide antigens are presented by proteins that are encoded by genes in a locus called the major histocompatibility complex (MHC).

There are two main types of MHC molecules, MHC class I and MHC class II. Both MHC molecules, in humans called HLA (human leukocyte antigen) molecules, function as peptide shuttles that transport peptides from the cytoplasm and display them at the cell surface. The peptides presented by MHC class I molecules are mostly derived from cytosolic proteins that were degraded in the proteasome,¹ whereas MHC class II molecules display peptides from extracellular antigens that have been endocytosed and degraded by endosomal and lysosomal

proteases. Class I molecules present peptides to CD8⁺ cytotoxic T lymphocytes (CTL) and class II molecules to CD4⁺ T helper cells.

The antigen receptors on T cells are specific for complexes of foreign peptides displayed on self MHC molecules. Up to six different MHC molecules can be displayed on the cell surface, which are the products of three different MHC class-I genes (A, B and C). These genes are particularly polymorphic in the region that encodes for the peptide-binding domain. Thus, different MHC molecules on APCs bind and display different peptides due to their unique binding motifs,² ensuring that a broad variety of antigens can be presented and recognized by T cells. In addition to displaying the antigen-MHC complexes, professional APCs also express costimulatory molecules to optimally activate T lymphocytes.

Naive CD8⁺ T cells, after binding to the MHC-peptide complex on APCs, can develop into CTL that are capable of lysing peptide-presenting cells. This mechanism allows detecting foreign antigens that are displayed by MHC class I molecules when cells harbor intracellular infections, but also the detection of subtle changes in the repertoire of "normal" cells after mutation into cancer cells.

1.1.1 T Cell Responses to Tumors

Effector mechanisms of both humoral and cell-mediated immunity have been shown to kill tumor cells in vitro. However, the principal mechanism of tumor immunity appears to be the lysis of tumor cells by CD8⁺ CTL. CTL may perform a surveillance function by recognizing and killing potentially malignant cells that express peptides derived from mutant cellular proteins or oncogenic viral proteins, which are presented in association with MHC class I molecules. However, most tumor cells are not derived from APCs and therefore do not express costimulatory receptors that are necessary to optimally initiate primary T cell responses. Therefore, besides the direct presentation of tumor antigens by tumors, tumor antigens might additionally be presented by host APCs. Tumor antigen presentation by host APCs, so-called cross-presentation, would provide adequate costimulation to activate CD8⁺ T cells. It has become clear in several studies that professional

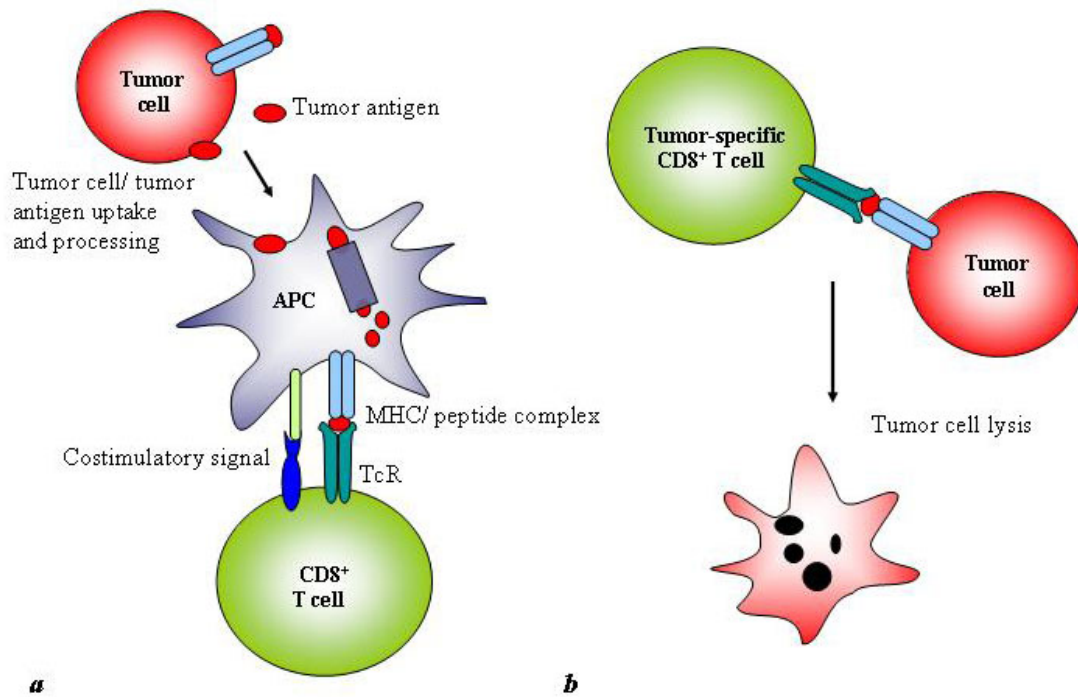


Figure 1.1: **Induction of T cell responses to tumors.** CD8⁺ T cell responses to tumors might be induced by cross-presentation through host APCs after ingestion of tumor cells or tumor antigens (a). This leads to differentiation of tumor-specific CD8⁺ T cells that are capable of tumor cell lysis (b).

APCs are indeed responsible for tumor antigen cross-presentation to CD8⁺ T cells.^{3,4}

Thus, tumor cells or their antigens might be ingested by APCs, processed inside the APC and peptide antigens presented on MHC class I molecules to CD8⁺ T cells, as illustrated in Figure 1.1. The costimulator ligands on APCs can provide the second signal for T cell differentiation and proliferation. These differentiated tumor-specific effector CTL are capable of tumor cell lysis through recognition of the peptide/MHC complex on the tumor cell.

However, cancer cells developed strategies to evade the recognition by T cells and to induce antigen-specific tolerance, for example by evading the host immune system through the downregulation of MHC molecules or molecules that are involved in antigen processing.⁵

The goal in tumor immunotherapy is to overcome this tumor-specific T cell

tolerance and to achieve an effective T cell-mediated anti-tumor immune response. Therapeutic vaccines against cancer mainly aim at inducing CTL capable of recognizing and eliminating tumor cells.

Studies were initiated to vaccinate tumor patients against tumor antigens and the ability of vaccines to induce anti-tumor T cell immunity has been shown in various clinical studies.⁶ However, despite some progress, the overall success of cancer vaccines has been somewhat limited. A major limitation of the success of cancer vaccines is the capability of cancerous cells to make themselves resistant to the immune system. Also, many questions as to how to improve tumor immunotherapy still remain to be answered, for example regarding the right choice of antigen, the right choice of adjuvant and regarding the type of desired induced immune response.

To answer one of the remaining questions, which proteins and peptides are the most suitable targets for a tumor vaccine, it is necessary to identify and characterize novel tumor-associated peptides. Thus, our lab utilized a new method for the identification of HLA-peptides as tumor vaccine candidates, the recovery of peptides from soluble MHC molecules secreted by tumor cell lines. The recovered peptide pool was sequenced and peptides derived from potential tumor-antigens were synthesized, as previously described by our group.⁷ Here, in the first part of this study, we tested a selection of novel sHLA-A2-derived peptides for their binding affinities to HLA-A2, their immunogenicity and their ability to induce a tumor-directed cytotoxic T cell response, aiming to identify a new candidate for a peptide cancer vaccine.

However, even with the use of immunogenic tumor antigens as cancer vaccines, it proved very difficult in clinical studies to overcome the tolerance to tumor antigens and to overcome tumor immune evasion mechanisms.

Therefore, it is necessary to develop tools to make vaccines more efficient e.g. by enhancing T cell activation. One approach to generate more effective immune responses to tumors is through the manipulation of costimulatory molecules that control T cell reactivity through both positive and negative signaling mechanisms.

To fully use the capacities of costimulatory molecules, their mechanism of action has to be understood. Thus, in the second part of this study we investigated one of the major costimulatory systems on T cells, CD28 and CTLA-4, which are critical costimulatory receptors that determine the outcome of stimulation through the T cell receptor. We addressed the question as to how CTLA-4 exerts a negative effect on CD8⁺ T cell activation and whether CTLA-4 negatively affects the expression of CD28.

Knowledge about mechanisms by which inhibitory receptors influence T cell activation might prove helpful for the design of cancer vaccines that are capable of inducing an effective anti-tumor immune response.

1.2 Questions addressed

Part I

The major goal of this study was the identification and characterization of tumor-associated antigens, which were recovered from soluble MHC molecules. The chosen peptides were characterized for their affinity to membrane bound HLA molecules and the question addressed whether the peptides are capable of inducing a CD8⁺ T cell response and whether the generated CTL are HLA-restricted, peptide-specific and capable of tumor cell lysis.

The identification of novel immunogenic tumor antigens might provide new candidates for a cancer vaccine.

Part II

In order to improve the efficacy of either tumor immunotherapy or the immunosuppressive treatment in transplantation and autoimmune disease, basic understanding of T cell inhibition and activation is crucial. The major costimulatory pathway in T cell activation involves two molecules: CD28 and CTLA-4. Thus, we addressed the question as to how CTLA-4 exerts a negative effect on CD8⁺ T cell activation and whether CTLA-4 negatively affects the expression of CD28.

Understanding the molecular mechanisms, by which these costimulators regulate T cell activation, might prove helpful to generate more effective immune responses to tumors through manipulation of these molecules.

Part I

Induction of Cytotoxic CD8⁺ T Cells by a
Soluble HLA-derived Tumor-associated Peptide

CHAPTER 2

INTRODUCTION

2.1 Tumor Immunity

The immune system attempts to identify peptide antigens derived from foreign proteins, e.g. viruses that are presented by MHC class I molecules and eliminate the pathogen by killing the infected cell. Self antigens, which are also presented by MHC molecules, are not recognized by T cells, thus avoiding autoimmunity. Self reactive T cells are eliminated in the thymus during embryonic development, a phenomenon called central tolerance. However, a small number of self reactive T cells escapes thymic selection and is found in the periphery in normal adults. These cells are controlled by peripheral tolerance mechanisms that prevent autoimmunity.

A major function of the immune system is the elimination of transformed cells before they develop into tumors and the elimination of developed tumor cells. Even though tumors are in many cases not eradicated by the immune system, there is evidence for T cell dependent immunity against tumors. This became evident in a mouse tumor-transfer model. Mice that were injected with irradiated tumor cells and re-challenged with non-irradiated tumor cells survived, whereas control mice that directly received tumor cells died. Pre-treatment of mice with irradiated tumor cells only protected from tumor development of the same tumor cell line, indicating a tumor-specific immune reaction.⁸

However, it is until now not clear whether the immune system recognizes tumors as 'self' or 'foreign', which is crucial for understanding tumor immunity. In the 1950s the immune surveillance hypothesis was formed, postulating that the immune system surveys the body for specific antigens expressed by newly arising tumors which are then rapidly eliminated. This hypothesis was questioned when it became evident that non-virus-associated cancers do not occur more frequently in humans with a defective immune system and not in mice, which have

impaired T cell and B cell immunity, compared to normals. The only tumors that appeared more frequently in immunodeficient mice were virus-associated tumors. This showed that immune surveillance plays a role in virus-associated tumors but possibly not for spontaneously developing tumors that formed due to genetic mutation. Therefore, for a long time little interest was paid to the possibility that the immune system could also prevent the development of non-virally induced tumors.

However, between the 1970s and 1990s several attempts were made to resurrect the immune surveillance concept, which revealed two major findings: first, the protection against growth of transplanted tumors by interferon-gamma (IFN- γ)⁹ and second the increased tumor formation in mice lacking perforin, a component of the cytolytic granules of cytotoxic T cells and natural killer (NK) cells.¹⁰ Altogether these observations clearly indicated that components of the immune system are indeed involved in controlling primary tumor development.

Finally, human studies revealed that individuals with severe deficits of immunity, such as transplant patients,¹¹ have a higher probability of developing a variety of cancers with and without viral etiology, clearly supporting the physiological relevance of cancer immune surveillance.

2.1.1 Tumor Evasion

The fact that cancer immune surveillance does exist leads to the question as to how cancers do occur in immuno-competent individuals. One explanation is the ability of cancerous cells to make themselves resistant to the immune system. Defects in the MHC class I antigen-processing machinery, leading to inactivation or downregulation of the cell's MHC complex have been described in a variety of tumors,¹² thus enabling the tumor to escape recognition and destruction by CTL (Figure 2.2*a*). The loss or mutation of β_2 -microglobulin, which is a common and necessary component of all class I HLA molecules, is a common cause of HLA class I loss in human melanomas.¹³ An alternative mechanism, leading to total loss of HLA class I in tumors, is the hypermethylation of the MHC class I region leading to HLA class I gene silencing.¹⁴ Haplotype loss caused by deletions on chromosome

6, as well as loss of single HLA alleles caused by genetic defects have also been described in a variety of different carcinomas.¹⁵ Tumor cell recognition by CTL is also dependent on an intact antigen-processing machinery that allows cytoplasmic degradation of tumor-associated proteins and the antigenic peptide transport in the endoplasmic reticulum, where peptide loading on HLA molecules takes place. Thus, in tumors different components of the antigen processing machinery are frequently lost or downregulated to escape recognition by T cells, as for example the peptide transporters associated with antigen processing, TAP-1 and TAP-2.⁵

Another escape mechanism involves direct deletion of immune effector cells by induction of apoptosis through different mechanisms: through the expression of apoptosis-inducing Fas ligand, resulting in apoptosis of tumor-reactive T cells,¹⁶ through the expression of the RCAS1 (receptor-binding cancer antigen) receptor on tumor cells, which has been shown to induce apoptosis in T and NK cells¹⁷ and the expression of human B7-H1 on tumor cells, a member of the B7 family of costimulatory molecules which also leads to induction of T cell apoptosis¹⁸ (Figure 2.2*b*).

In addition, the above described way of tumor cell destruction through cross-priming by host APCs can also lead to the development of T cell tolerance, if immature DCs that fail to provide adequate costimulation, present the tumor antigen¹⁹(Figure 2.2*c*). This phenomenon underscores the relevance of the activation state of the APC with regard to the induction of T cell tolerance or activation.

Another way to escape immune surveillance is the production of immunosuppressive cytokines such as transforming growth factor- β (TGF- β) and interleukin-10 by tumor cells,²⁰ leading to induction of anergy (Figure 2.2*d*).

Regulatory or suppressor T cells have been the focus of many studies during the past years and have been shown to play a crucial role in preventing autoimmunity. Recent evidence suggests that such regulatory T cells are present within certain tumors and could be involved in dampening the T cell response against the tumor²¹ (Figure 2.2*e*).

In order to overcome these different types of immune evasion, strategies have

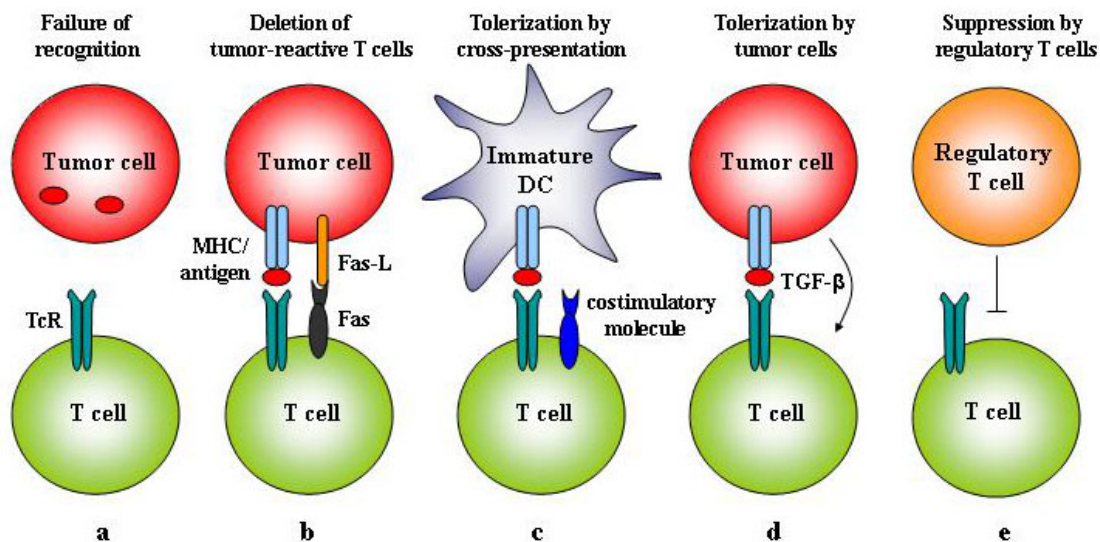


Figure 2.2: **Mechanisms of tumor immune evasion.** (a) Failure of tumor recognition through MHC class I loss or downregulation. (b) Deletion of tumor-reactive T cells through death-ligand expression. (c) Tolerization by cross-presentation of tumor antigens on immature DCs. (d) Tolerization through expression of suppressive cytokines. (e) Suppression of tumor-reactive T cells by regulatory or suppressor T cells.

been developed to actively stimulate the host immune response to the tumor. This treatment would be of great advantage compared to the still unsatisfactory form of treatment with surgery, irradiation or chemical intervention. The classical pharmaceutical reagents, like for example alkylating agents and antimetabolites that unspecific target dividing cells, are necessary tools for tumor chemotherapy, but unfortunately cause severe side effects and are in many cases not able to heal the patients. Due to the specificity of cytotoxic T cells for a particular antigen, tumor immunotherapy might provide an alternative way of cancer treatment without causing severe side effects.

2.2 Tumor Immunotherapy and Tumor Antigens

The achievement of an effective T cell-mediated immune response against tumor antigens is one of the major goals in cancer immunotherapy. In tumor cells, mutated self genes or oncogenes often produce protein antigens that are

recognized by MHC class I-restricted CTL. These antigens are called tumor associated antigens (TAA) or tumor specific antigens (TSA) and can be used as cancer vaccines. TAA are peptides from tumor cell proteins, which are presented to T lymphocytes via MHC molecules. These peptides are targets for a tumor specific T cell reaction since they are ideally not presented on the surface of normal cells, or not abundant enough to be recognized by T cells.

One can distinguish between active and passive ways of vaccination in cancer therapy. The use of killed tumor cells or tumor antigens e.g. by injection of antigen-pulsed dendritic cells displays an active vaccination. A passive way of tumor immunotherapy is the adoptive transfer of tumor-specific CTL, which have been activated against the tumor antigen and expanded *in vitro*.

TAA can be categorized into four main groups. The first group contains tumor specific antigens that are derived from genes involved in oncogenesis. These peptides might induce a T cell response by being able to bind MHC class I or through mutation in the peptide sequence that now displays a new T cell epitope, e.g. β -catenin in melanoma.^{22,23} A second group of TAA includes proteins, which are encoded by genes that are normally only expressed in germ-cells that don't express MHC class I and are now expressed on MHC class I-expressing cells. Examples for such antigens are the melanoma antigens MAGE-1 and MAGE-3, which are normally only expressed in the testis. A third group consists of differentiation antigens that are expressed in specific tissues, for example the tyrosinase gene that is involved in the melanin-synthesis. Another group contains genes encoding for proteins that are also expressed in normal cells but over-expressed in certain tumors. By over-expressing a protein, the density of the peptide-MHC complexes presented on the cell surface is increased and might therefore enable T cells to recognize the antigen, the peptide becomes immunogenic.²⁴

Some studies showed successful elimination of tumor cells that occurred after eliciting an antigen-specific anti-tumor CTL response^{25,26,27,28} and human clinical trials for a variety of malignant diseases using T cell therapy proved to be effective and curative for some individuals.^{29,30,6} However, despite some progress, the

overall success of tumor immunotherapy has been limited.

2.2.1 Tumor Antigen Discovery

In order to develop new cancer vaccines, many strategies have been developed to identify TAA. One of these strategies is the genetic approach, pioneered by Boon et al.²⁷ This approach is based on transfection of expression-libraries made from cDNA of tumor cells into cell lines expressing the same MHC haplotype as the tumor. The transfected cells are tested for sensitivity to lysis by an anti-tumor CTL clone. Genes that sensitized the cell line for CTL lysis are analyzed to identify the protein antigen that is recognized by the anti-tumor CTL. Several melanoma protein antigens, such as the above mentioned antigens MAGE³¹ and tyrosinase³² were discovered using this approach.

A different technique, which has led to identification of a large number of TAA, is based on the humoral immune response to an antigen (SEREX: serological analysis of recombinant cDNA expression libraries).³³ This technique uses patient antibodies to identify tumor antigens from a cDNA expression library made from mRNA of tumor specimens.

The direct biochemical approach for the identification of HLA peptides as cancer vaccine candidates is based on the elution of endogenous peptides from immunoaffinity purified MHC molecules isolated from detergent extracts of tumor cells with monoclonal antibodies specific for the desired MHC haplotypes.³⁴ The resulting mixtures of peptides are further fractionated using reversed-phase high-performance liquid chromatography (HPLC). The fractions that are able to sensitize MHC-matched non-tumor target cells for lysis by a tumor-specific CTL clone are then analyzed using mass spectrometry (MS)^{35,36} to identify their amino acid sequences.

Another approach is the consensus peptide binding motif approach, also known as epitope deduction or reverse immunology approach. This approach is based on identification of sequences that correspond to MHC peptides within the amino acid sequence of known TAA. The amino acids observed at high levels at

particular positions along the peptides were defined as consensus residues, also known as anchor residues of the peptides to the binding pockets in the groove of MHC class I molecules. These peptide binding motifs are now well established for the most common MHC alleles.^{34,37} Software is used to predict the sequence of MHC peptides from amino acid sequences of proteins known to be TAA using the established consensus.³⁷ The predicted peptides are then synthesized and tested by reconstitution into empty MHC molecules, followed by testing their stabilization as well as their capability to elicit a T cell response.

The T cell-independent approach for identification of MHC peptides is also based on predicting the sequences of MHC peptides based on sequences of proteins expressed in the tumors and does not require pre-existing CTL lines or clones.³⁸ Potential epitopes are predicted from the sequences of known TAA and the corresponding synthetic peptides analyzed by HPLC/MS. In parallel, peptides are extracted from fresh solid tumors or tumor cell lines and analyzed the same way. The analyzed peptides are then searched for those with masses and fragmentation patterns similar to those predicted.

However, to date most identified TAA are melanoma derived and no real major breakthroughs have been reported. Thus, to improve on peptide-based therapy there is the need to develop novel strategies for the identification of new cancer related peptides that are ideally suitable for different types of cancers and a broad group of patients.

2.3 Application of Soluble MHC Molecules for the Discovery of TAA

Membrane bound MHC molecules are hard to purify in large amounts as needed for peptide analysis, especially from a already limited number of cancer cells. In addition, the purification of MHC molecules by detergent extraction of cell membranes with subsequent MS/MS analysis is hampered by contamination of the MHC preparations with cellular debris and detergents.

It is known for decades that MHC molecules not only exist as membrane

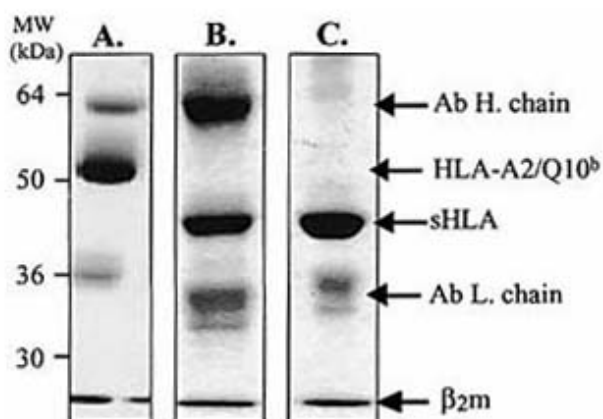


Figure 2.3: **Purification of soluble HLA molecules from cancer cell lines.** sHLA were purified by immunoaffinity from the growth medium of transfected cells, as previously described by our lab.⁷ Purified proteins were analyzed by SDS-PAGE and gels stained with Coomassie Blue. (A) sHLA-A2/Q10b purified from MCF-7 cells. (B) sHLA-A2 purified from UCI-101 cells, (C) sHLA-B7 purified from UCI-107 cells.

bound proteins but also in a soluble form.^{39,40} Thus, an approach to improve MHC peptide preparations was taken previously by our group, based on transfection of cancer cells with vectors encoding for soluble, secreted MHC molecules lacking a functional transmembrane domain.⁷ Using this approach, relatively large numbers of MHC peptides were purified from relatively small numbers of cells. Also, most cancer cell lines could be transfected with a desired MHC haplotype, regardless of their innate MHC haplotype.

Prostate, breast and ovarian cancer cell lines were transfected with HLA vectors lacking functional transmembrane domains (sHLA-A2, sHLA-B7) or by using a non-functional transmembrane domain (Q10b),⁴¹ as previously described.⁴² Peptides were recovered from soluble, secreted HLA (sHLA) that were purified by immunoaffinity from the growth medium of the transfected cell lines. Purified sHLA molecules were analyzed by SDS PAGE.

Figure 2.3 shows the SDS PAGE of soluble HLA-A2 and HLA-B7 molecules

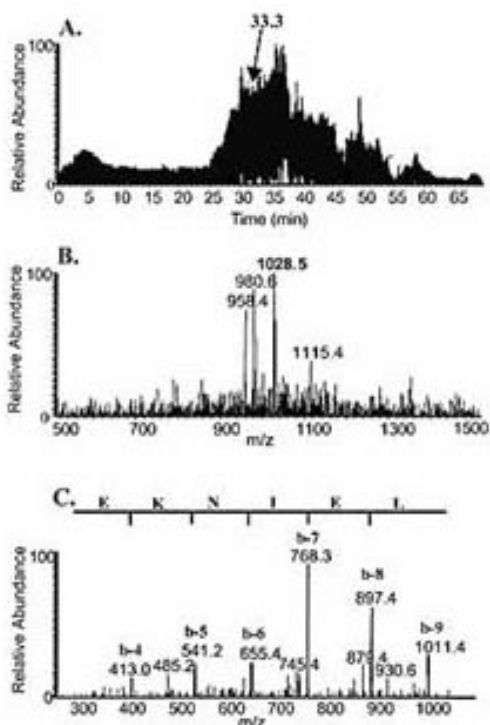


Figure 2.4: **HPLC of peptides eluted from soluble HLA-A2.** A typical nano-capillary reversed-phase chromatography of MHC peptides purified from soluble MHC recovered from 5×10^7 MCF-7 breast cancer cells. (A) The total-ion-current chromatogram (TIC). (B) Full spectrum taken at time point of 33.3 min. (C) Spectrum of the collision-induced-disintegration (CID) of the dominant peptide in (B) having a mass/charge ratio (m/z) of 1028.5 with the sequence GLIEKNIEL derived from DNA-methyl transferase.

that were purified from sHLA-A2/Q10b-transfected breast cancer cell line MCF-7 and sHLA-A2 or sHLA-B7-transfected ovarian cancer cell lines UCI-101 and UCI-107, respectively.⁷

Peptides recovered from sHLA produced by about 5×10^7 cells were used for each nano-capillary electrospray ionization (ESI)-MS/MS analysis. The HPLC profile revealed a multitude of peptides that were estimated to be between 2000-3000 as shown in Figure 2.4.⁷ The profile not only revealed that the number of peptides is very high, but also that there are concentration differences between the various identified peptides. From the large number of peptides, about 220 peptides, which were with very high certainty derived from known proteins, were

identified for HLA-A2. After purification of the secreted soluble HLA by affinity chromatography, peptides were eluted and sequenced. Novel peptides unique to the soluble MHC variants were identified and the discovered peptides were screened for tumor-relevant precursor proteins. The detection of these peptides on the soluble MHC indicate that these peptides are properly processed and displayed on the soluble transfected MHC molecules similarly to those displayed on the membrane bound MHC molecules. Similar to membrane bound MHC, soluble MHC class I molecules bound peptides which were 8-10 amino acids long, with a few exceptions having a length of 6, 11 and 12 amino acids.⁷ Peptides recovered from the sHLA molecules also included already known MHC peptides.

Therefore, the repertoire of MHC peptides recovered from the soluble MHC molecules is a good representation of the naturally processed and displayed peptides of the transfected cells. Peptides detected this way should be presented at many copy numbers per cell and are likely to be bound at relatively high affinity to the HLA.

The use of soluble HLA as a source of peptides enabled our lab to identify large numbers of MHC peptides presented by human cancer cells and to screen these for peptides possibly derived from TAA as candidates for tumor vaccines. The goal of this study was to select and characterize candidate peptides that were eluted from soluble HLA-A2 and were derived from putative tumor-associated antigens and other proteins to evaluate their usefulness as tumor vaccine candidates.

2.4 Specific Aims

1. To select candidate peptides from potential TAA derived from soluble HLA-A2 molecules based on their relative abundance and to determine their affinity to HLA-A2 molecules.
2. To test peptides for their immunogenicity in vitro by inducing peptide-specific CTL from blood of healthy donors using generated autologous dendritic cells as antigen presenting cells.
3. To characterize CTL for cytotoxicity against tumor cell lines and to evaluate their specificity by tetramer binding assays and intracellular cytokine expression analysis.

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell lines and culture conditions

Breast cancer cell line MDA-231, prostate cancer cell line PC-3 and the TAP-transporter deficient lymphoblastic T2 cell line were maintained at 37°C in a 5% CO₂ incubator in RPMI-1640 medium plus 25 mM HEPES (Gibco, Grand Islands, NY, USA) supplemented with 100 U/ml streptomycin/penicillin (Gibco). Breast cancer cell line MCF-7 was maintained in DMEM medium, containing 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1.5 g/l sodium bicarbonate and 10% FCS. All cell lines were purchased from American Type Culture Collection (ATCC). Adherent cell lines (PC-3, MCF-7 and MDA-231) were passaged every 2-3 days by rinsing cells with PBS followed by addition of 2 ml 0.25% trypsin-EDTA. 10 ml complete growth medium was added after the cell layer was dispersed and cells subcultured according to their growth rate. Medium of suspension cells (T2 cell line) was renewed every 2 or 3 days and cells subcultured in a ratio of 1:3.

3.2 Antibodies

Antibodies were used for immuno-staining of CD (cluster of differentiation) surface markers as well as MHC molecules. FITC-labeled anti-CD80, -CD83, -CD86, -CD11c and PE-labeled anti HLA-DR antibodies were used for characterization of dendritic cells. The FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies were used for phenotyping of T cells. All antibodies were purchased from BD Pharmingen.

PA2.1 (anti-HLA-A2) mouse anti-human monoclonal antibody, used for analyzing HLA-A2 expression, was purified by affinity chromatography from the supernatant of the PA2.1 hybridoma cell line (purchased from ATCC). As secondary antibody, the FITC-conjugated goat anti-mouse IgG was used (BD Pharmingen).

A goat polyclonal anti-DNMT-1 antibody (Santa Cruz Biotechnologies) and a secondary HRP-conjugated bovine anti-goat IgG (Santa Cruz) were used for staining of western blots.

3.3 Flow cytometry

Flow cytometry uses instruments that scan single cells flowing past excitation sources in a liquid medium. Measurement of visible and fluorescent light emission allows quantitation of antigenic characteristics of individual cells. The light that emerges from the sample is analyzed for forward and side scatter (representing cell size and cell granularity, respectively) as well as fluorescent light. The fluorescent light intensity can be used as measure for quantitative analysis of receptor or cytokine expression. This method was used to phenotype dendritic cells, to analyze MHC class I expression on tumor cell lines, to measure HLA-A2 reconstitution on T2 cells and to evaluate tetramer staining as well as intracellular cytokine staining.

For phenotyping of the generated dendritic cells, $2-5 \times 10^5$ mature or immature DCs were stained with 10 μl antibody in 100 μl cell suspension (PBS, 0.5% BSA) for 30 minutes in the dark at 4°C. The antibodies used are FITC-labeled anti-CD80, -CD83, -CD86, -CD11c and PE labeled anti HLA-DR antibodies. After staining, cells were washed three times with PBS and surface receptors detected with a FACScan (Becton Dickinson). Data was analyzed using the WinMDI software (Joseph Trotter, URL: <http://facs.scripps.edu>).

For analysis of HLA-A2 expression on tumor cell lines MDA-231 and MCF-7, cells were stained with a monoclonal anti-HLA-A2 antibody (PA2.1) for 30 minutes in the dark at 4°C. After staining, cells were washed three times with PBS and subsequently incubated with a FITC-conjugated goat anti-mouse IgG secondary antibody for another 30 minutes at 4°C. After additional three washes, HLA-A2 expression was analyzed by flow cytometry.

3.4 Peptide binding assay

To evaluate the binding affinities of the sHLA-derived peptides to HLA-A2, they were tested for binding to T2 cells in an HLA-A2 stabilization assay. The TAP-deficient T2 lymphoma cell line was utilized for this HLA-A2 stabilization assay. This cell line has a defect in MHC class I expression and cannot effectively present class I-associated antigens to T cells. The low level of peptides bound in the MHC of this cell line allows most MHC class I molecules to remain empty, thus allowing for association with high affinity binding peptides. High affinity peptides can also compete with peptides in preloaded MHC class I molecules at the cell surface, whereas low affinity peptides require empty MHC class I molecules within cells.⁴³ Upon exposure to exogenously added HLA-A2-binding peptides, enhanced expression of conformational HLA-A2/peptide complexes on the cell surface of T2 cells can be measured by flow cytometry. The ability of peptides to reconstitute HLA-A2 on the T2 cell surface can be used as a measure for their binding affinities to HLA-A2.

Thus, TAP deficient T2 hybridoma cells were incubated with 25 $\mu\text{g}/\text{ml}$ peptide and 15 $\mu\text{g}/\text{ml}$ β_2 -microglobulin, or β_2 -microglobulin alone (RD Research Diagnostics) at 37°C for 24 h. β_2 -microglobulin was used for stabilization of the peptide/MHC complex. After incubation, cells were analyzed for HLA-A2 expression. Cells were incubated with anti-HLA-A2 antibody PA2.1 for 30 minutes on ice and subsequently washed three times with PBS. Secondary antibody was a FITC-conjugated goat anti-mouse IgG. Cells were stained for 30 minutes on ice and again washed as described above. Cell fluorescence was analyzed by flow cytometry.

3.5 Preparation of cell lysates

For the preparation of cell lysates, $1\text{--}5 \times 10^6$ cells were used. Cells were washed in PBS and cell pellets resuspended in 500 μl lysis buffer (1% NP-40, 0.15 M NaCl, 1M Tris pH 7.4), containing protease and phosphatase inhibitors (1 mM sodium monovanadate, 10 mM sodium fluoride, 1 mM sodium pyrophosphate,

1 mM phenylmethylsulfonyl fluoride, 5 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin) to prevent cleavage of proteins by proteases as well as dephosphorylation by phosphatases. After 30 min of incubation at 4°C, cell lysates were spun at 10000 rpm and the supernatant harvested. Protein concentration of cell lysates was measured using a colorimetric standard protein assay (DC Protein Assay, BioRad), which is based on the Lowry protein assay.⁴⁴ A protein standard curve was established using BSA as standard and absorbance measured at 655 nm in a Benchmark microplate reader (BioRad). Cell lysates were analyzed by SDS-PAGE and Western blotting.

3.6 SDS polyacrylamide gel electrophoresis

Equal protein amounts of each lysate were denatured in reducing sample buffer (3 \times solution: 6% SDS, 30% glycerine, bromphenol-blue, 4% 2-ME, 125 mM Tris HCl pH 6.8, ddH₂O) for 3 min at 100°C. Denatured samples were electrophoresed on SDS-polyacrylamide gels (5% stacking gel, 10% resolving gel) using the Mini-Protean II system (BioRad). As running buffer a Tris/glycine/SDS 10x concentrate from BioRad, diluted with ddH₂O, was used. Gels were run under constant voltage of 100 V for about 15 minutes (until the proteins entered the resolving gel). Voltage was then increased to 200 V and the gel run until the tracking dye reached the bottom of the gel.

3.7 Western blotting

Separated proteins were transferred from polyacrylamide gels to nitrocellulose membranes (Amersham) for 2-5 h at a constant current of 0.2-0.8 A (transferbuffer: 25 mM Tris, 150 mM glycine, 20% methanol, pH 8.3). After protein transfer, unspecific binding sites on the transfer membrane were blocked by incubating the membrane with 5% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS for 1 h at RT under continuous shaking. The membrane was incubated with the primary antibody, goat polyclonal anti-DNMT-1, diluted 1:10000 in PBS-T (0.1% Tween in PBS) for 2 h at RT. The membrane was subsequently washed three

times with PBS-T and incubated with a peroxidase-conjugated bovine anti-goat IgG as secondary antibody. After additional washes, proteins were visualized on the membrane after chemiluminescence-labeling using the ECL detection reagent (Amersham). The membrane was incubated for 1 minute in the dark with 3 ml ECL detection reagent. Immediately after incubation, Hyperfilm ECL (Amersham) was exposed to the membrane in the darkroom for 30 seconds for detection of chemiluminescence.

3.8 Preparation of HLA-A2 positive PBMC and DCs

To generate dendritic cells and to obtain T cells, PBMC derived from leukapheresis products of healthy individuals were purified by centrifugation in Ficoll-Histopaque (Sigma-Aldrich). The cell suspension was diluted with 2-4 volumes of PBS. 7 ml diluted cell suspension was carefully layered over 3 ml Ficoll-Histopaque (1.077 density, 20°C) in a 15 ml conical tube and centrifuged without brake at 2400 rpm for 20 minutes at RT. The interphase, containing lymphocytes and monocytes, was carefully transferred into a new tube and washed twice with PBS. Cells were then used for dendritic cell generation or CD8⁺ T cell separation with magnetic beads. Autologous DCs were used as APCs for in vitro generation of peptide-specific CTLs.

DCs were generated from fresh PBMCs in 2 steps. To enrich the PBMC for monocytes, 6.67×10^6 PBMCs/ml were plated with a density of 0.2 ml medium/cm² in 75 cm² culture flasks (Corning) for 2 h at 37°C. After 2 h, the semi-adherent cells (containing mostly B lymphocytes) and the cells in free suspension (mostly T lymphocytes) were washed off with culture medium and further utilized for T cell separation. Adherent cells, containing mostly monocytes, were cultured for 7 days in X-VIVO-15 medium (Biowhittaker) supplemented with 1% autologous plasma, 800 U/ml GM-CSF and 500 U/ml IL-4 to start their differentiation into DCs. On day 7, the non-adherent cell population, comprising of immature dendritic cells, was harvested and either analyzed by flow cytometry or further used for maturation. For further maturation, DCs were transferred to fresh tissue culture flasks

and resuspended in fresh culture medium containing 800 U/ml GM-CSF, 500 U/ml IL-4 and the pro-inflammatory cytokines IL-6 (1000 U/ml), IL-1 (10 ng/ml) and TNF- α (10 ng/ml). All cytokines were purchased from PeproTech. On day 10, non-adherent cells were harvested and analyzed for expression of CD11c, CD80, CD83, CD86 and MHC class II (HLA-DR) by flow cytometry to determine their phenotype.

3.9 Purification of CD8⁺ T Cells

Cell separation can be achieved by allowing cells to attach to antibodies bound to magnetic beads that can be separated in a strong magnetic field. Here, CD8⁺ T cells were enriched using an anti-CD8 monoclonal antibody that is conjugated to magnetic beads (Miltenyi Biotec).

First, PBMC from healthy donors were purified by centrifugation in Ficoll-Histopaque as described above and subsequently filtered through a nylon mesh (pore size 70 μm) to remove cell clumps. Cells were resuspended in MACS buffer (containing 0.5% BSA and 2 mM EDTA in PBS) and labeled with anti-CD8 conjugated magnetic micro beads for 15 min at 4°C. CD8⁺ cells were positive selected on a MS column using a MidiMACS Separator by loading the magnetically-labeled cell suspension on the MS column, which was placed into the magnetic field. Non-labeled, therefore CD8⁻ cells were rinsed out of the column by washing three times with MACS buffer. CD8⁺ cells were eluted with buffer after removing the column from the magnetic field. Monoclonal fluorochrome-conjugated antibodies for CD4 and CD8 (BD Pharmingen) were used to monitor cell purity of separated cells by flow cytometry.

3.10 Induction of cytotoxic T cells

For the induction of CTL, magnetic bead-separated CD8⁺ T cells were stimulated with peptide-pulsed DCs from the same blood donor. DCs were pulsed for 4 h at 37°C with 50 $\mu\text{g}/\text{ml}$ of peptide (p1028, p1145, p981 or p1258) in the presence

of 3 $\mu\text{g}/\text{ml}$ β_2 -microglobulin (Research Diagnostics) and subsequently washed. Before use as stimulators, DCs were inactivated by irradiation (50 Gray) to inhibit their growth. CD8^+ T lymphocytes were co-cultured at 2×10^6 cells/well with 6.7×10^5 peptide-pulsed DCs/well in 24-well plates in 2 ml RPMI-1640 (supplemented with 10% autologous plasma, 25 mM HEPES, 0.05 mg/ml gentamicin, 20 U/ml IL-2 and 5 U/ml IL-4 (PeproTech)) to induce CTL directed against the presented peptide. Medium was added or exchanged as necessary and on day 12 and weekly thereafter, CTL were restimulated with peptide-pulsed DCs in the same ratio as described above.

3.11 Cytotoxicity assay

To test whether the generated CTL are capable of target cell lysis and display specificity, we used a standard 4 h- ^{51}Cr -release assay. To test for CTL cytotoxicity, target cells are labeled with ^{51}Cr and are co-cultured with the CTL. In case of target cell lysis, the ^{51}Cr is released into the supernatant and can be detected and quantified with a γ -scintillation counter.

1.25×10^5 target cells (T2 cells pulsed with 25 $\mu\text{g}/\text{ml}$ peptide or unpulsed, MCF-7, MDA-231, K562, DC) were labeled for 1 h at 37°C with 1.85 MBq of $\text{Na}_2^{51}\text{CrO}_4$ (PerkinElmer), washed three times with 5 ml medium, resuspended in RPMI-1640 medium (containing 10% FCS) and plated in conical 96-well plates (1000 cells in 25 $\mu\text{l}/\text{well}$).

CTL were added to target cells at indicated effector:target (E/T) ratios to a total volume of 200 $\mu\text{l}/\text{well}$. After 4 h co-incubation of effectors and targets at 37°C , the supernatants were harvested using harvesting frames (Molecular Devices) and the chromium-release detected in a γ -scintillation counter. Spontaneous release was determined from the wells to which 100 μl of medium was added instead of effector cells (media control). Total releasable radioactivity was obtained after treating the target cells with detergent (1% Triton X-100). Percent specific lysis was calculated as $(\text{cpm experimental counts} - \text{cpm media control}) / (\text{cpm TritonX-100} - \text{cpm media control}) \times 100$.

To test for HLA-A2 restriction of the CTL, MCF-7 cells were incubated for 30 min with anti-HLA-A2 Ab PA2.1 (ATCC) and washed subsequently. Incubation of these cells with PA2.1 leads to binding of the antibody to surface HLA-A2 molecules, which are therefore masked and cannot be recognized by HLA-A2-restricted CTL. After staining, cells were labeled with $\text{Na}_2^{51}\text{CrO}_4$ and used as target cells in the cytotoxicity assay as described above.

3.12 Intracellular IFN- γ assay

The specificity and effector function of the p1028 CTL was further tested by measuring intracellular IFN- γ release by the CTL upon exposure to peptide p1028. 1×10^5 CTL were co-incubated with 1×10^3 peptide-pulsed T2 cells in 96-well plates in 100 μl medium/well. Control wells contained CTL alone or CTL in the presence of unloaded T2 cells. Intracellular IFN- γ -release by CTL was measured after 24 h incubation of CTL with stimulator cells at 37°C.

Before staining with the anti-IFN- γ antibody, cells had to be permeabilized to enable the antibody to pass through the cell membrane. Here, the Cytofix/Cytoperm solution from BD Pharmingen was used for permeabilization and fixation of the cells. Cells were first stained for surface CD8-expression with anti-CD8-FITC for 30 min at 4°C. Cells were washed three times with PBS and subsequently resuspended in 250 μl Cytofix/Cytoperm solution for 20 minutes at 4°C to permeabilize the cells. After permeabilization cells were washed in Perm/Wash solution to keep them permeabilized. Cells were incubated with PE-conjugated IFN- γ -antibody (antibodies and Cytofix/Cytoperm Kit was purchased from BD Pharmingen) for 30 minutes at 4°C and washed subsequently. Cell fluorescence was analyzed by flow cytometry.

3.13 Tetramer staining

To further determine the specificity of the CTL, tetramers were generated (tetramers were produced by ProImmune, Oxford, UK). Tetramers are tetrameric MHC/peptide complexes that are capable of binding stably to their specific TcR

on T cells. Tetrameric peptide-MHC complexes, compared to monomeric complexes, have the advantage of being able to bind more than one TcR on a specific T cell, therefore have correspondingly slower dissociation rates, which makes them more suitable for immunological staining. The tetramers purchased are fluorescence-labeled in order to monitor staining by flow cytometry. A FITC-conjugated tetramer, loaded with peptide p1028 and another irrelevant tetramer, presenting HLA-A2 derived peptide p898, were used for CTL analysis. $2-5 \times 10^5$ CTL were washed with PBS and resuspended in 50 μ l PBS/1% BSA. The cells were incubated for 15 min on ice with 1 μ g/ml FITC-labeled tetramer, while PE conjugated anti-CD8 Ab (BD Pharmingen) was added for 20 min. After washing, stained cells were detected with a FACScan.

CHAPTER 4

RESULTS

4.1 Selection of soluble HLA-A2-derived peptides

Four peptides from the pool of identified soluble HLA-A2 peptides were selected on the basis of their relative abundance and their tumor-associated precursor proteins. Theoretical binding affinity for these peptides was calculated using the affinity calculation program designed by Rammensee.³⁷ This program uses motif-based algorithms to predict affinities to MHC class I molecules, based on the assumption that each amino acid of the peptide contributes independently to the overall binding, and that certain positions (anchor residues) in the peptide are critical to its ability to bind to certain MHC molecules. The scoring system evaluates every amino acid within a given peptide. Individual amino acids are given values from 1-15, with 15 for an optimal anchor residue. The allocation of values is based on the frequency of the respective amino acid in natural ligands, T-cell epitopes, or binding peptides.

Peptide	Sequence	Precursor Protein	Score MHC Database ³⁷	NCBI Accession Number
p981	SLIGHLQTL	Protein Tyrosine Phosphatase	32	U16996
p1028	GLIEKNIEL	DNA Methyl Transferase I	28	X63692
p1258	FLFDGSPTYVL	Fatty Acid Synthase	N/A	U29344
p1145	FLFDGSPTYV	Fatty Acid Synthase	N/A	U20344

Table 4.1: sHLA-A2-eluted peptides

The four selected sHLA-A2-derived peptides characterized here are 8 to 11 amino acids long (Table 4.1), representing the natural length of peptides presented by membrane-bound MHC class I molecules. Theoretical binding affinities were calculated for peptide p981 and peptide p1028, derived from protein tyrosine phosphatase and DNA methyl transferase I, respectively. Both peptides have a high theoretical binding affinity (scores 32 and 28, respectively), considering the maximal score for HLA-A2 peptides being 36. The binding affinities for the two fatty acid derived peptides p1258 and p1145 could not be calculated. These four peptides were synthesized for in vitro analysis of their binding affinities to HLA-A2 and to test their immunogenicity.

4.2 Peptide p1028 has the highest binding affinity to HLA-A2

The stability of the peptide-MHC complex is critical in determining the immunogenicity of the peptide. It is therefore crucial to confirm the predicted binding affinities of peptides to HLA-A2 experimentally.

Binding affinities of peptides p981, p1028, p1258 and p1145 to HLA-A2 were evaluated using the human TAP-deficient T2 cell line, a T cell leukemia/B cell hybrid with an HLA-A2 haplotype. This cell line has a deletion in the MHC class II region encompassing the peptide transporter genes TAP1 and TAP2. TAP1 and TAP2 are responsible for the transport of MHC class I-restricted peptides into the ER, where they would be loaded onto MHC class I molecules. The absence of ER-localized peptides in TAP-deficient cells therefore results in accumulation of empty MHC class I molecules in the ER-Golgi intermediate compartments as well as on the cell surface.⁴⁵

Exogenous peptides can potentially be loaded onto MHC complexes at the cell surface without the requirement for peptide-uptake into the cell. However, they could also be internalized and loaded onto MHC molecules in the ER, where high affinity peptides can bind to newly synthesized MHC class I molecules in a TAP-independent way. High affinity peptides can also compete with peptides in preloaded MHC class I molecules at the cell surface, whereas low affinity peptides

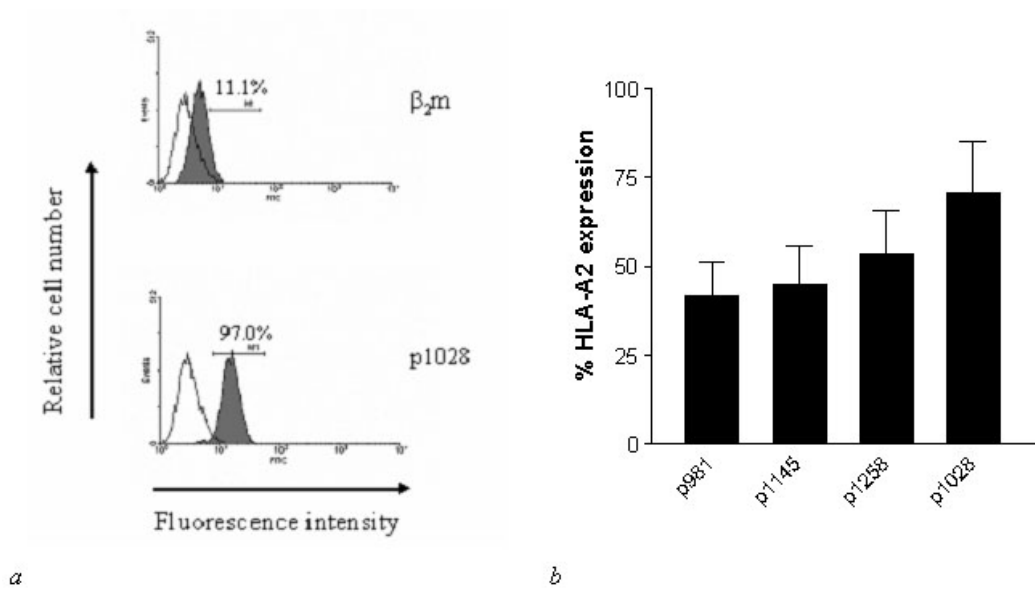


Figure 4.5: **Soluble HLA peptides induce reconstitution of HLA-A2.** T2 cells were pulsed with 25 $\mu\text{g/ml}$ peptide and 15 $\mu\text{g/ml}$ β_2 -microglobulin, or β_2 -microglobulin alone at 37° C for 24 h. After incubation, cells were analyzed for HLA-A2 expression after staining with anti-HLA-A2 antibody PA2.1 for 30 minutes. Secondary antibody was a FITC-conjugated goat anti-mouse IgG. (a) β_2 -microglobulin alone induces little reconstitution of conformational HLA-A2 complexes on the cell surface of T2 cells (11.1%), whereas the addition of peptide p1028 significantly increases HLA-A2 expression (97%). (b) Pulsing with each peptide led to HLA-A2 reconstitution, but was most pronounced after addition of peptide p1028, as shown in the bar graph, where percentage of HLA-A2 reconstitution is indicated on the y-axis. The error bars represent standard deviations from three independent experiments.

require empty MHC class I molecules within cells.⁴³ The affinity of peptides to their MHC is proportional to their ability to reconstitute MHC molecules on the surface of the T2 cell line, making this cell line a useful tool for the evaluation of peptide binding affinities.

Thus, T2 cells were exposed to sHLA-A2-derived peptides and expression of conformational HLA-A2 was detected by flow cytometry after staining with monoclonal anti-HLA-A2 antibody PA2.1. β_2 -microglobulin was used in addition to peptides for stabilization of the HLA/peptide complex. β_2 -microglobulin alone, as seen in Figure 4.5a, leads to reconstitution of conformational HLA-A2 complexes on the cell surface of T2 cells (11.1%). However, the addition of each

peptide led to significant increase in HLA-A2 expression, most pronounced after addition of peptide p1028. The percentage of HLA-A2 reconstitution for all four peptides is illustrated in Figure 4.5*b*, representing the results of three independent experiments. These data show that peptide p1028 (GLIEKNIEL) had the highest affinity to HLA-A2 compared to the other three chosen peptides.

The binding of peptides to MHC molecules is a non-covalent interaction that is mediated by residues in the peptides and in the clefts of the MHC molecules. The amino acids of the peptide fit in the pockets of the particular MHC molecule and bind to their complementary peptides in the MHC molecules. These residues of the peptide that are crucial for binding to a certain MHC allele and are called anchor residues. Peptide p1028 has the typical length of a MHC class I ligand (9 residues) and contains the characteristic anchor residues for the HLA-A2 allele, a leucine at position 2 and 9 of the peptide.

The relatively high affinity of p1028 for HLA-A2 is also consistent with the theoretical score in the database for MHC ligands and peptide motifs SYFPEI-THI³⁷ where this epitope is scored with 28, compared to a maximal theoretical score for HLA-A2 peptides of 36. In these experiments, maximal HLA-A2 expression was achieved with a peptide concentration of 25 $\mu\text{g}/\text{ml}$ after peptide pulsing for 24 hours. We identified peptide p1028 as the highest affinity binder, compared to peptides p981, p1145 and p1258.

4.3 Peptide p1028-pulsed DCs induce CD8⁺ T cell proliferation

To analyze the immunogenicity of the peptides, we utilized the capabilities of dendritic cells to induce a T cell response. Dendritic cells are so-called professional antigen presenting cells with unique costimulatory properties and an exceptional migration capacity.

In vivo, DCs acquire a mature phenotype after activation via CD40 ligation or through microbial stimuli. This state is characterized by high levels of costimulatory molecules such as CD80 and CD86, and upregulation of MHC class I and II molecules on their cell surface. The type of DC used for stimulation is crucial

for the fate of the naive lymphocyte. Resting or immature DCs are capable of tolerance induction^{46,47} due to non-adequate costimulation, whereas activated or mature DCs provide full costimulation and are therefore potent inducers of T cell immunity.⁴⁸ Thus, mature DCs represent an ideal tool for the stimulation of naive T cells.

Dendritic cells can be generated from monocytes or bone marrow cells (CD34⁺ cells) and can be further distinguished by their abilities to induce Th1, Th2 or tolerogenic immune responses (named DC1, DC2 and DC3, respectively). Here, we are differentiating monocytes from peripheral blood into mature dendritic cells of type DC1.

First, we isolated PBMC from leukapheresis products of HLA-A2 positive healthy blood donors and cultivated the adherent cell population in the presence of the growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) and cytokine IL-4. GM-CSF induces DC progenitor expansion and promotes differentiation and survival of DCs.⁴⁹ IL-4, on the other hand, functions to inhibit macrophage colony formation in addition to its role in inducing DC growth and maturation from monocytes.⁵⁰ This treatment produces dendritic cells with an immature phenotype, as shown in Figure 4.6 by flow cytometry. The generated DCs highly express adhesion molecule CD11c, costimulator ligand B7.2 (CD86) as well as MHC class II (HLA-DR), whereas the expression of DC marker CD83 as well as costimulator ligand B7.1 (CD80) is still limited, thus confirming the immature phenotype of those DCs. Immature DCs are able to capture and process antigen, but are not efficient in presentation of the antigen with subsequent T cell stimulation.

Thus, to further mature the DCs, the non-adherent cell population was harvested on day 7 and further cultivated in the presence of GM-CSF, IL-4, IL-6, IL-1 and TNF- α . This pro-inflammatory combination of cytokines has been described in various protocols to induce DC maturation. After full maturation, DCs expressed high levels of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) as well as MHC class II (Figure 4.6, lower panel) and CD11c. In addition,

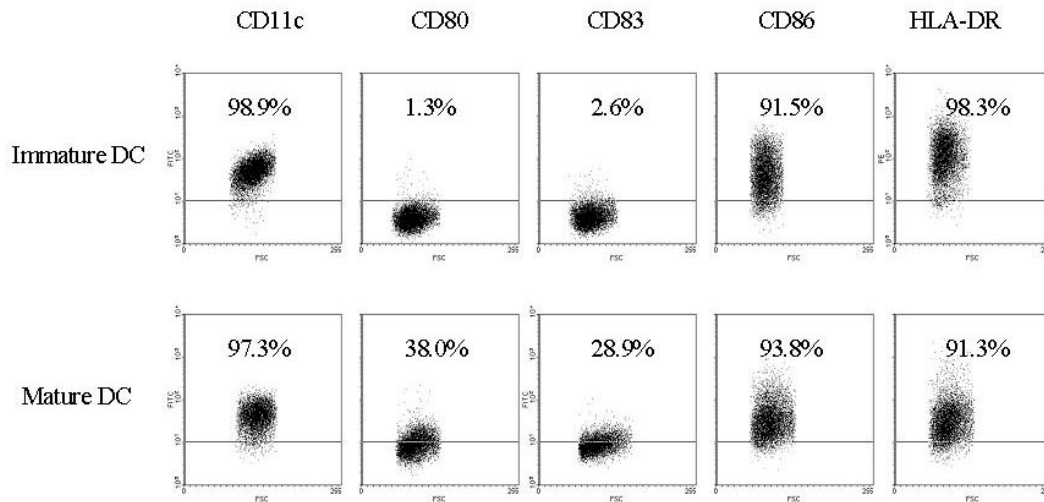


Figure 4.6: **Phenotyping of immature and mature dendritic cells.** Adherent PBMC were co-cultured in the presence of GM-CSF and IL-4. At day 7, non-adherent cells were harvested and cultured for three days with additional cytokines (TNF- α /IL-1/IL-6). Mature and immature DCs were stained with mAbs for 30 min at 4°C and analyzed by flow cytometry. Characteristically, mature DCs showed enhanced CD80 and CD83 expression.

the expression of CD83, a mature DC marker, was elevated compared to the immature DCs.

Mature DCs were used to generate cytotoxic T cells against sHLA-A2 derived peptides. As responder cells, CD8⁺ T cells were separated from PBMC of the same blood donor using anti-CD8-labeled magnetic beads. Separated CD8⁺ T cells were stained with anti-CD4 and anti-CD8 antibodies to evaluate their purity. The positive-selected cell fraction contained 93% CD8⁺ T cells, as shown in Figure 4.7.

Mature DCs were pulsed with peptide p1028, p981, p1145 or p1258 and co-cultured with autologous separated CD8⁺ T cells to induce CD8⁺ T cell proliferation. Peptide p1028-loaded dendritic cells induced clonal expansion of T cells after the first restimulation, indicating that peptide p1028 can activate T cells and induce their differentiation and proliferation. However, no expansion was observed for the lower affinity peptides p981, p1145 and p1258, substantiating the

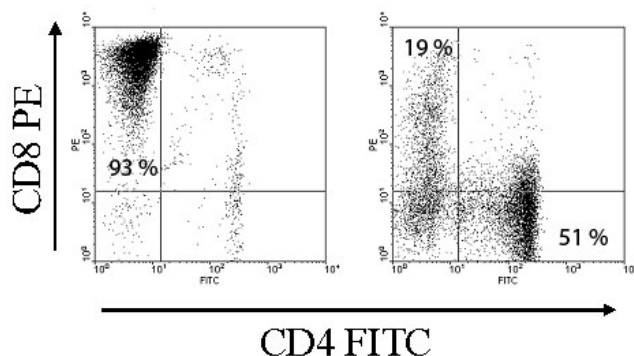


Figure 4.7: **Magnetic bead separated CD8⁺ T cells from PBMC.** PBMC were labeled with CD8 magnetic beads for 15 minutes at 4°C. CD8⁺ cells were selected on a MS column using a MidiMACS Separator. Positive selected cells (left panel) and the CD8⁻ flow through (right panel) were stained with monoclonal antibodies for CD4 and CD8 and analyzed by flow cytometry. Positive selected cells are 93% CD8⁺.

importance of peptide affinity.

These findings indicate that the exogenously added sHLA-A2 derived peptide p1028 bears the HLA-A2 motif that is efficiently presented by the MHC of DCs and is able to induce T cell expansion, suggesting immunogenicity of the peptide. Therefore, in the following experiments, peptide p1028 was used for the generation of peptide-specific T cells and the CTL further analyzed.

4.4 Cancer cell lines highly express DNMT-1

The selected peptide, p1028, is derived from DNA methyl transferase I (DNMT-1), which is a highly abundant protein found in tumors. DNMT-1 is an enzyme, involved in the maintenance of DNA-methylation activities and is required to maintain DNA methylation and aberrant CpG island methylation in cancers. Elevated levels of DNMT-1 mRNA and DNMT-activity have been observed in many cancer cells in vitro and tumors in vivo.⁵¹ However, it has also been shown that DNMT-1 mRNA is highly expressed in most fetal tissues as well as adult tissues containing continuously renewing cell populations (e.g. liver).

Thus, these results suggest a relationship between DNMT mRNA levels and



Figure 4.8: **DNMT-1 expression of tumor cell lines compared to activated and naive PBL.** Peptide p1028 is derived from DNA methyl transferase-1, an enzyme associated with cancer. Cell lysates derived from tumor cell lines T2, MCF-7, MDA-231, PC-3, peripheral blood lymphocytes (PBL) as well as ConA-activated human T lymphocytes (ConA blasts) from four healthy blood donors were analyzed for DNMT-1 expression by western blotting using an anti-human DNMT-1 antibody. DNMT-1 is highly expressed in all tumor cell lines, whereas a moderate expression was observed for PBL as well as ConA blasts of all 4 donors.

the proliferative state of the tissue.⁵¹ DNMT-1 has already been identified as a target for cancer therapy, since it is well known that DNA methylation patterns change in malignancy, leading to genetic instability and the repression of tumor suppressor genes.⁵² Pharmacological DNA methyl transferase inhibitors exerted anti-tumor effects in in vitro and in vivo laboratory models, reviewed by Goffin et al.⁵³

Here, we analyzed the protein expression of DNMT-1 in tumor cell lines in comparison to normal cells. The tumor cell lines analyzed here are a selection of cell lines that were previously used for transfection with soluble HLA-A2 and the lymphoma cell line T2. Expression of DNMT-1 was compared between the two breast cancer cell lines, MCF-7 and MDA-231, the T2 lymphoma cell line and the prostate cancer cell line PC-3, in addition to peripheral blood lymphocytes (PBL) and Conavalin A (ConA)-activated T lymphocytes from four healthy blood donors (Figure 4.8). Equal amounts of protein were loaded on the gel, analyzed by a standard spectrophotometry-based protein assays. This experiment revealed that the enzyme is highly expressed in all tumor cell lines used in this study and expressed at very low levels in PBL as well as activated T lymphocytes (ConA blasts).

The relative over-expression of DNMT-1 in many tumor cell lines in comparison to non-malignant cells and the capability of peptide p1028 to induce T cell proliferation suggest that the DNMT-1 derived peptide p1028 may be suitable as candidate for a peptide cancer vaccine. However, as mentioned above, the enzyme is highly expressed in non-cancerous proliferative tissue such as the liver. Thus, a DNMT-1-directed tumor vaccine would bear the risk for the induction of autoimmunity.

4.5 Effector CTL specifically recognize p1028 loaded T2 cells

The cytotoxic T cells that were generated against peptide p1028 were phenotyped and subsequently analyzed for lytic activity in a standard cytotoxicity assay to test their functionality and specificity.

The major feature of cytotoxic T cells is the ability of target cell lysis. After the recognition of an antigen on the surface of a target cell, cytotoxic T cells release the contents of their lytic granules through a calcium-dependent process. These granules contain two major classes of cytotoxic effector proteins - perforin and proteases known as granzymes. Perforin is released through exocytosis at the point of contact and polymerizes within the membrane of the target cell, producing a cylindrical structure in the lipid bilayer that is lipophilic on the outside and hydrophilic in its hollow center. Water and salts are then able to enter the cell through these pores, destroying the integrity of the target cell membrane.

In addition to water and salts, granzymes (specifically granzyme A and B), which have also been released from the lytic granules, can now enter the target cell. Granzyme A and B are introduced into the target cell and activate the caspase family of proteases, which leads to cleavage of the DNA into 200 base pair fragments.

A standard assay to analyze these lytic capabilities of CTL is the chromium release assay, in which ^{51}Cr -labeled target cells are mixed with effector CTL in different ratios for 4 hours. During the co-incubation time, CTL recognize and lyse the target cells. The amount of ^{51}Cr released into the supernatant by killed

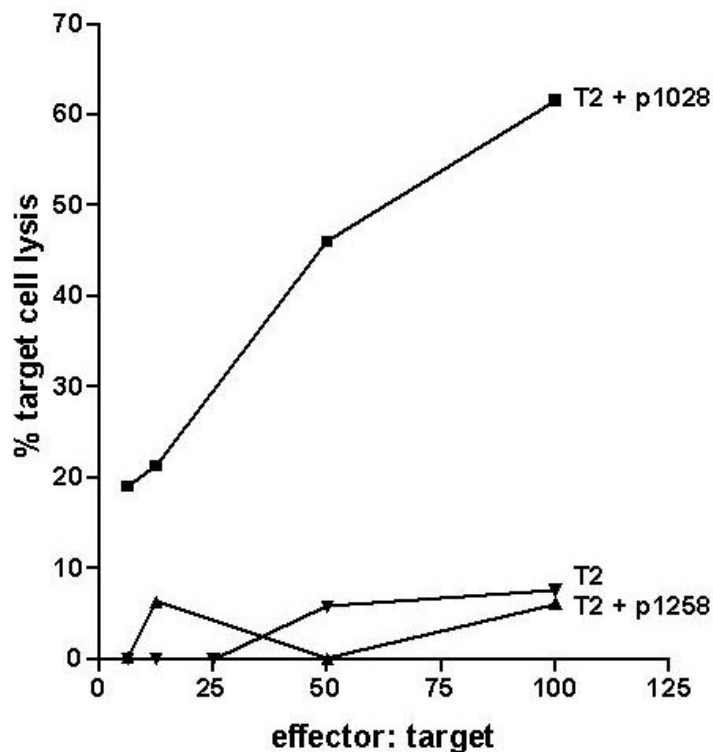


Figure 4.9: **Effector CTL specifically recognize p1028 loaded T2 cells.** Peptide-pulsed (25 $\mu\text{g}/\text{ml}$) and unpulsed HLA-A2 positive T2 cells were labeled with ^{51}Cr and used as target cells in a 4 h ^{51}Cr -release assay. CTL specifically lysed p1028 pulsed T2 cells, while no significant lysis was detected in the unpulsed T2 cells, as well as for T2 cells pulsed with p1258, an irrelevant sHLA-A2 derived control peptide.

target cells is quantitated using a γ -scintillation counter. The amount of chromium released is a measure for the lytic activities of the effector CTL.

As a measure for maximum release of chromium, representing 100% target cell lysis, target cells are incubated with the detergent TritonX-100. Spontaneous ^{51}Cr release is measured in wells containing only target and no effector cells (medium control). The percentage of target cell lysis can be calculated by: $(\text{cpm experimental counts} - \text{cpm medium control}) / (\text{cpm TritonX-100} - \text{cpm medium control}) \times 100$.

To test whether the CTL specifically lyse p1028 presenting cells, T2 cells

were used as target cells to present the desired peptides. T2 cells were incubated overnight with 25 $\mu\text{g}/\text{ml}$ peptide 1028 or the irrelevant control peptide p1258 and β_2 -microglobulin. Pulsed and unpulsed T2 cells were subsequently used as target cells (labeled with ^{51}Cr) in a 4 hour ^{51}Cr -release assay. After co-incubation of targets and effectors the supernatant was harvested and analyzed for released ^{51}Cr in a γ -scintillation counter. Percentage of target cell lysis was calculated as described above and plotted in Figure 4.9. CTL clearly lysed peptide p1028 presenting T2 cells, but not p1258 loaded or unloaded T2 cells.

These data demonstrate that target cell killing by p1028 CTL is specifically dependent on the presentation of peptide p1028.

4.6 Peptide p1028-specific CTL lyse tumor cells

Besides being able to lyse peptide-pulsed T2 cells, we aimed to analyze whether p1028 CTL are capable of lysing tumor cells that naturally present p1028 in the context of HLA-A2.

In order to investigate whether the p1028 CTL line is capable of tumor cell lysis, we had to utilize an HLA-A2 expressing tumor cell line that expresses the enzyme DNMT-1 at high level. We predicted that this cell line presents peptide p1028 in an HLA-A2-restricted manner. MDA-231 and MCF-7 are two of the tumor cell lines previously used for the identification and purification of soluble HLA-A2-derived peptides after transfection with the truncated HLA-A2 gene. Both cell lines also naturally present MHC class I molecules on their cell surface and are known to have the HLA-A2 haplotype. However, since tumor cells have been described to downregulate their MHC expression in order to evade immune surveillance, we confirmed HLA-A2 surface expression on these cells by flow cytometry.

Breast cancer cell lines MCF-7 and MDA-231 were stained with anti-HLA-A2 antibody PA2.1 and HLA-A2 surface expression analyzed by flow cytometry. As shown in Figure 4.10, both breast cancer cell lines, MCF-7 as well as MDA-231, expressed HLA-A2 on their cell surface. In addition, MCF-7 and MDA-231

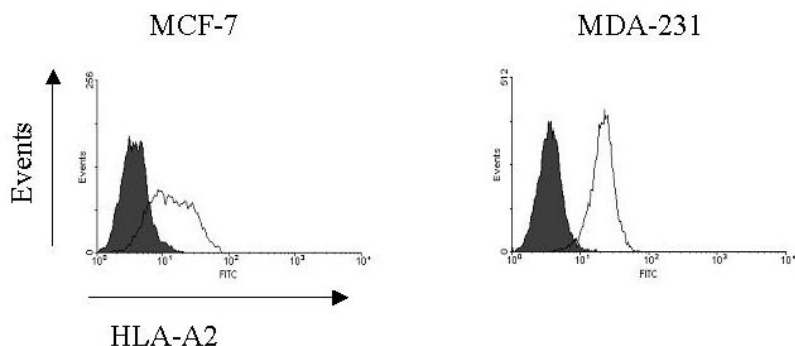


Figure 4.10: **Breast cancer cell lines MDA-231 and MCF-7 express membrane-bound HLA-A2.** MCF-7 and MDA-231 cells were analyzed for the expression of membrane-bound HLA-A2. Cells were stained with monoclonal anti-HLA-A2 antibody PA2.1 and analyzed by flow cytometry. Both cell lines express membrane-bound HLA-A2.

expressed high levels of DNMT-1, as described above (Figure 4.8).

If peptide p1028 is potentially presented by membrane-bound MHC molecules, the expression of HLA-A2 should make these tumor cell lines susceptible for lysis by p1028 specific CTL. Furthermore, MCF-7 and MDA-231 were originally used for the discovery of TAA peptides and displayed p1028 in the transfected soluble HLA-A2 molecules. This suggests that DNMT-1 was degraded partially into peptide p1028 which should, besides being presented by the soluble HLA-A2 molecule, be presented on membrane bound HLA-A2 molecules on these cell lines. Therefore, both cell lines should be susceptible to lysis by p1028 CTL.

Thus, MCF-7 and MDA-231 were used as target cells in a ⁵¹Cr-release assay. Pulsed T2 cells were included as positive control for lysis by p1028-specific CTL and unpulsed DCs were used as negative control, representing non-oncogenic cells. As an additional control for unspecific lysis, K562 cells were used as target cells. K562 cells lack MHC class I expression, which makes them susceptible to NK cell lysis. Thus, they were used as target cell line to exclude unspecific natural killer cell-like activity of the CTL.

As expected, peptide p1028-specific CTL efficiently lysed T2 cells that were pulsed with p1028 (Figure 4.11). No significant NK-like activity of the generated

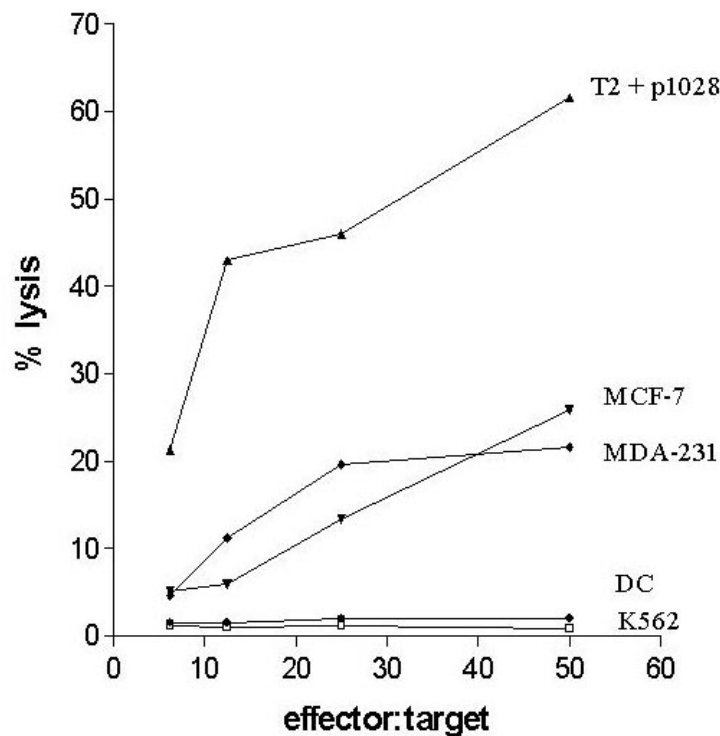


Figure 4.11: **Peptide p1028-specific CTL lyse HLA-A2 positive tumor cell lines.** MCF-7, MDA-231, K562, DC and p1028-pulsed T2 cells were used as target cells in a 4 h ^{51}Cr -release assay. Peptide-pulsed T2 cells were highly susceptible to CTL killing. In addition, both breast cancer cell lines, MCF-7 and MDA-231, were lysed by p1028 specific CTL. K562 target cells were not susceptible to cell lysis. Also, no lytic activity was detectable against unpulsed dendritic cells used as target cells, indicating that the tumor cells, but not primary human DCs display peptide p1028 on their surface MHC.

CTL could be observed, as K562 cells were not lysed.

In addition, the CTL were able to lyse the two HLA-A2-positive breast cancer cell lines MCF-7 and MDA-231, although at a lower percentage than pulsed T2 cells (20% breast cancer cell lysis compared to 60% T2 cell lysis at the highest E:T ratio). As control for lysis of primary human cells, we used mature dendritic cells as target cells. These dendritic cells were not susceptible to lysis, suggesting low expression of peptide p1028 on their surface MHC. This result also confirms the poor physiological expression of DNMT-1 in primary PBMC, as already proposed in Figure 4.8.

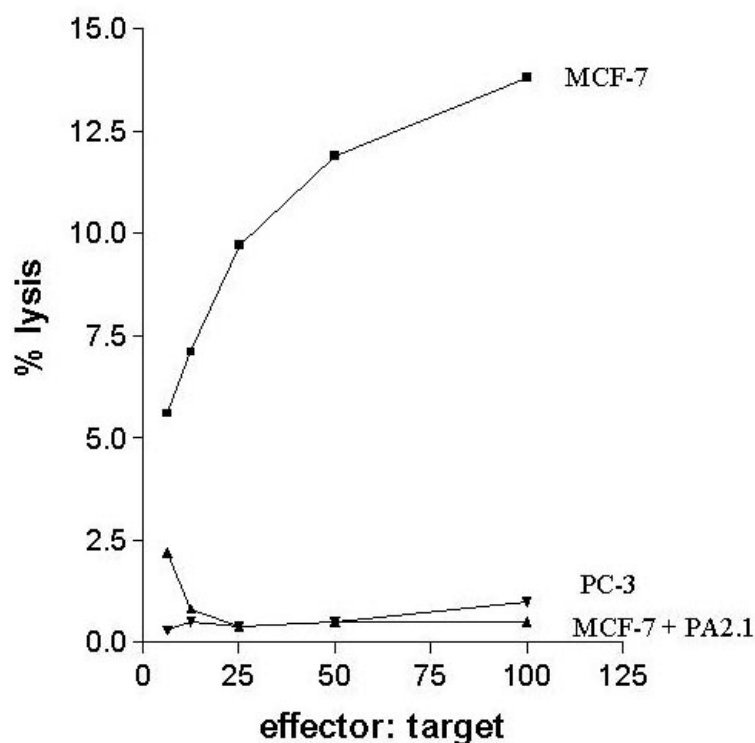


Figure 4.12: **HLA-A2 restriction of p1028 CTL.** HLA-A2⁻ prostate cancer cell line PC-3 and HLA-A2⁺ breast cancer cell line MCF-7 were used as targets for p1028 CTL in a ⁵¹Cr-release assay. The HLA-A2-negative, DNMT-1 expressing prostate cancer cell line PC-3, was not susceptible to lysis by p1028-specific CTL, whereas HLA-A2-expressing breast cancer cell line MCF-7 was susceptible. The anti-HLA-A2 antibody inhibited lysis of MCF-7. Both results prove HLA-A2 restriction of the generated CTL.

Thus, peptide p1028 is capable of inducing a cytotoxic T cell response towards tumor cells.

4.7 p1028 CTL are HLA-A2-restricted

To test whether the tumor cell lysis by p1028 CTL is HLA-A2 restricted, we utilized prostate cancer cell line PC-3 as HLA-A2 negative tumor cell line that highly expresses DNMT-1. Breast cancer cell line MCF-7 was used as HLA-A2 positive tumor cell line. In addition, we masked HLA-A2 molecules on the surface of MCF-7 cells using the anti-HLA-A2 mAb PA2.1. Binding of mAb PA2.1 to

HLA-A2 surface molecules on tumor cell line MCF-7 should inhibit the binding of the specific TcR to HLA-A2 and therefore prevent recognition by HLA-A2-restricted CTL.

Therefore, anti-HLA-A2 stained MCF-7 cells should not be susceptible to lysis by p1028 CTL, if the CTL are HLA-A2 restricted. Unstained and stained MCF-7 cells, as well as PC-3 cells were used as target cells in a chromium-release assay. As expected, p1028 CTL specifically lysed the A2-expressing tumor cell line MCF-7, whereas the A2-negative cell line PC-3 as well as the HLA-A2-masked MCF-7 cells were not susceptible to CTL killing (Figure 4.12).

Thus, this experiment proves that HLA-A2 expression was a requirement for p1028 CTL recognition of target cells.

4.8 p1028 CTL produce IFN- γ

To further characterize the p1028-generated CTL, they were tested for interferon gamma (IFN- γ) production on interaction with p1028-pulsed T2 cells. IFN- γ is a cytokine, which is involved in both the adaptive and innate immune responses. However, its main functions include macrophage activation, upregulation of certain T helper cells, and enhanced antigen presentation by MHC molecules. IFN- γ is secreted by cytotoxic T cells in response to activation and has been shown to inhibit the growth of Th2 cells and to promote the growth of Th1 cells. Therefore the measurement of IFN- γ production is a means to analyze the effector function of cytotoxic T cells.

Here, IFN- γ production of p1028 CTL was measured intracellularly after 24 h co-incubation with peptide-loaded or native T2 cells, Figure 4.13.

CTL were first stained with a FITC-conjugated anti-CD8 antibody, then permeabilized to allow the anti-IFN γ -antibody to enter the cell and subsequently stained with a PE-conjugated IFN- γ antibody. Clearly, p1028-pulsed T2 cells induced significantly higher IFN- γ production by p1028 CTL (68%) than did native T2 cells (28%). This experiment therefore shows a specific effector T cell reaction of p1028-CTL towards the peptide p1028. The observed background after

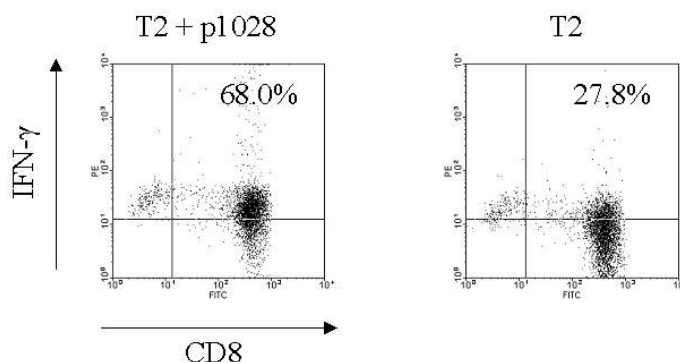


Figure 4.13: **p1028 specific CTL express intracellular IFN- γ to p1028 peptide-loaded T2 cells.** IFN- γ was measured after 24 h incubation with either pulsed or unpulsed T2 cells. Cells were stained with anti-CD8, permeabilized and incubated with a PE-conjugated IFN- γ antibody, respectively. Data were analyzed by flow cytometry. 68% of the CTL specifically produced IFN- γ to peptide p1028 loaded T2 cells, compared to only 28% of those responding to unloaded T2 cells.

restimulation with unpulsed T2 cells was also observed in CTL that were cultured without T2 cells, and is therefore most likely due to the stimulated state of the CTL created during the initial CTL culture, which causes the CTL to unspecifically produce IFN- γ .

4.9 p1028 CTL are stained by a p1028-specific tetramer

To confirm the peptide-dependent cytotoxicity and specificity of p1028 CTL, we stained the CTL with newly generated MHC-peptide tetramers. The staining of specific T cells with their ligand, the MHC-peptide complex, has proven to be difficult due to the fast dissociation rate from the T cell antigen receptor.^{54,55} Multimeric peptide-MHC complexes are able to bind more than one T cell receptor on a specific T cell and have correspondingly slower dissociation rates than monomeric MHC molecules. These complexes are therefore more suitable for immunological staining of antigen specific T cells, as first described by Altman et al.⁵⁶ In addition, the binding of tetrameric MHC-peptide complexes to a specific T cell receptor correlates well with cytotoxicity of CTL, making tetramer staining a useful technique for the analysis of CTL specificity and cytotoxicity.

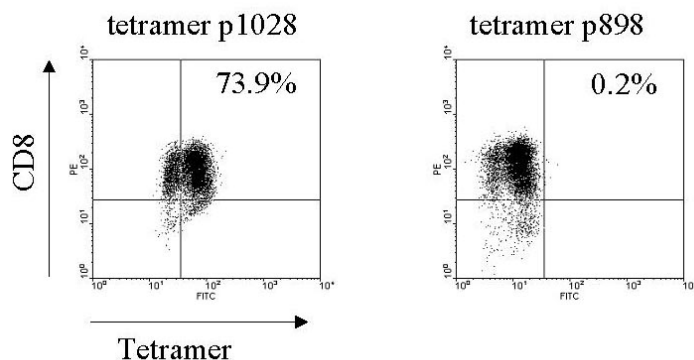


Figure 4.14: **CTL were stained by a p1028-specific tetramer.** CTL were co-stained with FITC-conjugated A2.1/p1028 or control A2.1/p898 tetramers and anti-CD8-PE. The p1028 tetramer stained approximately 74% of the CTL, while the irrelevant tetramer showed no significant binding, showing that the generated CTL are highly specific for peptide p1028.

Therefore, FITC-conjugated tetramers expressing either peptide p1028, or the irrelevant peptide p898, were commercially acquired and used for CTL staining. Peptide p898 is derived from an IFN- γ -inducible protein, which was also recovered from sHLA-A2 molecules, thus a peptide with the same characteristics as p1028, regarding MHC-restriction. CTL were co-stained with anti-CD8-PE and tetramer p1028 or p898, respectively. The p1028 tetramer stained 74% of the CTL, whereas the control tetramer failed to show any significant binding (Figure 4.14). These results confirm that the generated CTL are highly specific for peptide p1028.

Thus, here we describe a novel HLA-A*0201-eluted peptide that is immunogenic *in vitro* and able to induce tumor cell lysis by peptide-specific CTL, possibly making peptide p1028 a novel candidate for tumor immunotherapy.

CHAPTER 5

DISCUSSION

The elimination of transformed cells before they develop into tumors and the elimination of tumor cells is one of the major functions of the immune system. However, even though T cell dependent immunity against tumors exists, in many cases tumors are not eradicated by the immune system. The aim of cancer immunotherapy is therefore to exploit the capacity of the immune system to kill and eradicate tumor cells.

There are several reasons, as to why immune responses frequently fail to prevent the growth of tumors. One major obstacle is the fact that tumor cells are derived from the host and resemble normal cells in many aspects. Thus, they express only few antigens that could possibly be recognized as non-self. Therefore, most tumors prove to be of very low immunogenicity. Only tumors that are induced by viral infections, and therefore bear foreign antigens, are able to elicit a potent immune response.

Although the immune surveillance hypothesis, which states that the immune system can recognize and destroy transformed cells, has been generally confirmed, a majority of tumors induces very weak or even undetectable immunity.

Thus, there is a need for the development of strategies to activate the immune system to effectively lyse tumor cells and eradicate tumors.

Identifying tumor antigens has been an essential step in the progress towards the development of cancer vaccines. Since CD8⁺ T cells are able to directly lyse tumor cells and destroy tumor masses in vivo, most attention has been paid to TAA that are recognized by CTL. TAA recognized by CTL could potentially be effective tumor rejection antigens, since the MHC class I pathway ensures that any changes in the repertoire of antigens expressed by most somatic cells would be recognized by CTL. In addition, MHC class I molecules are expressed on most somatic cells and therefore on tumors arising from those cells. Also, there is a correlation between tumor progression and loss of HLA expression in tumor

patients,¹² implying that, in order to progress, tumors have to escape the MHC class I-restricted T cell response.

Thus, most emphasis has been put on the CD8⁺ T cell response against tumors and the identification of MHC class I-restricted TAA.

One can distinguish between four groups of TAA: (1) Tumor specific antigens that are derived from genes involved in oncogenesis, (2) antigens derived from proteins, which genes are only expressed in germ-cells that don't express MHC class I and therefore don't express the antigen on "normal" cells, (3) differentiation antigens that are expressed in specific tissues and (4) antigens encoded by genes that are also expressed in normal cells but over-expressed in certain tumors.

Early methods of identifying TAA were based on the identification of targets that were recognized by T cells of cancer patients. Several other strategies evolved during the past decade, including biochemical methods, gene expression analysis, reverse immunology, dendritic cell-based methods and the identification of viral antigens. However, since no major breakthroughs have been reported, there is the need for the development of novel strategies to identify new cancer related peptides. These peptides should be immunogenic and ideally suitable for different types of cancers as well as applicable to many patients.

The standard biochemical approach for the identification of HLA peptides as TAA involves the elution of endogenous peptides from immunoaffinity purified MHC molecules isolated from detergent extracts of cells with monoclonal antibodies specific for the desired haplotypes.³⁴ The resulting mixtures of peptides are analyzed and sequenced using reversed-phase HPLC connected to MS/MS.^{35,36} However, the purification of MHC molecules by detergent extraction of cell membranes leads to contamination of the MHC preparations with cellular debris and with detergents, which complicates the subsequent MS/MS analysis. Also, membrane bound MHC molecules are hard to purify in large amounts as needed for peptide analysis.

Thus, to improve the yields of MHC peptides from a limited amount of cancer cells and to avoid the contamination of the purified HLA by cell debris and

detergent, we utilized a soluble form of MHC molecules to purify MHC-presented peptides. In addition, the method of transfecting cancer cells with soluble HLA molecules gives us the opportunity to choose a desired MHC haplotype, regardless of the innate haplotype of the cancer cell. Here, we analyzed peptides derived from soluble HLA-A2 molecules, as HLA-A2 is a very common haplotype in the Caucasian population.

The most abundant peptides recovered from the sHLA-A2/peptide complexes that were also derived from TAA were evaluated for their binding affinity to HLA-A2. The lengths of peptides eluted from recombinant sHLA-A2 seem not to be different from peptides displayed on membrane bound MHC class I molecules,⁷ which usually are 8 to 10 amino acids long and display 2 to 3 primary anchor residues.⁵⁷ However, the high sensitivity analysis of MHC peptides based on recovery from soluble HLA followed by capillary HPLC and tandem mass spectrometry enabled the identification of few "outlier" peptides, such as p1258, with a length of 11 amino acids. This particular peptide (p1258), however, did not show high affinity to membrane bound HLA-A2 and was not able to induce T cell proliferation.

The peptide further characterized in this study, p1028 (GLIEKNIEL), displays the characteristic anchor residues for HLA-A2, with leucine at the second and ninth position. A high binding predictive value was calculated in the database for MHC ligands³⁷ for p1028, which was confirmed by our binding affinity studies on T2 cells. We observed slightly different results for peptide affinities using either the murine HLA-A2 expressing RMA-S-HHD cell line or the T2 cell line. This might be due to the higher basal expression of self-peptides presented by T2 cells at 37°C compared to RMA-S-HHD cells at 26°C, therefore creating a higher threshold for exogenously added peptides to bind to the surface MHC. Also, our results indicate a relatively low binding affinity for p981, which was sustained by the lack of ability to induce expansion of T cells in response to p981.

In contrast, the stimulation of CD8⁺ T cells with p1028 promoted the expansion of specific CTL. However, such a profound T cell response was only observed

for 1 out of 4 healthy individuals, probably due to differences between the T cell repertoire of each patient. Besides the differences between each patient regarding their T cell repertoire, the specificity of peptides for particular MHC class I molecules limits the use of peptide vaccines to patients who carry the specific HLA phenotype. In the case of peptide p1028, the situation is somewhat favorable, since about 50-60 % of the Caucasian population has the HLA-A2 subtype.

Some studies already showed successfully the elimination of tumor cells that occurred after eliciting a specific anti-tumor CTL response.^{25,26,27,28} T cell therapy proved to be effective in human clinical trials for a variety of malignant diseases, but mainly for melanoma patients.^{29,30} Overall, besides some success in the treatment of melanoma, the success of cancer vaccines has been only marginal. One of the major difficulties in cancer immunotherapy is the capability of cancer cells to evade immune recognition and to induce T cell tolerance.

One strategy, by which cancer cells can evade T cell recognition, is the inactivation or downregulation of the cell's MHC complex or its associated machinery.¹² Theoretically, these losses of MHC class I should make the tumor cells more susceptible to NK lysis, since NK effector cells monitor class I cell surface expression through their specific receptors (KIR), and eliminate those cells with downregulated HLA class I molecules.⁵⁸ To also evade NK cell lysis, some tumors developed that only downregulate the expression of a certain MHC class I molecule that expresses the tumor antigen. This way the tumor evades CTL and NK killing, which has been described in certain colon and ovarian cancers.^{59,60} Additional ways to escape immune surveillance are the expression of Fas-Ligand on tumor cells leading to Fas-induced apoptosis of activated T cells, the lack of costimulatory molecules and the release of immunosuppressive factors such as TGF- β and IL-10,²⁰ as described above.

In addition, one cannot overemphasize that it is still questionable whether patients that already developed a tumor can mount strong T cell responses to kill all tumor cells, since tumor-specific naive T cells might already be tolerant to tumor-antigens. In order to overcome T cell tolerance and immune evasion,

strategies have been developed to actively stimulate the host immune response to the tumor, e.g. by using dendritic cells as potent costimulators for presentation of peptides. In our study we used mature dendritic cells to present peptide p1028 and were able to induce peptide-specific CTL, using DCs and CD8⁺ T cells from healthy blood donors. However, in case of a tumor patient, tolerance towards this tumor-antigen might be already achieved, making it more difficult or even impossible to induce the peptide-directed T cell response.

CTL raised against p1028 presenting dendritic cells were capable of killing the p1028 loaded T2 cells, but not T2 cells loaded with an irrelevant sHLA-A2 derived control peptide p1258, showing p1028 dependent cytotoxicity. In addition, the CTL showed lytic activity against the breast cancer cell lines MDA-231 and MCF-7, suggesting that those cancer cell lines naturally present peptide p1028 on their cell surface. The lower cytotoxicity against those tumor cell lines compared to loaded T2 cells is likely to be due to a lower expression of the naturally processed peptide p1028 on membrane bound MHC molecules on those cell lines relative to the T2 cells loaded with a high concentration of the synthetic peptide. The natural levels of p1028 on these cells are, however, sufficient for recognition by the p1028 specific CTL.

Although high levels of DNMT-1 are expressed in the T2 cell line, unloaded T2 cells are not susceptible to CTL lysis. This is probably due to the TAP transporter defect in the T2 cell line, which impedes the natural presentation of endogenous peptides.

p1028 CTL were not capable to lyse HLA-A2 negative, DNMT-1 expressing cancer cell line PC-3, as well as MCF-7 cells incubated with an HLA-A2 blocking antibody. Those experiments proved the HLA-A2 restriction of the generated CTL. Peptide-dependent cytotoxicity was also confirmed by staining the CTL with the p1028 tetramer and by p1028-dependent IFN- γ production.

As mentioned above, peptide p1028 is derived from DNMT-1, an enzyme which is highly expressed in a variety of tumor cells, but has also been shown to be elevated in tissues containing continuously renewing cell populations, such

as fetal tissue.⁵¹ Here, we show that DNMT-1 is expressed in various tumor cell lines (breast cancer, prostate cancer and lymphoma cell lines). However, it is also expressed in primary PBL, even though at very low level.

A variety of tumors over-express physiological proteins, like DNMT-1, thereby presenting a different set of self-peptides associated with MHC class I molecules relative to normal cells. Due to the over-expression of a certain protein, the expression of the peptide-MHC complexes on the cell surface might be increased and might therefore enable T cells to recognize the antigen, making the peptide immunogenic.²⁴ Thus, p1028 can be categorized as an immunogenic peptide derived from a non-mutated tumor antigen that is over-expressed in a variety of tumors, but also expressed in some non-malignant tissues.

Besides being able to trigger tumor-specific CTL against self-antigens, the relative over-expression of a particular cancer associated protein, may also lead to autoimmunity induction. Autoimmunity induction did not seem to be a severe problem in most reported cases; even total mRNA or unfractionated peptide or protein extracts from tumors did not cause autoimmunereaction.^{61,62} However, some severe reactions using a dendritic cell based vaccine have been reported.⁶³ Thus, the expression of DNMT-1 in normal tissue might limit the use of peptide p1028 as cancer vaccine due to the risk of autoimmunity induction.

Therefore, there is a strong need to increase the specificity of CTL towards tumor cells. One approach to increase tumor-specificity utilizes CTL clones equipped with an NK cell inhibitory receptor, taking advantage of the usually low expression of MHC class I on tumor cells.⁵⁸ These CTL clones would not be able to lyse cells that do express MHC class I molecules on their surface, which is the case for most normal tissues.

Another major limitation to generate tumor-specific T cells against self-peptides for use in adoptive immunotherapy is that self-peptide specific CTL undergo negative selection and peripheral tolerance, which leads to elimination of these CTL. Since the tolerance to self-antigens is self-MHC restricted, one could generate allorestricted CTL against the peptide of interest. Those peptides would

be self-peptides, but bound by non-self MHC molecules on allorestricted CTL, thereby avoiding the MHC-restricted tolerance to self-antigens.^{64,65}

Put together, the over-expression of DNMT-1 in various tumors and the finding of a novel DNMT-1-associated immunogenic peptide that can elicit an immune response against tumor cells suggest that this epitope may be suitable for T cell-mediated immunotherapy.

Part II

Regulation of CD8⁺ T Cell Activation by CTLA-4

CHAPTER 6

INTRODUCTION

6.1 T Cell Tolerance

6.1.1 T Cell Tolerance in Tumor Immunity

T cell tolerance plays a major role in tumor immunity. Even though initial activation of tumor antigen-specific T cells is generally observed in tumor-bearing hosts, the activated state of T cells is typically not sustained, leading to failure of tumor elimination. Whether through direct presentation or cross-presentation, as described previously (Figure 1.1), the most common consequence of antigen recognition appears to be tolerance induction. Non-deletional tolerance mechanisms such as inadequate activation, leading to anergy, or active suppression, e.g. through regulatory cells, make T cells unable to respond to the antigens their T cell receptor recognizes and are involved in the above described tumor escape mechanisms (Figure 2.2). Deletional tolerance, the elimination of antigen-reactive cells either in the thymus or by peripheral mechanisms is known for self-antigen-specific T cells to prevent autoimmunity, but represents an obstacle to generating T cell responses to tumor-associated antigens, since most tumor antigens are non-mutated self-proteins that are also expressed in normal tissues.

Hence, the finding in most clinical trials using tumor-antigen-based vaccines is that vaccine-induced responses in patients with established cancer is relatively weak. However, many vaccine trials report the occasional dramatic clinical response even in patients with advanced metastatic disease. These patients represent examples in which immune surveillance and immune tolerance are almost equally balanced against each other. In this case, vaccination can effectively tip the balance toward immune activation, which is the aim of cancer immunotherapy. Based on the critical role of costimulatory signals expressed by APCs in

determining the outcome of T cell-dependent immune responses, the manipulation of inhibitory signals for costimulatory pathways is a promising attempt to enhance the cancer vaccine activity. It has been demonstrated for example that transient *in vivo* blockade of the inhibitory coreceptor cytotoxic T lymphocyte antigen-4 (CTLA-4) with a blocking antibody administered at the time of tumor vaccination can enhance vaccine potency and subsequent anti-tumor immunity.⁶⁶

Thus, understanding mechanisms of T cell tolerance, in particular the role of costimulatory molecules such as CTLA-4 and CD28, is crucial for the improvement of cancer vaccines and the induction of tumor immunity.

6.1.2 T Cell Tolerance in Transplantation

In contrast to tumor immunotherapy, the primary goal in transplantation is the induction of immune tolerance to prevent graft rejection, which is the major limitation in the success of transplantation. Rejection of allografts was first observed by Peter Medawar in 1944, trying to replace skin on burn patients with skin from unrelated donors. This approach was unsuccessful and showed the rejection of an allograft, which is caused by the adaptive immune response. Alloantigens presented by the allograft elicit both cell-mediated and humoral immune responses. However, MHC molecules are responsible for almost all immediate rejection reactions. The alloantigen is presented on the MHC of either donor-derived APC (direct presentation) or host APC (indirect presentation), leading to activation of alloreactive T cells and destruction of the graft by CTL in case of acute rejection.

In order to minimize graft immunity, MHC matching of donor and host is desired but not achievable in many cases. Thus, it is necessary to treat transplant patients with immunosuppressive agents, which unfortunately increases the risk of infections and the susceptibility to virus-induced tumors. Therefore there is a strong need for the development of strategies to induce graft specific T cell tolerance with minimal use of pharmacological immunosuppressive agents, e.g. through manipulation of costimulatory molecules.

Hence, the second part of this study addresses how CTLA-4 exerts a negative

effect on CD8⁺ T cell activation. The understanding of CTLA-4's inhibitory mechanisms might help to develop strategies to overcome T cell tolerance towards tumors, or to induce tolerance to prevent graft rejection or autoimmunity.

6.2 T Cell Anergy

Optimal stimulation of T cells is thought to involve at least two signaling events. Signal 1 originates from the ligation of the T cell receptor (TcR) and its coreceptors (e.g. CD8) with the peptide-MHC complexes. The second signal is dependent on the ligation of T cell surface molecules that provide the essential costimulatory signals complimentary to the TcR engagement.⁶⁷ The costimulatory signal is necessary for effective lymphocyte activation. A major costimulatory pathway involves the B7 and CD28 families of costimulators and receptors. CD28 interacts with costimulatory ligands B7.1 (CD80) and B7.2 (CD86) that are expressed on APCs. This interaction is essential for initiating antigen-specific T cell responses, upregulating cytokine expression and promoting T cell differentiation and expansion.⁶⁸

T cells which receive only a signal through the TcR in the absence of CD28 costimulation enter a hyporesponsive state, termed anergy, and become unresponsive to antigen re-challenge,⁶⁹ as illustrated in Figure 6.15. The term "unresponsiveness" was first established by Bretscher and Cohn in 1970 to describe the immunological state of an animal to which antigen has been administered and which could not subsequently respond to that antigen but to other noncross-reacting foreign antigens. This state of unresponsiveness is generally called T cell anergy and is the *in vitro* counterpart of *in vivo* tolerance. Anergy is defined by the inability of T cells to produce interleukin-2 (IL-2) and to proliferate following T cell receptor stimulation. T cells that are rendered anergic are incapable of responding to antigen even if the antigen is presented by costimulator-expressing APCs.

Besides the lack of costimulation, there is some evidence that anergy might also be induced by the inhibitory signal of CTLA-4. CTLA-4 is expressed on activated T cells and binds to the same ligands as CD28 (B7.1 and B7.2), as

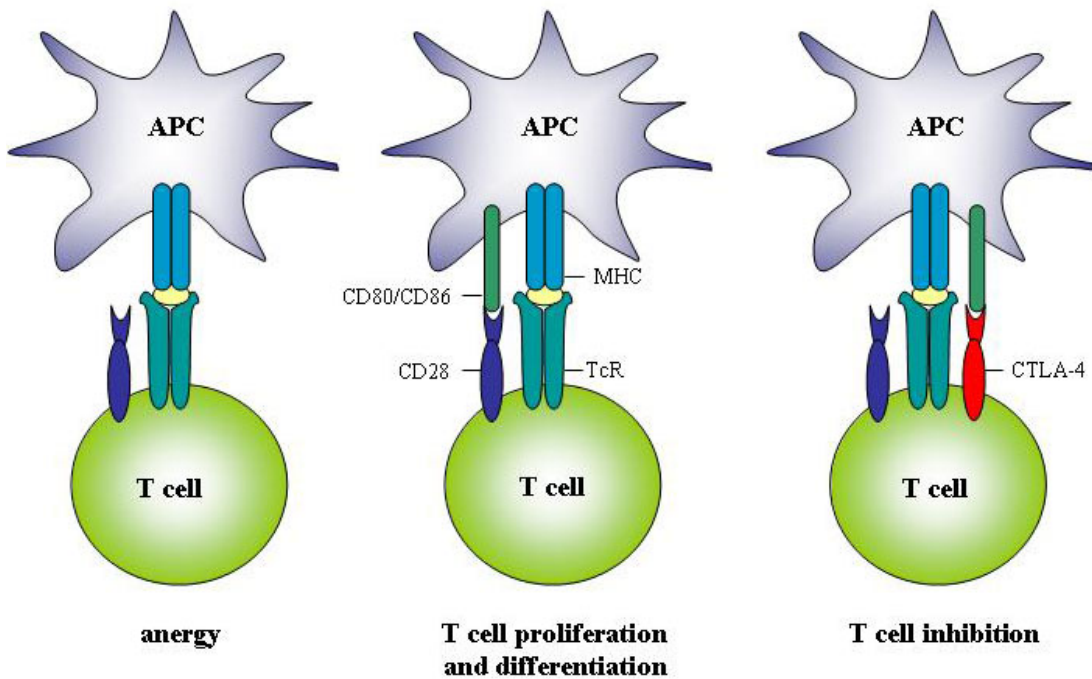


Figure 6.15: **T cell anergy.** T cell responses are induced when the T cell recognizes an antigen presented by the MHC on a costimulator-expressing antigen-presenting cell (APC). If the T cell receives both signals, the signal via the TcR and the signal via CD28 it becomes activated and can proliferate and differentiate (a). If the T cell recognizes the antigen without costimulation via CD28 the T cell fails to respond and becomes unresponsive to antigen, a state called T cells anergy (b). T cells that receive a signal via the TcR and a second signal via CTLA-4, which has the same ligands as CD28 (CD80 and CD86), might also become inhibited or anergic (c).

illustrated in Figure 6.15.

The administration of anti-CTLA-4 antibodies during anergy induction appears to block the induction of antigen-specific tolerance in several *in vivo* systems.^{70,71} However, anergy is not blocked by anti-CTLA-4 antibodies in other *in vivo* systems, despite close parallels to those in which CTLA-4 appears critical.⁷² Thus, there is a significant body of conflicting data regarding the requirements of CTLA-4 for anergy and tolerance induction. There is evidence that CTLA-4 is indeed required for the induction of acquired tolerance in CD4⁺ T cells, which has been shown using an *in vivo* TcR transgenic mouse model where CD4⁺ T cells

lack CTLA-4. These CTLA-4^{-/-} T cells are resistant to tolerance induction,⁷³ suggesting a central role for CTLA-4 for the induction of tolerance in this model. However, CTLA-4-deficient CD8⁺ T cells can be anergized in vitro⁷⁴ and CTLA-4 does not seem to be required for induction CD8⁺ T cell anergy in vivo.⁷⁵ These apparent differences have led to the suggestion that the mechanisms of anergy induction differ among model systems as well as between T cell subsets, such as CD4⁺ and CD8⁺ T cells.

6.3 Roles of CTLA-4 and CD28

As illustrated above, besides the ligation of the TcR, the outcome of T cell stimulation is dependent on the expression of a variety of coreceptors. One major receptor-ligand system involves CD28, CTLA-4 and their common ligands CD80 and CD86. CD28 and CTLA-4 are highly homologous molecules and bind the same ligands on APCs, although with different binding affinities. Even though both costimulatory molecules share the same ligands, they have opposing effects on the outcome of T cell responses,⁷⁶ with CD28 being a stimulatory and CTLA-4 being an inhibitory receptor. The dramatic inhibitory function of CTLA-4 became evident in CTLA-4^{-/-} mice, which exhibit severe lymphoproliferative disease.^{77,78} By contrast, CD28-deficient mice exhibit impaired T cell responses and reduced responses to infectious antigens as well as alloantigens.^{79,80,81} Both receptors also greatly differ in their expression on T cells. CD28 is constitutively expressed on most T cells whereas CTLA-4 is an activation-induced coreceptor.⁸²

The mechanism of activation through CD28 has been well established and involves upregulation of IL-2 production, progression through the cell cycle, prevention of anergy and inhibition of TcR-induced apoptosis.^{68,83} In addition, CD28 signaling appears to be important in prevention of cell death and promotion of cell survival.⁸⁴ In contrast, although the inhibitory role of CTLA-4 is generally accepted, the exact mechanism of T cell inhibition by CTLA-4 remains elusive.

Thus far, it is known that CTLA-4 can inhibit T cell responses by two different mechanisms. One is the antagonism of B7-CD28 mediated costimulatory

signals by CTLA-4,⁸⁵ due to a much higher affinity of CTLA-4 for B7.^{86,87} A second mechanism, by which CTLA-4 can inactivate T cells in the absence of CD28, involves the delivery of a negative signal by interfering with TcR-derived signals,^{88,89,90,91} possibly leading to a block of IL-2 transcription and prevention of cell cycle entry of T cells into the growth phase beyond G1/G0, thus leading to inhibition of T cell proliferation.^{76,92} The downregulation of the T cell response might favor the onset of antigen-specific T cell tolerance.^{67,92,86}

These observations indicate important roles of CD28 and CTLA-4 in immune regulation of T cells.

6.4 TcR Proximal Signaling

Engagement of the TcR leads to a series of signal transduction events that are critical for the activation of T cells. The TcR itself lacks intrinsic enzymatic activity and initiates signal transduction by recruiting and activating multiple cytoplasmic protein tyrosine kinases (PTK).⁹³ One of the earliest effects observed upon TcR engagement is the activation of members of the Src tyrosine kinase family, including both the tyrosine kinase p59^{fyn} (fyn) and the CD8/CD4 associated tyrosine kinase p56^{lck} (lck), as illustrated in Figure 6.16. The activation of lck and fyn is crucial for the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM).⁹⁴ ITAMs are found in the CD3 chains of the TcR/CD3 complex and are binding sites for SH2 domain-containing proteins.

One of the main proteins recruited to the ITAMs of the CD3 ζ chains is tyrosine kinase ZAP-70 (ζ -associated protein of 70 kD). After binding to one CD3 ITAM, ZAP-70 becomes activated after phosphorylation by the Src kinases. This leads to further recruitment and phosphorylation of adaptor proteins, such as the linker of activation of T cells (LAT). These adaptor proteins in turn become docking sites for enzymes such as phospholipase C γ 1 (PLC γ 1) and phosphatidylinositol-3-kinase (PI3K).

Phosphorylated PLC γ 1 catalyzes the hydrolysis of plasma membrane phospholipid PIP₂ (phosphatidylinositol 4,5-bisphosphate), generating the two products

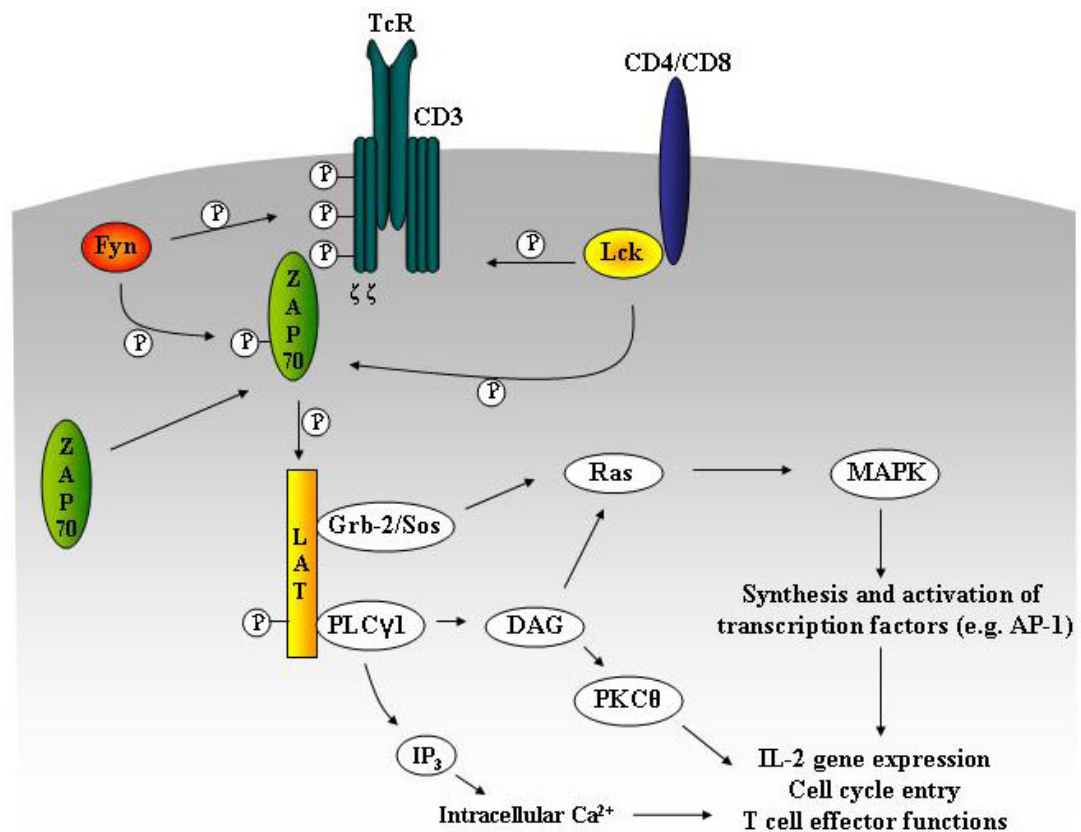


Figure 6.16: **TcR proximal signaling complexes.** Phosphorylation of the ITAMs of the CD3 ζ -chains by lck leads to recruitment of tyrosine kinase ZAP-70, which can in turn recruit other adaptor molecules such as LAT. The phosphorylation of LAT induces a variety of signal cascades and activation of enzymes such as PLC γ 1 and PI3K that ultimately lead to transcription factor activation and protein synthesis of IL-2.

IP₃ (inositol 1,4,5-triphosphate) and DAG (diacylglycerol). IP₃ causes rapid increase in cytosolic free calcium and DAG activates the enzyme protein kinase C (PKC). In addition, the phosphorylation of LAT induces MAP kinase signaling pathways.

All processes ultimately lead to the activation of transcription factors such as AP-1, NFAT (nuclear factor of activated T cells) and NF- κ B (nuclear factor κ B). These transcription factors function coordinately to regulate gene expression and are necessary for the transcription of the IL-2 gene.

6.4.1 T Cell Signaling in Anergy

Many groups have investigated the biochemical events leading to the state of anergy. It has been shown that anergic CD4⁺ T cells are unable to activate Ick and ZAP-70, to phosphorylate LAT and various kinases of the Ras/ MAP kinase pathways.^{95,96} These impairments lead to failure of induction of necessary transcription factors that are needed for transcription of the IL-2 gene. Our group reported previously that CD8⁺ T cells exhibit similar, but not identical, biochemical changes after anergy induction. Anergic cytotoxic T cells exhibited impaired tyrosine-kinase phosphorylation as well as impaired ZAP-70 activation.⁹⁷

Whether CTLA-4 affects the T cell signaling machinery has been investigated by a variety of groups, however, leading to controversial results. CTLA-4 can inactivate T cells in the absence of CD28, leading to the conclusion that it can actively deliver a negative signal by interfering with TcR-derived signals.^{88,89,90,91} One possibility for its negative signaling is that CTLA-4 facilitates the dephosphorylation of the TcR signaling machinery by recruiting phosphatases SHP-2 and PP2A to its cytoplasmic tail, thereby blocking T cell activation and expansion.^{89,90,98} In contrast to the studies where phosphorylated tyrosine residues in the cytoplasmic tail of CTLA-4 are required for recruitment of signaling molecules such as PP2A and SHP-2, there is some evidence that tyrosine phosphorylation of CTLA-4 is not essential for its function.^{90,91,99,100} Thus, the exact role of CTLA-4 in the induction of T cell anergy remains elusive.

6.5 Structure and Expression of CD28 and CTLA-4

CD28 as well as CTLA-4 exist as disulfide-linked homodimeric glycoproteins with high structural homology.⁶⁷ Both receptors are transmembrane proteins that include a immunoglobulin-like domain, which is essential for their binding to CD80 and CD86.¹⁰¹ Whereas CD28 interacts with a single binding domain, each CTLA-4 dimer binds two independent bivalent B7 molecules, possibly explaining the 50-fold higher binding avidity of CTLA-4.¹⁰² The relatively short cytoplasmic tails of CD28 and CTLA-4 include tyrosine residues that are important for the

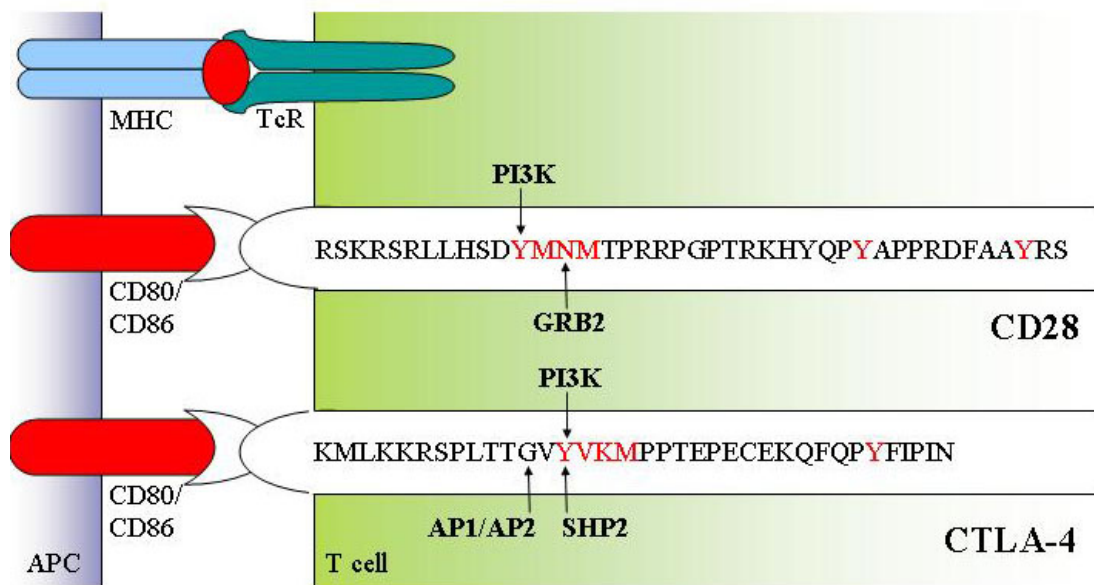


Figure 6.17: **Cytoplasmic tails of CD28 and CTLA-4.** The cytoplasmic tails of CD28 and CTLA-4 include tyrosine residues (Y) that are important for the recruitment of molecules that lead to signal transduction after receptor ligation. Common to both is a tyrosine motif that functions as binding site for lipid kinase PI3K. Besides common residues, CD28 has a binding site for GRB2, causing the recruitment of GTPase RAS. CTLA-4 on the other hand has a unique site for binding of the clathrin-adaptor protein complexes AP-1 and AP-2 and tyrosine phosphatase SHP-2.

recruitment of molecules that lead to signal transduction after receptor ligation, as illustrated in Figure 6.17.

Common to both is a tyrosine motif that functions as binding site for the SH2 domains of the adaptor subunit p85 of the lipid kinase PI3K.^{103,99} Besides common residues, CD28 has an asparagine residue, which is a binding site for GRB2 (growth-factor receptor-bound protein 2), causing the recruitment of GTPase RAS.¹⁰⁴ CTLA-4 on the other hand has a unique binding site for the clathrin-adaptor protein complexes AP-1 and AP-2, which are required for the rapid endocytosis of CTLA-4,¹⁰⁵ as well as for tyrosine phosphatase SHP-2, which might play an important role in the inhibitory function of CTLA-4 (Figure 6.17).

CD28 and CTLA-4 also significantly differ in their expression on T cells.

CD28 is expressed constitutively on almost all human CD4⁺ T cells and on approximately 50% of CD8⁺ T cells.^{106,107} However, it has been reported that CD28 surface expression is also not steady and is influenced by several mechanisms, including TcR-mediated activation,¹⁰⁸ in vitro replicative senescence,¹⁰⁹ and B7.1 and B7.2 engagement.¹¹⁰ By contrast, CTLA-4 is not constitutively expressed and its expression is tightly regulated on T cells. It is upregulated following T cell activation and its surface expression peaks between 24 and 48 h after activation.¹¹¹

Although an inhibitory function for CTLA-4 is generally accepted, views of its mechanism of action and its biological role in regulating immune responses remain controversial. In addition, most of the data regarding T cell proximal signaling and the role of CTLA-4 are obtained in CD4⁺ T cell models. As described above, there are significant differences in the requirements for tolerance induction as well as the biochemical changes after anergy induction between CD4⁺ and CD8⁺ T cells. There is little data regarding T cell signaling and the inhibitory effects of CTLA-4 in CD8⁺ T cells.

Thus, in the second part of this study, we analyzed the inhibitory function of CTLA-4 and its effect on the expression of CD28 in CD8⁺ T cells. The understanding of stimulation and inhibition via coreceptors on T cells is important for the development of strategies to overcome or to induce tolerance in tumor immunotherapy or transplantation, respectively.

6.6 Specific Aims

1. To establish a model to investigate the inhibitory function of CTLA-4 in CD8⁺ T cells.
2. To analyze whether the expression of CTLA-4 in CD8⁺ T cells leads to development of T cell anergy.
3. To investigate the effects of CTLA-4 upregulation on the expression of co-stimulatory molecule CD28.

CHAPTER 7

MATERIALS AND METHODS

7.1 Cell lines and culture conditions

The Jurkat CD8 $\alpha\alpha$ cell line, that will be referred to hereafter as WT (kindly provided by Dr. Thorsten Witte, Division of Clinical Immunology, University of Hannover, Germany) was maintained at 37°C in a 5% CO₂ incubator in RPMI-1640 plus 25 mM HEPES (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml streptomycin/penicillin and 0.2 mg/ml hygromycin B (Gibco).

The OKT-3 hybridoma cell line was maintained in Iscove's modified Dulbecco's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 20% FBS. Cultures were maintained between 1-10 \times 10⁵ cells/ml and the medium renewed every 2 to 3 days.

7.2 Generation of CTLA-4 and HLA-B7-transfectants

The cDNA of human CTLA-4 was obtained from human PHA-blasts and kindly provided by Dr. Judith Welke (University of Iowa, IA, USA). Human CTLA-4 cDNA was ligated into the pcDNA3.1⁺ expression vector (Invitrogen Life technologies), which contains the neomycin-resistance gene for selection of stable cell lines, using standard recombinant DNA techniques.

Jurkat CD8 $\alpha\alpha$ cells were transfected using LipofectAMINE TM 2000 (Gibco). 5 \times 10⁵ Jurkat CD8 $\alpha\alpha$ cells/well were plated in a 24-well-plate containing 500 μ l medium/well without antibiotics. For 1 well, the following amounts of DNA and Lipofectamine were used: 1 μ g hCTLA-4 pcDNA3.1 and 3 μ l Lipofectamine were diluted in 50 μ l medium each (RPMI 1640 without FCS) and mixed gently. After 5 minutes of incubation, diluted DNA was combined with the diluted Lipofectamine for 20 minutes to build the DNA/Lipofectamine complex. 100 μ l of complex were then added to each well (containing cells and medium) and gently mixed. The cells were subsequently incubated for 24 hours.

Positive clones harboring the neomycin-resistance gene were selected in the presence of 1.5 mg/ml G418 (Genitacin disulfate). Stable transfectants were maintained in medium containing 0.5 mg/ml G418 and cultured under the same conditions as WT cells. CTLA-4 expression in transfectants was monitored after 24 h stimulation on anti-CD3 coated plates by flow cytometry.

As control for unspecific transfection effects, Jurkat CD8 α cells were also transfected with human leukocyte antigen HLA-B7. The cDNA of the HLA-B7 was kindly provided by Dr. Shukla (Department of Genetics, Yale University, New Haven, CT) and ligated into the pcDNA3.1 expression vector (Invitrogen Life technologies). Cells were transfected as described above and cultured in G418 selection medium. HLA-B7^{high} transfectants were selected and purified by cell sorting.

7.3 Cell sorting

Flow cytometry can be used to separate distinct subpopulations of cells on the basis of quantitative difference in expression of ligands detectable with labeled probes. Labeled cells are separated by electrostatic deflection and can be cultured further after separation. This technique was utilized for purification of HLA-B7^{high} transfectants.

One weeks after transfection of Jurkat cells with HLA-B7, cells were stained with monoclonal antibody ME.1-FITC (anti-HLA-B7) and sorted for HLA-B7^{high} using a FACS DiVa (Becton Dickinson). Sorted cells were subsequently cultured in RPMI-medium containing 0.5 mg/ml G418.

7.4 OKT-3 purification

Supernatant of OKT-3 producing hybridoma cell line was collected weekly and filtered (0.45 μ m) before monoclonal antibody purification by affinity chromatography. A HiTrap Protein G HP 1 ml column (Amersham) was used for IgG purification. Protein G is a bacterial cell wall protein which is isolated from group G streptococci. It is a type III bacterial IgG Fc receptor that binds to the Fc

region of IgG by a non-immune mechanism.

Columns were packed with 1 ml Protein G Sepharose. The sample was adjusted to the composition of the binding buffer (20 mM sodium phosphate, pH 7.0). The elution buffer contained 0.1 M glycine-HCl, adjusted to a pH of 2.7. Binding buffer was applied to the column and the sample applied subsequently. After washing of the column with 10 column volumes of binding buffer the IgG was eluted with two column volumes elution buffer. The purified fractions were tested by Western blotting and protein concentration measured by spectrophotometry.

7.5 Antibodies

The following antibodies were used for stimulation of Jurkat cells: the anti-CD3 monoclonal antibody was purified from the supernatant of hybridoma cell line OKT3 (ATCC CRL 8001) as described above, whereas the anti-CD28 mAb (CD28.2, BD Pharmingen) and anti-mouse-IgG (Santa Cruz Biotechnology) were purchased. Anti-CTLA-4-PE, anti-CD28-FITC/ PE, anti-CD69-FITC, anti-CD45-FITC and anti-CD25-FITC (all purchased from BD Pharmingen) were used for flow cytometric analysis, internalization assays and confocal microscopy. Monoclonal mouse anti-CD28 from BD (CD28.2) was also used for immunoprecipitation of CD28. Staining of western blots was performed with anti-phosphotyrosine mAb (PY99), anti-ZAP-70 mAb, rabbit-anti-CD28 (H-93), rabbit-anti-cbl (C-15) and the appropriate secondary antibody: HRP-conjugated anti-mouse-IgG or anti-rabbit-IgG, obtained from Santa Cruz Biotechnologies. Monoclonal mouse-anti-ubiquitin antibody (P4D1) was purchased from Covance Inc.

7.6 Stimulation via OKT-3

In order to mimic TcR stimulation, the anti-CD3 antibody has to be either cross-linked by a secondary antibody or immobilized on a plate. Here, we immobilized the antibody on 6-, 12-, or 24-well culture plates by coating the plates with 10 $\mu\text{g}/\text{ml}$ purified OKT-3 in PBS. Plates were incubated over night at 4°C and subsequently washed with PBS. Jurkat cells were plated at a density of $2\text{-}4 \times 10^5$

cells/ml for 24 hours in OKT-3-coated plates. Cells were washed with RPMI after stimulation and used for the appropriate experiments.

7.7 Determination of IL-2 production using an ELISA

WT cells and CTLA-4 mutants were stimulated for 24 h on a anti-CD3 (OKT-3)-coated plate as described above. Subsequently, cells were cultured for restimulation on anti-CD3-coated 96-well plates in a density of 2×10^5 cells per well in 200 μ l RPMI culture medium. Supernatants were harvested after 48 hours and analyzed using a commercially available human IL-2 ELISA Kit (Biosource).

96-well flat bottom plates were coated with 100 μ l/well coating antibody at 2 μ g/mL for 18 h at 2-8 °C, aspirated and then blocked for 2 h at room temperature by adding 300 μ l blocking solution to each well. After 3 washes, diluted standards or samples were added at 100 μ l/well followed immediately by addition of 50 μ l of biotinylated antibody and incubated for 3 h at room temperature with continual shaking (700 rpm). After 3 washes, 100 μ l/well of diluted Streptavidin-HRP (1:2500) was added and incubated for 30 minutes at room temperature with continual shaking. After additional washes, 100 μ l/well of the Chromogen TMB (tetramethylbenzidine) was added and incubated for 30 min at room temperature. Then, 100 μ l/well stop solution was added. The optical density of samples and standards was measured at 450 nm (reference filter 650 nm) on a Benchmark Microplate Reader (BioRad). IL-2 concentrations of samples were calculated based on the established IL-2 standard curve.

7.8 Preparation of cell lysates

For preparation of cell lysates $1-5 \times 10^6$ cells were used and treated as described in section 3.5. Briefly, cells were washed in PBS and cell pellets resuspended in 500 μ l lysis buffer. After 30 min incubation at 4°C, cell lysates were spun at 10000 rpm and the supernatant harvested. Protein concentration of cell lysates was measured using a colometric standard protein assay (DC Protein Assay, BioRad). A protein standard curve was established using BSA as standard

and absorbance measured at 655 nm. Cell lysates were analyzed by SDS-PAGE and Western blotting.

7.9 SDS polyacrylamide gel electrophoresis and Western blotting

SDS-PAGE and western blotting were performed as described under section 3.6 and 3.7. Briefly, equal protein amounts of each lysate were denatured in reducing sample buffer for 3 min at 100°C. Denatured samples were electrophoresed on SDS-polyacrylamide gels.

Separated proteins were transferred from gels to nitrocellulose membranes (Amersham) for 2-5 h at constant current (0.2-0.8 A). The transfer membrane was blocked with 5% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS for 1 h at RT. Membranes were incubated with the primary antibody of choice for 1 h to overnight at 4°C. The membranes were subsequently washed three times with PBS-T, incubated with an appropriate peroxidase-conjugated secondary antibody (Amersham) and developed by using ECL western blot detection reagents (Amersham).

For restaining of the same membrane, bound antibodies were removed by incubating the membrane for 30 min at 56°C in stripping solution (2% SDS, 1 M Tris HCl pH 6.8 and 100 mM 2-ME) under continuous shaking. After three washes with PBS-T the staining procedure was repeated with the antibody of choice.

7.10 Cytoplasmic tyrosine kinase phosphorylation assay

Anti-phosphotyrosine blotting is used for detection of tyrosine-phosphorylated substrates by the use of anti-phosphotyrosine monoclonal antibodies that recognize a variety of proteins. Here, it is used to detect the tyrosine-phosphorylation of CD3 ζ -chains and tyrosine kinases ZAP-70, fyn and lck in resting and stimulated WT and CTLA-4 transfectants. Here, 5×10^5 Jurkat cells (WT and JM8A-4 cells) were activated for 24 h on plate-bound OKT-3 to induce the expression of CTLA-4. Cells were washed three times with PBS to remove FCS and subsequently restimulated for 2 min at 37°C in RPMI (without FCS) alone or supplemented

with 10 $\mu\text{g/ml}$ anti-CD3, 5 $\mu\text{g/ml}$ anti-CD28 and 2 $\mu\text{g/ml}$ anti-mouse IgG (Santa Cruz Biotechnology) to provide adequate costimulation, thus inducing tyrosine phosphorylation of TcR proximal tyrosine kinases. In order to avoid dephosphorylation and proteolysis of proteins, cells were immediately transferred into lysis buffer containing phosphatase and protease inhibitors and put on ice. Cells were incubated with lysis buffer for 30 min on ice and spun at 10000 rpm. The cell lysate (supernatant) was harvested and resolved by SDS-PAGE as described above. Phosphorylated proteins were analyzed by western blotting using the anti-phosphotyrosine (py99) antibody. The same membrane was reprobred with an anti-ZAP-70 antibody to assure equal protein loading.

7.11 Immunoprecipitation of CD28

Immunoprecipitation is used to isolate an antigen by binding to a specific antibody which is attached to a sedimentable matrix. To analyze the protein expression of CD28 by immunoprecipitation, we used monoclonal mouse anti-human CD28 IgG and as matrix protein-G sepharose.

5×10^6 WT cells and CTLA-4 mutants were stimulated over night with anti-CD3. For proteasome inhibition assays, cells were treated for 16 h or 12 h during stimulation with 2 μM lactacystin (Calbiochem) or 10 μM MG-132 (Calbiochem), respectively. Both reagents are inhibitors of the 26S proteasome.

Cells were subsequently lysed as described above and lysates used for CD28 immunoprecipitation. Lysates, containing 0.5 mg of protein, were first subjected to preclearing with protein G-Sepharose (Amersham), to remove proteins from the lysate that bind to protein G sepharose. After preclearing, the lysate was incubated with anti-CD28 mAb (28.2) for 2 h at 4°C (rotating). The immunocomplex was subsequently precipitated with Protein G Sepharose beads for 2 h. The precipitate was washed 4 times in lysis buffer and proteins were detached and denatured in reducing sample buffer. Electrophoresis and western blotting were performed as above. The membranes were incubated with antibodies against CD28 (H-93), cbl (C-15) or ubiquitin, and subsequently visualized with an anti-rabbit or anti-mouse

HRP-conjugated secondary antibody and ECL detection reagent (Amersham).

7.12 Flow cytometry

For flow cytometry, 5×10^5 Jurkat cells were incubated for 30 minutes at 4°C with one of the following antibodies: PE-labeled anti-CTLA-4, FITC-labeled anti-CD45, anti-CD69, anti-CD25, anti-CD28 or FITC-conjugated ME.1 antibody. Cells were subsequently washed three times with PBS and surface receptor expression analyzed with a FACScan (Becton Dickinson). Data was analyzed using the WinMDI software.

7.13 Analysis of CD28 internalization by flow cytometry and confocal microscopy

To analyze the internalization of CD28 in WT cells and CTLA-4 transfectants, flow cytometry and confocal microscopy were used. Both cell lines were stimulated with the anti-CD3 antibody for 24 h or left untreated. The cells were subsequently incubated with 2 $\mu\text{g}/\text{ml}$ of a FITC-conjugated anti-CD28 mAb or 1 $\mu\text{g}/\text{ml}$ of a PE-conjugated anti-CTLA-4 mAb at 37°C for 2 h, leading to internalization of the receptor-antibody-complex as well as to surface staining of the receptor. After incubation, cells were washed once with ice cold PBS, and divided into two aliquots. One aliquot was left untreated on ice, while the other was incubated on ice for 1 min in 0.2 M acetic acid solution (0.5 M NaCl solution adjusted to pH 2.5, supplemented with 10% FCS). This procedure removes cell surface-bound antibodies and therefore allows for monitoring of the internalized antibody. Samples were additionally treated during the staining procedure with 0.45 M sucrose medium to block clathrin-dependent endocytosis, as indicated. Samples were then washed in a large excess of RPMI 1640 medium supplemented with 10% FCS and 100 mM HEPES buffer and analyzed by flow cytometry. Confocal microscopy was performed after cytospin preparation of WT or CTLA-4 mutants that were stained with anti-CD28-FITC for 30 min at 4°C or for 2 h with anti-CD28-PE at 37°C, respectively. Cells were either left untreated or acid-stripped as described

above. After staining, cells were washed two times with PBS and 100 μ l cell-suspension containing 10^5 cells was cytopun. Samples were examined under a Bio-Rad 1024 confocal microscope using a 60 \times objective lens.

7.14 Analysis of CD28 RNA expression by RT-PCR

Both cell types, WT and CTLA-4 transfectants, were analyzed for CD28 RNA expression in resting and stimulated conditions using RT-PCR. 5×10^6 stimulated or resting Jurkat WT and JM8A-4 cells were lysed and RNA extracted using the Qiagen RNA extraction kit (Qiagen, Valencia, CA, USA). RNA was treated with recombinant DNase I to remove contaminating DNA from RNA preparation.

The primers for the PCR reaction were purchased from Integrated DNA Technologies, Inc. (IDT, Coralville, IA, USA) and their sequences were as follows:

- hCD28 5' primer: 5' TCAAGTAACAGGAAACAAGATT 3'
- hCD28 3' primer: 5' ACTCCTCACCCAGAAAATAATA 3'
- GAPDH 5' primer: 5' TGAAGGTCTGGAGTCAACGGATTTGGT 3'
- GAPDH 3' primer: 5' CATGTGGGCCATGAGGTCCACCAC 3'

RT-PCR was performed according to standard procedures using the following conditions: 0.3 μ M GAPDH primer, 0.6 μ M hCD28 primer, 500 ng RNA, annealing temperature: 55 $^{\circ}$ C. Temperature protocol: 5 to 3 loop , 25 cycles

1. reverse transcriptase phase: 30 minutes at 50 $^{\circ}$ C
2. inactivation of reverse transcriptase: 15 minutes at 95 $^{\circ}$ C
3. denaturation: 30 seconds at 94 $^{\circ}$ C
4. annealing: 30 seconds at 55 $^{\circ}$ C
5. polymerization: 60 seconds at 72 $^{\circ}$ C
6. polymerization: 10 minutes at 72 $^{\circ}$ C

7. amplification ending: 4 °C

A 'no-template'-control, lacking the RNA, as well as a control for genomic DNA are included (lane 5 and 6). We controlled for genomic DNA contamination by adding the RNA sample after step 2 (after inactivation of the reverse transcriptase).

Samples were run on 2% agarose gels. For the gels, 2 g agarose was suspended in 100 ml TAE buffer and boiled until it dissolved. 5 μ l ethidium bromide (10 mg/ml) was added and the gel poured into the gel chamber. Samples were run under constant voltage (100 V) and visualized under UV-light.

7.15 In vitro anergy induction in primary T lymphocytes

To analyze the effects of CTLA-4 on CD28 expression in primary T cells, we anergized PBMC from healthy blood donors, as CTLA-4 is highly expressed on anergic T cells. PBMC were obtained from healthy volunteers and separated by density gradient centrifugation over Ficoll-Histopaque (Sigma-Aldrich). Briefly, the cell suspension was diluted with 2-4 volumes of PBS. 7 ml diluted cell suspension was carefully layered over 3 ml Ficoll-Histopaque in a 15 ml conical tube and centrifuged without brake at 2400 rpm for 20 minutes at RT. The interphase, containing lymphocytes, was carefully transferred into a new tube and washed twice with PBS. The obtained PBL were used for anergy induction by multiple superantigen stimulation. PBL were stimulated with 5 μ g/ml SEB (staphylococcal enterotoxin B) for 3 days in RPMI 1640. Cells were harvested, resuspended in fresh medium and three times restimulated. For restimulation, irradiated (50 Gray) PBMC from the same blood donor were used as antigen presenting cells in addition to 5 μ g/ml SEB. Cells were cultured for three days between restimulations.

After multiple stimulations, cells were harvested, and rechallenged with SEB alone or left untreated (resting) for 3 days. Surface expression of CD28 and CTLA-4 was assessed by flow cytometry.

CHAPTER 8

RESULTS

8.1 CTLA-4 transfectants highly express CTLA-4 after stimulation

In order to develop a model to analyze the inhibitory effects of CTLA-4 in CD8⁺ T cells, we utilized a Jurkat T lymphoma cell line, which was previously transfected with T cell coreceptor CD8. Jurkat CD8 $\alpha\alpha$ cells (referred to hereafter as WT) constitutively express CD8 on their cell surface, as shown by flow cytometry in Figure 8.18.

WT cells were analyzed for surface expression of CTLA-4 by flow cytometry in resting conditions and after 24 hours of activation via CD3. WT cells do not express CTLA-4 in resting conditions and its surface expression is not inducible

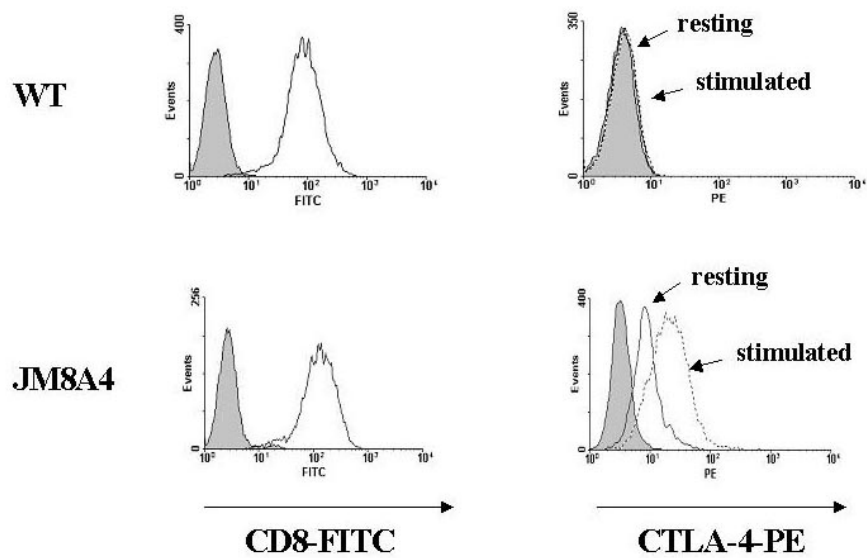


Figure 8.18: **Activated CTLA-4 mutants highly express CTLA-4.** Wild type Jurkat CD8 $\alpha\alpha$ cells (WT) were transfected with human CTLA-4 cDNA (JM8A-4). Both cell lines, WT and JM8A-4, constitutively express CD8. CTLA-4 surface expression was assessed on WT cells and CTLA-4 mutants in resting conditions and after 24-hour stimulation with 10 $\mu\text{g}/\text{ml}$ immobilized anti-CD3 (OKT-3). CTLA-4 transfectants highly expressed CTLA-4 after activation. The full line represents resting and the broken line stimulated cells.

by T cell activation. Therefore, to analyze the inhibitory effects of CTLA-4 in these cells, we stably transfected WT cells with human CTLA-4 cDNA (referred to as JM8A-4). CTLA-4 was already detectable at low levels on the cell surface of resting JM8A-4 cells, but its surface expression was highly elevated after 24 hours of activation via CD3 ligation (Figure 8.18), as has been described for physiological CTLA-4 expression.¹¹¹ These CTLA-4 mutants also constitutively express CD8 on their cell surface, thus providing a model to study CTLA-4 in CD8⁺ T cells.

8.2 CTLA-4 impairs IL-2 production in CD8⁺ T cells

IL-2 is produced and secreted by activated T-cells and is the major interleukin responsible for clonal T-cell proliferation. The impairment of IL-2 production is the hallmark of T cell anergy, as described above. Anergy is induced by lack of costimulation of T cells, but might also be induced by the inhibitory signal of CTLA-4, which has been shown to be expressed on anergic T cells.⁹⁷

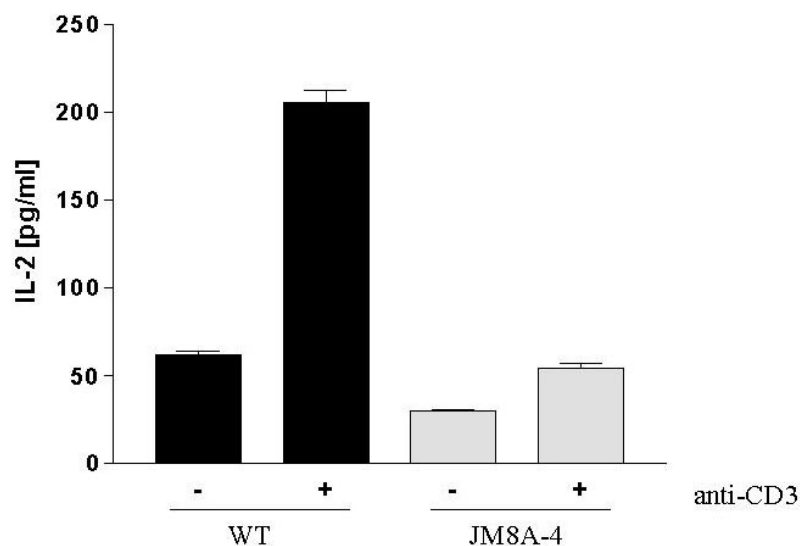


Figure 8.19: **CTLA-4 abrogates interleukin-2 production in CD8⁺ T cells.** WT and CTLA-4 transfected cells were stimulated with anti-CD3 for 24 hours. After restimulation for additional 48 hours with 10 $\mu\text{g}/\text{ml}$ immobilized anti-CD3 in 96-well plates, supernatants of triplicates were assessed for human interleukin-2 (IL-2) by ELISA.

Therefore, to determine whether CTLA-4 mutants develop an anergic phenotype, we quantitatively compared IL-2 production by WT cells versus that by CTLA-4 mutants after CD3 stimulation. If CTLA-4 induces T cell anergy in CD8⁺ T cells, we expect diminished IL-2 production in CTLA-4 transfectants in comparison to WT cells. Therefore, WT cells and CTLA-4 transfectants were stimulated for 24 hours on immobilized anti-CD3 antibody to induce CTLA-4 expression. IL-2 production was measured in the supernatant after 48 hours of restimulation. Figure 8.19 shows that indeed the ability of CTLA-4 mutants to secrete IL-2 is significantly reduced compared to WT cells. This finding indicates that signaling through the TcR, which ultimately leads to IL-2 production, might be impaired in CD8⁺ CTLA-4 transfectants, similar to what has been described previously for CD4⁺ T cells.¹¹²

8.3 CTLA-4 impairs the phosphorylation of TcR proximal tyrosine kinases

To investigate whether the signaling through the TcR is impaired in CTLA-4 transfectants we analyzed the phosphorylation state of TcR proximal tyrosine kinases, which are required in the TcR-mediated activation cascade. The tyrosine kinases analyzed here, ZAP-70, p59^{lyn} and p56^{lck}, are involved in early TcR-signaling, as illustrated in Figure 6.16.

Analysis of the phosphorylation state of these kinases and of the CD3 ζ chains in stimulated CTLA-4 mutants and WT cells will reveal whether early T-cell signaling is negatively influenced by CTLA-4. CTLA-4 mutants and WT cells were costimulated with an anti-CD3 and anti-CD28 antibody (cross-linked with anti-mouse-IgG) to induce tyrosine kinase phosphorylation. The phosphorylation of tyrosine kinases ZAP-70, p59^{lyn}, p56^{lck} and CD3 ζ chains was studied by western blotting, using an anti-phosphotyrosine monoclonal antibody (py99) for staining.

Indeed, in comparison to WT cells, phosphorylation of ZAP-70, p59^{lyn}, p56^{lck} and CD3 ζ chains was significantly reduced in CTLA-4 mutants after costimulation via CD3 and CD28, suggesting that CTLA-4 interfered with the activation

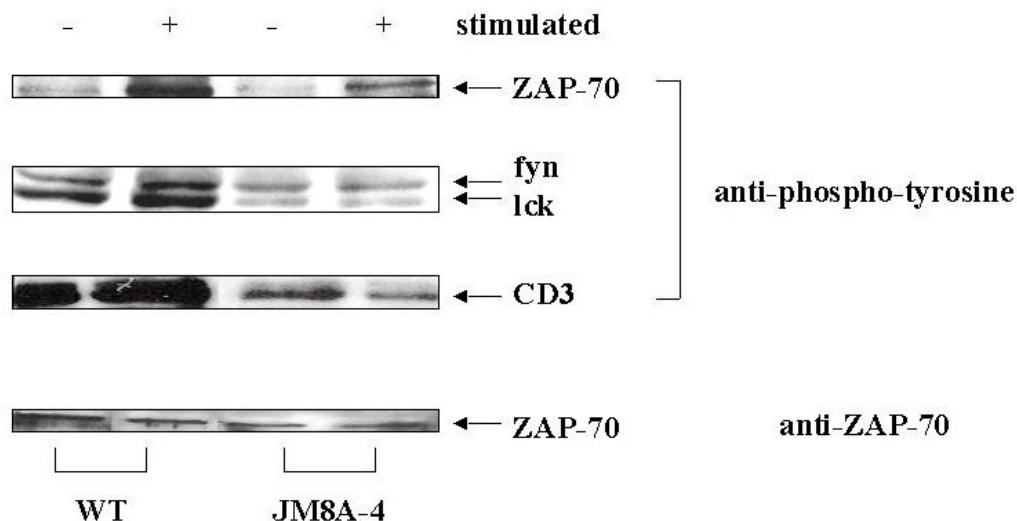


Figure 8.20: **Phosphorylation of p59^{fyn}, p56^{lck}, ZAP-70 and CD3 ζ chains is impaired in CTLA-4 mutants.** WT and CTLA-4 mutants were stimulated via CD3 ligation for 24 hours. Cells were restimulated for 2 minutes using anti-CD3 (10 $\mu\text{g}/\text{ml}$) and anti-CD28 (5 $\mu\text{g}/\text{ml}$) monoclonal antibodies, crosslinked with 2 $\mu\text{g}/\text{ml}$ anti-mouse IgG. Cells were directly lysed and the cell lysate analyzed by western blotting. Phosphorylated proteins were visualized by an anti-phospho-tyrosine antibody (py99). The same membrane was reprobed with an anti-ZAP-70 antibody to compare protein abundance (bottom).

of these T cells (Figure 8.20). Equal protein loads are shown on the membrane after reprobing with an anti-ZAP-70 antibody.

Thus, expression of CTLA-4 led to the development of an anergic phenotype of CD8⁺ T cells, since lack of IL-2 production as well as impaired tyrosine kinase phosphorylation of TcR proximal tyrosine kinases are characteristics of T cell anergy.

Ligating CTLA-4 with an anti-CTLA-4 antibody did not significantly further decrease the IL-2 production or phosphorylation of tyrosine kinases compared to non-ligated CTLA-4 mutants, suggesting that the high expression of CTLA-4 molecules on the cell surface of activated CTLA-4 mutants led to close proximity of CTLA-4 receptors on the cell surface, therefore creating a state similar to that achieved by CTLA-4 ligation. Thus, in the following experiments, CTLA-4

mutants (JM8A-4), are compared with the WT cells without the requirement for CTLA-4 ligation.

8.4 CTLA-4 promotes downregulation of CD28 expression

The effect of CTLA-4 on T cell activation has been related to negative signaling through its cytoplasmic tail and B7 sequestration. In addition, CTLA-4 negatively influences T cell activation due to competition for B7.1 and B7.2, since it has about 50 fold higher affinity for both molecules than CD28. We hypothesized that CTLA-4 could negatively influence the expression of CD28, thereby negatively influencing T cell responses to stimulation.

CD28 is expressed constitutively on almost all human CD4⁺ T cells and on approximately 50% of CD8⁺ T cells.^{106,107} However, it has been reported that CD28 surface expression is also not steady and can be influenced by several mechanisms, including TcR-mediated activation,¹⁰⁸ which leads to elevated CD28 expression. Therefore, we expected CD28 surface expression in WT cells to be elevated upon CD3 ligation.

WT cells were activated with anti-CD3 for 24 hours and CD28 surface expression was measured by flow cytometry. Indeed, CD28 surface expression was enhanced upon CD3-stimulation in WT cells compared to untreated cells, shown in Figure 8.21*a*. In addition, we analyzed the protein expression of CD28 in lysates of stimulated and resting WT cells by CD28 immunoprecipitation and western blotting. CD28 protein expression was clearly enhanced upon stimulation, compared to resting cells.

To analyze whether CTLA-4 upregulation has an effect on the expression of CD28, we stimulated CTLA-4 transfectants for 24 hours to upregulate CTLA-4 expression and compared CD28 expression in resting and stimulated CTLA-4 mutants. CD28 surface staining of resting and stimulated CTLA-4 transfectants revealed that CD28 expression was downregulated after stimulation, in contrast to WT cells, where stimulation led to increased CD28 expression.

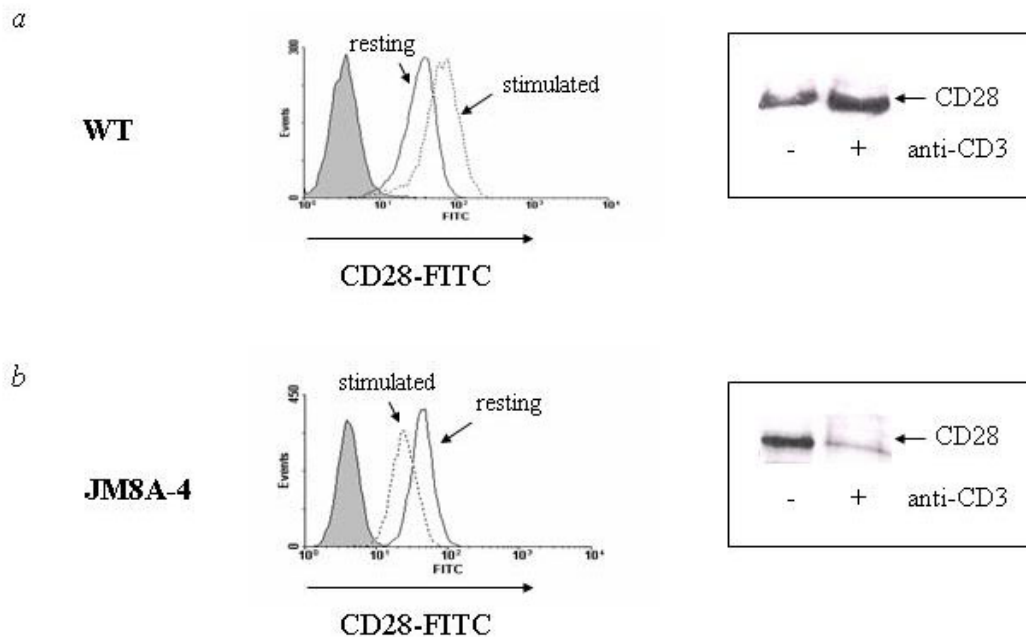


Figure 8.21: **CD28 expression is downregulated after stimulation in CTLA-4 transfectants.** Cell surface expression of CD28 was assessed by flow cytometry in resting and 24 hour stimulated ($10 \mu\text{g/ml}$ immobilized anti-CD3) WT cells. Also, CD28 was immunoprecipitated with an anti-CD28 mAb (CD28.2) from 0.5 mg cell lysate of resting and CD3-stimulated WT cells to analyze CD28 protein expression. The western blot was stained with an anti-CD28 rabbit polyclonal antibody (H-93). CD28 cell surface expression as well as protein concentration is enhanced after stimulation in WT cells (*a*).

Surface expression and CD28 protein concentration of CD28 was analyzed in stimulated and resting CTLA-4 mutants as described for WT cells. In contrast to WT cells, CD28 cell-surface expression as well as protein concentration are downregulated upon stimulation in CTLA-4 mutants (*b*).

Also, the protein level of CD28 was significantly reduced in stimulated CTLA-4 transfectants compared to resting cells, as detected by immunoprecipitation and western blotting (Figure 8.21*b*), again displaying the opposite to enhanced CD28 protein expression in stimulated WT cells. This unexpected observation indicates that CD28 expression is negatively influenced by CTLA-4.

In order to monitor CTLA-4 and CD28 expression over time, we stimulated CTLA-4 transfectants via anti-CD3 and measured the expression of CD28 as well as CTLA-4 from 30 minutes up to 24 hours of activation. Comparing the total

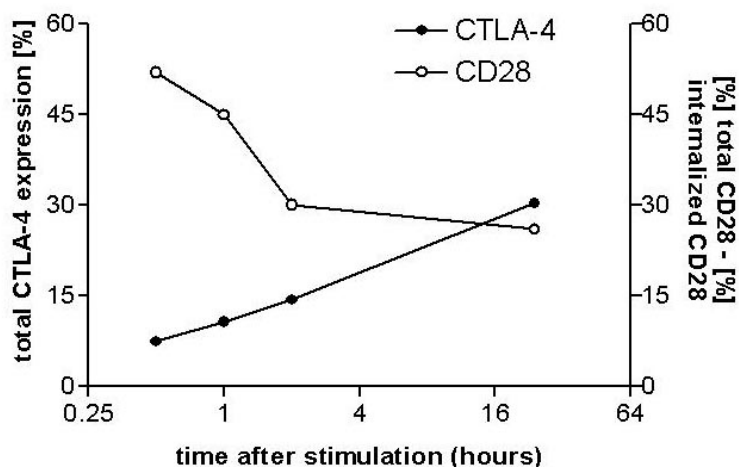


Figure 8.22: **CD28 downregulation correlates with increased CTLA-4 expression in CTLA-4 mutants.** The left y-axis shows total CTLA-4 expression, while the right y-axis displays the surface expression of CD28 [% total CD28 - % CD28 internalized] in CTLA-4 mutants. The decrease of CD28 surface expression correlates with increasing expression of CTLA-4. Receptor expression was measured during anti-CD3 stimulation, beginning with 30 minutes after stimulation. The highest total expression of CTLA-4 was reached after 24 h of stimulation, whereas CD28 surface expression diminished over time.

expression of CTLA-4 (left y-axis) with surface expression of CD28 (right y-axis) in CTLA-4 mutants over time revealed that the decrease of CD28 surface expression correlates with increasing expression of CTLA-4 (Figure 8.22). The highest total expression of CTLA-4 was reached after 24 hours of stimulation, which has been described previously.¹¹¹ However, the expression of surface CD28 diminished over time.

These data suggest that CTLA-4 upregulation leads to decreasing surface and protein expression of CD28.

8.5 CTLA-4 and CD28 co-localize in CTLA-4 transfectants

To further study the expression and location of CTLA-4 and CD28 in CTLA-4 mutants, we utilized confocal microscopy. WT and CTLA-4 mutants were co-stained with anti-CD28-FITC and anti-CTLA-4-PE at 37°C for 2 hours, leading to surface staining as well as internalization of the receptor-antibody-complex. The

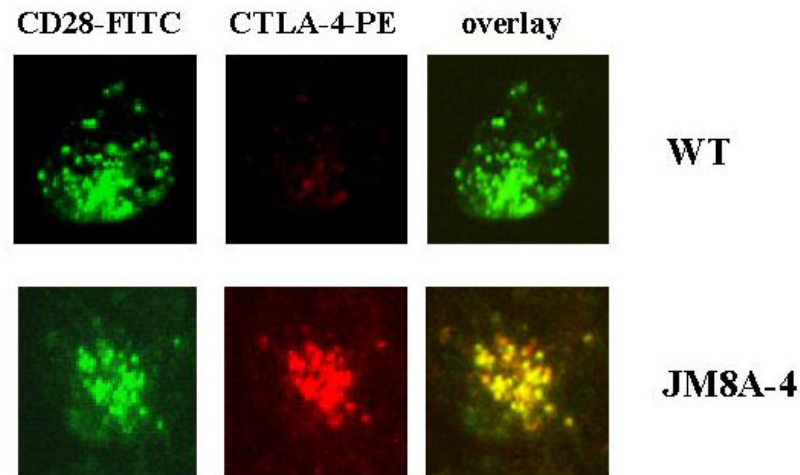


Figure 8.23: **CD28 co-localizes with CTLA-4 in CTLA-4 mutants.** CD28 and CTLA-4 expression was analyzed in CD3-stimulated WT and CTLA-4 mutants by confocal microscopy (60 \times objective). Samples were immunostained with anti-CD28-FITC (green) and CTLA-4-PE (red) at 37 $^{\circ}$ C for 2 hours. Yellow indicates overlay of red and green signals.

upper panel in Figure 8.23 shows WT cells stained with anti-CD28-FITC (green fluorescence) and anti-CTLA-4-PE (red fluorescence), as well as the overlay of both channels (yellow fluorescence).

Staining of WT cells with anti-CD28 at 37 $^{\circ}$ C reveals CD28 molecules on the cell surface as well as in the cytosol. As expected, no CTLA-4 was detectable in WT cells (upper panel). In contrast, CTLA-4 transfectants reveal mostly internalized CD28 as well as CTLA-4 receptors (lower panel). Interestingly, both receptors, CD28 and CTLA-4, entirely co-localized, as illustrated in the overlay (Figure 8.23). In addition, CD28 seems to be relocated from the surface into the cytosol in CTLA-4 mutants more so than in WT cells, suggesting that CTLA-4 facilitates the internalization of CD28.

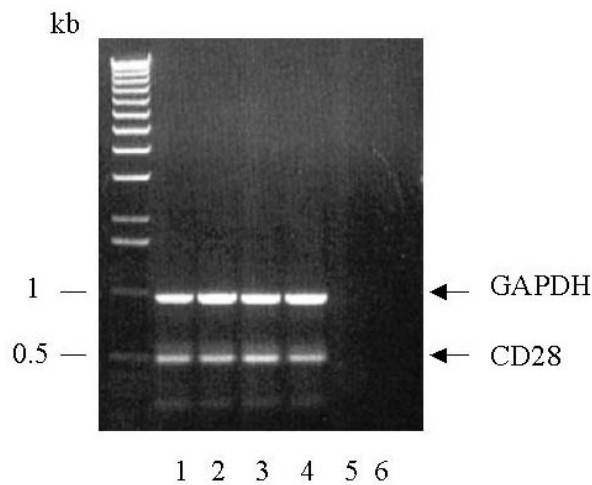


Figure 8.24: **WT and CTLA-4 mutants express equal amounts of CD28 RNA.** Resting and stimulated JM8A-4 cells (lanes 1 and 2) and WT cells (lanes 3 and 4, respectively) were analyzed by semi-quantitative RT-PCR. Lane 5 and 6 are genomic DNA (sample added after RT-step) and "no template" controls, respectively.

8.6 WT and CTLA-4 mutants express equal amounts of CD28 RNA

To rule out the possibility that the observed downregulation of CD28 was due to interruption of gene transcription, a semi-quantitative RT-PCR for CD28 was performed. WT and CTLA-4 mutants were stimulated for 24 hours or left untreated (resting). RNA was subsequently isolated and analyzed for CD28 expression by RT-PCR. Samples were run on agarose gels and a no-template as well as a genomic DNA control were included. There was no detectable quantitative difference in CD28 RNA levels between the WT and CTLA-4 mutants neither in resting nor activated conditions, lanes 1-4 (Figure 8.24).

This result implies that the differences in CD28 surface and protein expression between WT and CTLA-4 mutants are most likely due to post-translational processes, which are either directly or indirectly influenced by CTLA-4.

8.7 CTLA-4 does not influence the expression of CD25, CD45 and CD69

To rule out the possibility that CTLA-4 has a general negative influence on other activation-dependent T cell surface molecules, we analyzed WT cells and CTLA-4 transfectants for their expression of CD69, CD25 as well as CD45.

CD69 is generally known as a T cell activation marker, but its function is still unclear. CD25 functions as IL-2 receptor α -chain, whose expression is induced after lymphocyte activation. CD45, a tyrosine phosphatase, controls levels of tyrosine activity in lymphocytes and is also induced upon T cell stimulation.

We therefore compared the cell-surface expression of these activation-induced T cell surface markers on resting and 24 hour CD3-stimulated WT cells and CTLA-4 mutants. Resting as well as stimulated cells were stained with FITC-conjugated anti-CD25, anti-CD45 and anti-CD69 antibodies. CD69, CD25 and CD45 were equally elevated in stimulated WT cells as well as stimulated CTLA-4 mutants (Figure 8.25), compared to resting cells. These data indicate that CTLA-4 upregulation specifically induced the reduction of CD28 cell surface expression, whilst the expression of other activation-dependent T cell surface molecules remained unaffected.

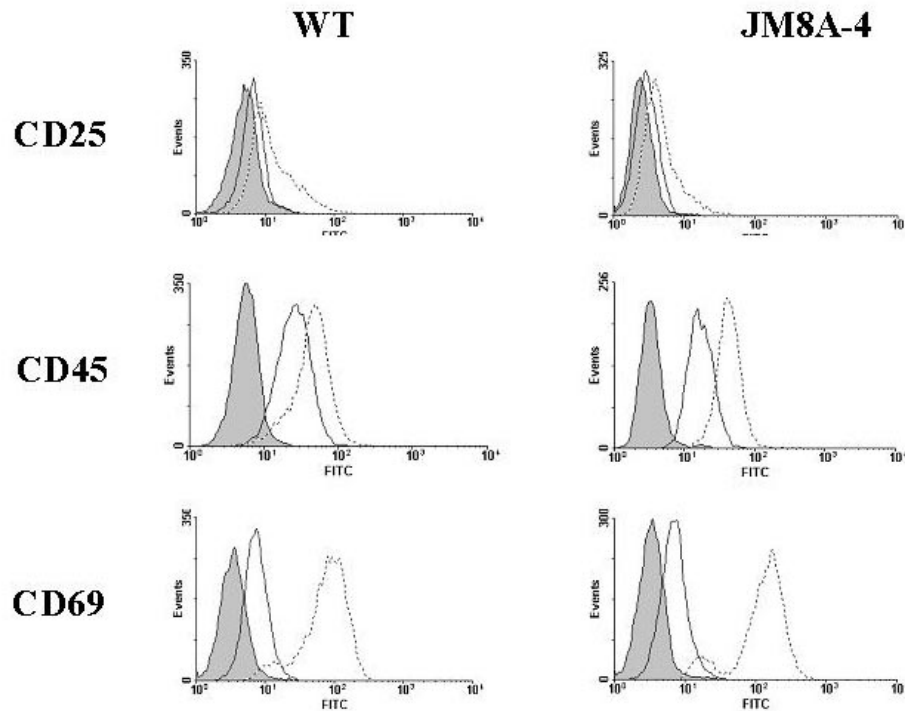


Figure 8.25: **CTLA-4 does not influence the expression of CD25, CD45 and CD69.** WT and CTLA-4 mutants were analyzed by flow cytometry for expression of IL-2 receptor α -chain (CD25), phosphatase CD45 and T cell activation marker CD69. The full line represents resting cells and the broken line 24 h CD3-stimulated cells. In WT cells as well as CTLA-4 mutants CD25, CD45 and CD69 are upregulated after stimulation.

8.8 CD28 expression is enhanced upon CD3-stimulation in HLA-B7 transfectants

In order to rule out that the downregulation of CD28 was due to unspecific effects of the transfection of WT cells with pcDNA3.1, we transfected WT cells using the same plasmid as used for transfection with CTLA-4 cDNA, now containing cDNA encoding for membrane bound HLA-B7 (JM8-B7).

Surface expression of HLA-B7 on HLA-B7 transfectants was detected by flow cytometry after staining with ME.1 monoclonal antibody (anti-HLA-B7), as shown in Figure 8.26*a*. JM8-B7 cells, as WT cells, constitutively express CD8,

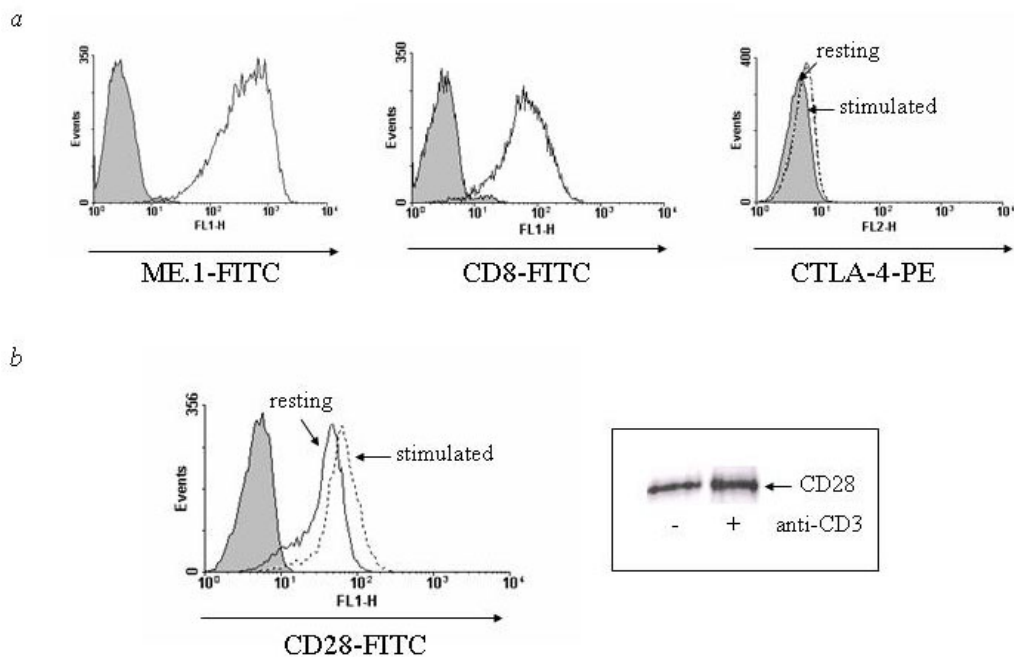


Figure 8.26: **CD28 expression is enhanced upon CD3-stimulation in HLA-B7 transfectants.** Jurkat WT cells were transfected with cDNA encoding for membrane bound HLA-B7 (JM8-B7). Surface expression of HLA-B7 was detected by flow cytometry after staining with FITC-conjugated ME.1 monoclonal antibody (anti-HLA-B7/B27). HLA-B7 is constitutively expressed on HLA-B7 transfectants. JM8-B7 cells also constitutively express CD8, but do not express CTLA-4, neither in resting condition nor after stimulation (a). CD28 expression was assessed by flow cytometry and immunoprecipitation in resting and 24 h anti-CD3 stimulated JM8-B7 cells, revealing upregulation of CD28 surface expression as well as protein concentration upon CD3-stimulation (b).

but do not express CTLA-4, neither in the resting condition nor after stimulation (Figure 8.26a).

The stimulation experiment in which CD28 was detected by both, flow cytometry and immunoprecipitation revealed the same result as in WT cells. CD28 surface expression as well as protein concentration was enhanced upon CD3-stimulation in HLA-B7 transfectants (Figure 8.26b). This result confirmed that CD28 downregulation was indeed induced by CTLA-4 upregulation and not due to unspecific vector effects.

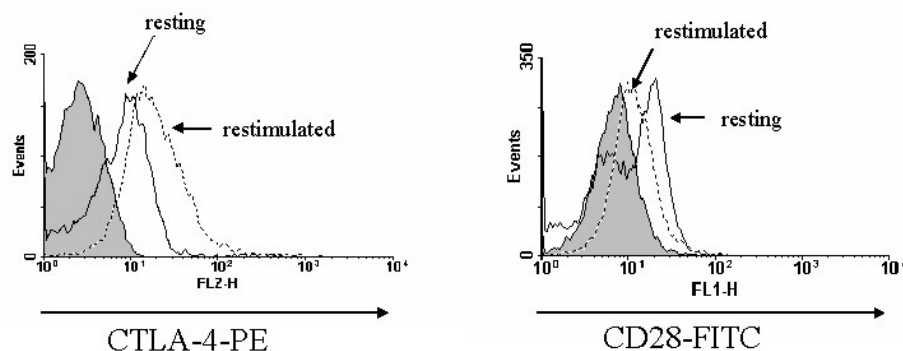


Figure 8.27: **CD28 surface expression is reduced after stimulation of primary anergized T cells.** CD28 and CTL-4 surface expression were analyzed in stimulated and resting anergized T cells. Multiple SEB (staphylococcal superantigen B) stimulations were used to anergize PBMC. After anergization, cells were either left untreated (full line) or were rechallenged (broken line) with 5 $\mu\text{g}/\text{ml}$ SEB. CTLA-4 was already expressed in resting anergized T cells but its expression even further enhanced after SEB rechallenge, whereas CD28 expression was decreased upon restimulation.

8.9 CD28 surface expression is reduced after stimulation on primary anergized T cells

CTLA-4 has been described to be elevated in anergic primary T cells. Thus, to analyze the effects of CTLA-4 upregulation in primary T cells, we obtained lymphocytes from healthy blood donors and anergized those using the superantigen SEB (staphylococcal superantigen B).

Staphylococcal enterotoxins belong to a family of bacterial proteins described as superantigens. Superantigens are proteins that bind and activate all CD4^+ and CD8^+ T cells that express a particular set or family of V_{β} TcR genes.¹¹³

CD8^+ T-cells exhibit a reduced capacity to proliferate in response to the staphylococcal enterotoxin in vitro after repeated stimulation in vivo¹¹⁴ and multiple superantigen stimulations in vitro are known to induce a state of anergy in superantigen-reactive T cells.¹¹⁵

Thus, we used multiple SEB stimulations in vitro to anergize primary T cells from healthy blood donors. Primary T cells were anergized by multiple stimulations (three restimulations) with 5 $\mu\text{g}/\text{ml}$ SEB, using irradiated PBMCs as APC.

Anergized cells were either left untreated or were rechallenged with SEB alone and analyzed for surface expression of CD28 and CTLA-4 by flow cytometry. CTLA-4 was already expressed in resting anergized T cells, but its expression was even further enhanced after SEB rechallenge (Figure 8.27, left histogram). This represented a comparable situation as observed in Jurkat CTLA-4 mutants that expressed low levels of CTLA-4 in resting conditions, which was further enhanced after CD3-stimulation. Analyzing CD28 surface expression on resting and restimulated anergized primary T cells revealed that CD28 expression was decreased upon restimulation with SEB (Figure 8.27, right histogram).

Thus, these data confirmed the phenomenon observed in CTLA-4 mutants, the downregulation of CD28 surface expression upon upregulation of CTLA-4, in a primary T cell model.

8.10 CTLA-4 promotes internalization of CD28

Cell surface receptors can be divided into two groups based on their modes of action. One group functions by internalizing bound ligands through receptor-mediated endocytosis,¹¹⁶ the other group, which includes CD28, functions by transmitting signals across the membrane upon ligand binding. However, many receptors of the second group also undergo internalization. Even if this internalization does not play a role in signal transduction, it may alter the biology of the receptor system by removing the receptor from the cell surface, thereby diminishing the responsiveness of the cell to the receptor ligand. This process might explain the surface downregulation of CD28 in CTLA-4 transfectants. Therefore, to investigate whether CD28 receptor internalization is involved in CD28 downregulation in CTLA-4 transfectants, we studied the internalization of CD28 in WT cells and CTLA-4 mutants by flow cytometry. Surface receptor internalization can be measured by staining the receptor with a fluorescein-conjugated antibody at 37°C, which leads to internalization of the antibody/receptor complex as well as surface staining of the receptor. In contrast, staining at 4°C leads to surface receptor staining only.

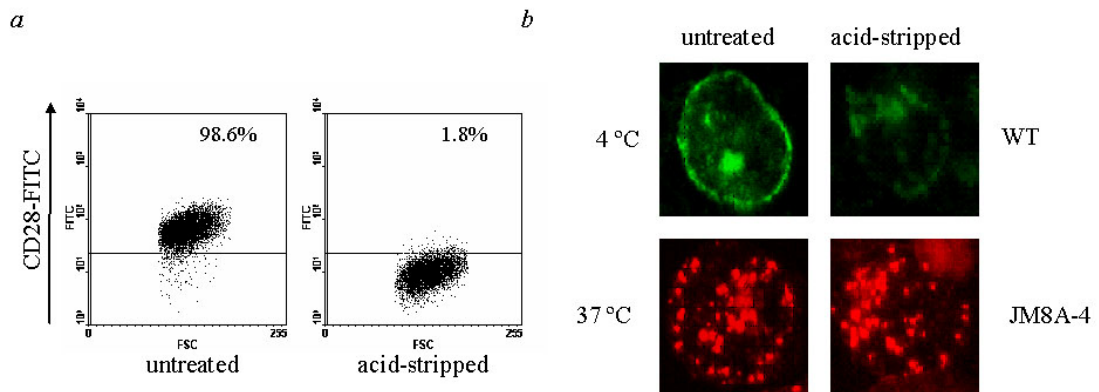


Figure 8.28: **Acid-treatment completely removes surface-bound antibody, but does not affect internalized antibody.** WT cells were stained with an anti-CD28-FITC mAb at 4°C and subsequently treated with 0.2 M acetic-acid solution to remove surface-bound antibody. The left panel shows anti-CD28-stained cells before acid-treatment, whereas the right panel shows anti-CD28 stained cells after removal of surface antibody with acetic acid. Acid-treatment completely removed surface-bound anti-CD28-antibody (a).

WT cells were stained with an anti-CD28-FITC mAb at 4°C and left untreated or underwent acid-treatment, as indicated (upper two pictures). CTLA-4 mutants were stained with an anti-CD28-PE mAb at 37°C for 2 hours and were left untreated or underwent acid-treatment. Stained cells were analyzed by confocal microscopy using a 60× objective. Surface-bound antibody was removed by acid-treatment (upper panel), whereas internalized antibody remained acid-resistant and could not be removed (lower panel). Thus, internalized antibody is not affected by acid treatment (b).

The internalized receptor-antibody complex can be quantified by measuring fluorescence intensity using flow cytometry. In order to distinguish between internalized and surface bound antibody, surface-bound antibody can be stripped off by acetic acid. The internalized antibody/receptor complex is not affected by acid treatment. This method was optimized and used to analyze internalization of CD28 and CTLA-4 in resting and stimulated WT and CTLA-4 transfectants.

Resting WT cells were stained with a FITC-conjugated anti-CD28 antibody at 4°C and subsequently stripped with acetic acid or left untreated. Acid-stripped cells were completely FITC-negative, confirming the complete removal of surface-bound antibody after acid-treatment and the lack of internalization of the receptor/antibody complex at 4°C (Figure 8.28a). This result was further confirmed by

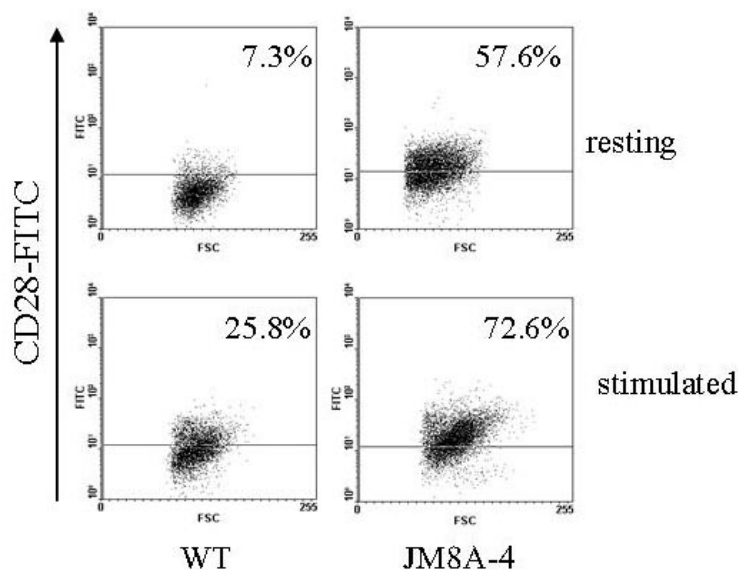


Figure 8.29: **CD28 internalization is enhanced in CTLA-4 mutants.** The left panel shows WT cells and the right panel CTLA-4 mutants (resting and 24 h anti-CD3-stimulated) that were stained with anti-CD28-FITC at 37°C for 2 hours and subsequently acid-stripped.

confocal microscopy (Figure 8.28*b*, upper panel), showing only surface staining of CD28 on WT cells and the removal of surface bound antibody after acid-stripping. The internalization of CD28 is shown by confocal microscopy in CTLA-4 mutants after staining of cells with anti-CD28-PE at 37°C for 2 hours. The internalized antibody (red fluorescence) could clearly not be removed by acid-treatment (Figure 8.28*b*, lower panel).

To quantitate CD28 internalization in resting and stimulated WT cells and CTLA-4 mutants, both cell lines were stained with anti-CD28-FITC at 37°C for 2 hours, subsequently treated with acetic acid to remove surface-bound antibody and analyzed by flow cytometry. In both cell types, increased CD28 internalization was measured after T cell activation compared to resting cells (Figure 8.29). However, comparing the internalization of CD28 between WT cells and CTLA-4 mutants reveals that internalization of CD28, especially upon stimulation, is indeed enhanced in CTLA-4 mutants (mean fluorescence intensity, MFI 18) compared to the WT (MFI 10).

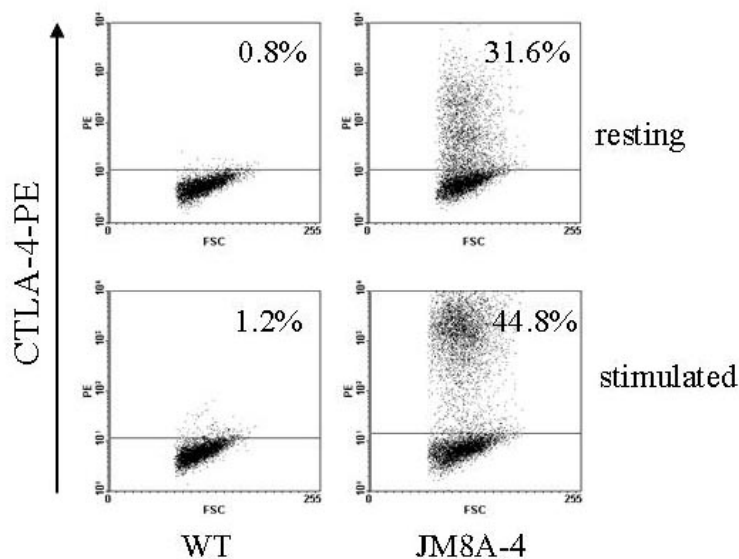


Figure 8.30: **CTLA-4 internalization is enhanced after stimulation.** WT and CTLA-4 mutants (stimulated for 24 hours with anti-CD3 or resting) were stained with anti-CTLA-4-PE at 37°C for 2 hours. Cells were acid-stripped as described above and samples subsequently analyzed by FACS.

The same method was used to analyze CTLA-4 internalization in CTLA-4 mutants. The expression and subsequent internalization of CTLA-4 was significantly elevated after anti-CD3 stimulation (untreated: MFI 15 versus stimulated: MFI 53), demonstrating the high trafficking rate of CTLA-4 in activated T cells (Figure 8.30). As expected, CTLA-4 antibody was not internalized in WT cells due to lack of CTLA-4 expression.

Thus, CTLA-4 might facilitate the internalization of CD28, possibly by heterodimerization of CD28 with CTLA-4, as further addressed in the discussion.

8.11 CD28 internalization is partially clathrin-dependent

To determine, whether the observed CD28 endocytosis in CTLA-4 mutants is based on a clathrin-dependent mechanism, we repeated the above described internalization experiment with the addition of an inhibitor of clathrin-dependent endocytosis. Hypertonic sucrose medium inhibits clathrin-mediated endocytosis by perturbing the association of clathrin with the adaptor protein AP-2.

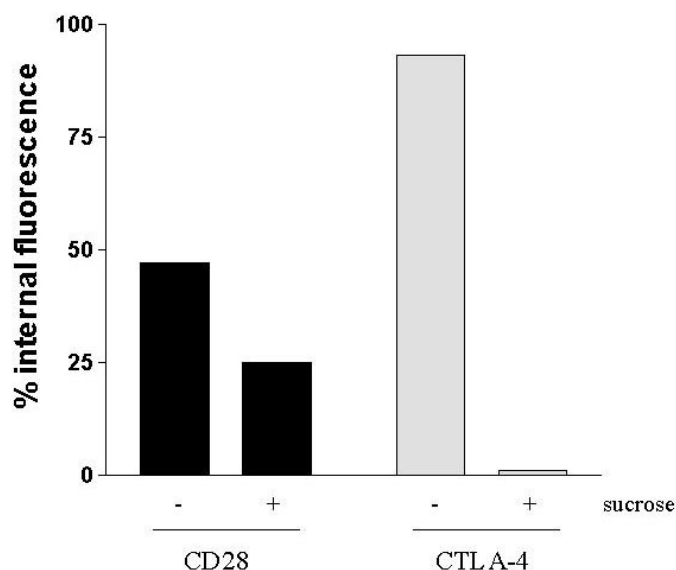


Figure 8.31: **CD28 internalization is partially clathrin-dependent.** CTLA-4 mutants were stimulated with anti-CD3 for 24 hours. Cells were subsequently stained with anti-CD28-PE or CTLA-4-PE, but additionally incubated with 0.45 M sucrose (as indicated) during the staining procedure to inhibit clathrin-dependent endocytosis. Samples were acid stripped (accounting for internal fluorescence) or left untreated (total fluorescence). The x-axis shows the percentage of internal fluorescence, calculated as a ratio of internal to total fluorescence.

It is known that CTLA-4 internalization occurs in a clathrin-dependent manner through association with clathrin-adaptor molecules AP-1 and AP-2.¹¹⁷ Thus, the internalization of CTLA-4 should be entirely inhibited with hypertonic sucrose medium.

Although CD28 internalization has been described as clathrin-dependent by some groups,¹¹⁸ others have observed that CD28 does not interact with AP-1 and AP-2.¹⁰⁵ Here, we analyzed the endocytosis of CD28 as well as CTLA-4 in CTLA-4 mutants.

Our data indicate that CD28 is endocytosed partially in a clathrin-dependent manner, as its internalization was reduced (lower levels of internal fluorescence), but not entirely blocked, after treatment with hypertonic sucrose medium (Figure 8.30). In contrast, CTLA-4 internalization, as expected, was completely abrogated with hypertonic sucrose medium (Figure 8.31). These data suggest that

overexpression and high trafficking of CTLA-4 coincide with an increased, partially clathrin-dependent internalization of CD28.

8.12 CTLA-4 promotes degradation of CD28

Besides the downregulation of CD28 surface expression, we observed decreased protein levels of CD28 in stimulated CTLA-4 transfectants, Figure 8.21. The decreased surface expression is most likely due to the enhanced internalization of CD28 in CTLA-4 mutants, as seen above. However, in order to decrease the protein expression of CD28, a protein degradation process must be involved.

The major mechanism for protein degradation is the ubiquitin pathway. Degradation of a protein via the ubiquitin-pathway involves the covalent attachment of multiple ubiquitin molecules to a protein substrate and the subsequent degradation of the tagged protein by the 26S proteasome complex, a multisubunit protease. Besides the 26S proteasome the ubiquitin conjugation system comprises of ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3), as illustrated in Figure 8.32. The conjugation of ubiquitin to the target protein involves three steps: first, the activation of the ubiquitin molecule by E1, second the transfer of ubiquitin to E2 and third the conjugation to the target protein by E3.

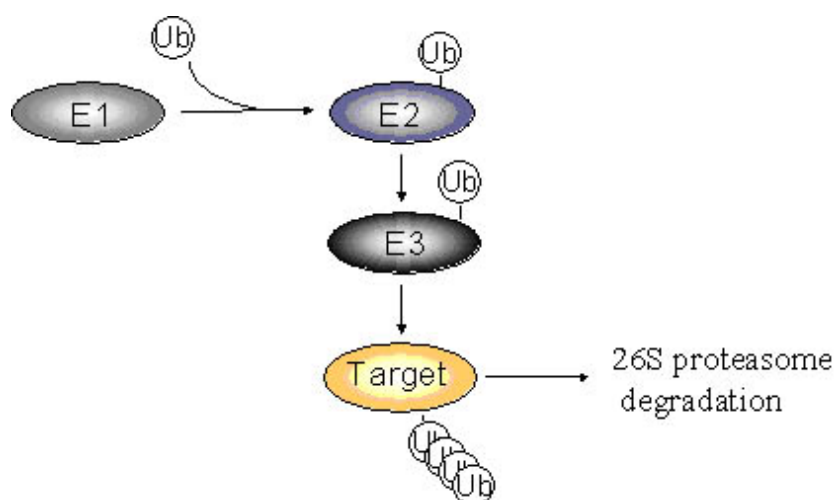


Figure 8.32: Ubiquitin degradation pathway

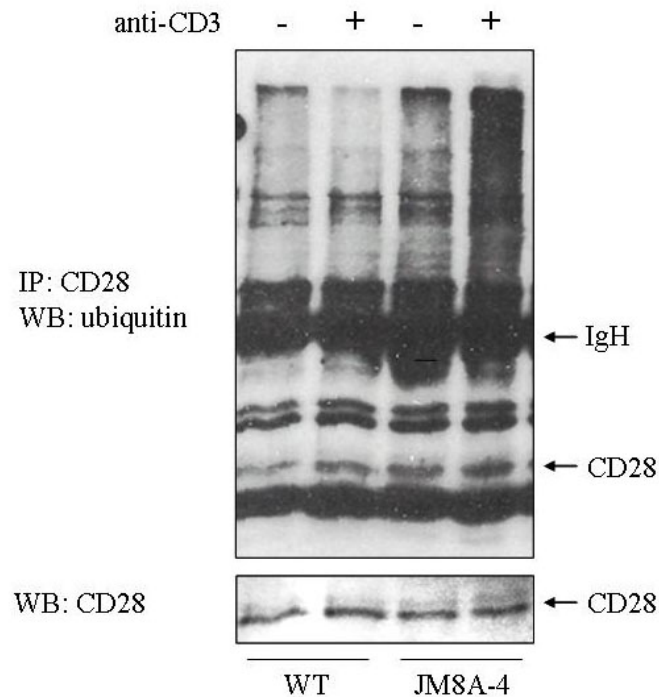


Figure 8.33: **CD28 is ubiquitinated in CTLA-4 mutants.** CD28 was immunoprecipitated from resting and stimulated (anti-CD3) WT cells and CTLA-4 mutants that were treated with 10 μ M MG-132, a proteasome inhibitor, during the last 12 h of stimulation. The western blot was stained with anti-ubiquitin monoclonal antibody (upper panel) and the same membrane reprobred with polyclonal anti-CD28 antibody (lower panel). Ubiquitination of CD28 was most significant in stimulated CTLA-4 mutants.

The E3 ubiquitin ligases are responsible for specific substrate recognition and for promoting ubiquitin ligation to the target protein, followed by degradation of the targeted protein by the 26S proteasome.

To determine whether the observed reduction of CD28 protein expression in CTLA-4 mutants is due to ubiquitination and subsequent degradation in the 26S proteasome complex, we analyzed whether CD28 is indeed ubiquitinated after TcR stimulation.

WT Cells and CTLA-4 transfectants were stimulated with anti-CD3 or left untreated. Cells were additionally treated with proteasome inhibitor MG-132 during the stimulation to increase the level of ubiquitinated proteins. CD28 was

immunoprecipitated from the cell lysate and analyzed by western blotting.

The membrane was first probed with an anti-ubiquitin antibody (upper panel), and reprobed with an anti-CD28 antibody (lower panel), Figure 8.33. The anti-CD28 staining did not imply any degradation of CD28 in CTLA-4 mutants, even after TcR stimulation, probably due to the inhibition of CD28 degradation by the proteasome inhibitor MG-132.

The ubiquitin staining clearly revealed that CD28 was polyubiquitinated in stimulated CTLA-4 mutants, indicated by a smear in the high molecular weight region on the western blot.

If CTLA-4 promotes proteasome-dependent CD28 proteolysis, the downregulation of CD28 as observed in CTLA-4 mutants should be abrogated by blocking proteasomal degradation with a proteasome-inhibitor, as already indicated in Figure 8.33 after MG-132 treatment. To confirm this observation we used another proteasome inhibitor, lactacystin, which inhibits the proteolytically active 20S core complex of the 26S proteasome, and compared CD28 protein expression in lactacystin-treated and untreated WT and CTLA-4 mutants.

WT and CTLA-4 mutants were treated for 16 hours with lactacystin during anti-CD3 stimulation. If the CD28 protein downregulation in CTLA-4 mutants is indeed proteasome-dependent, the CD28 protein concentration should not be decreased in CTLA-4 mutants after lactacystin-treatment.

Figure 8.34 shows an immunoprecipitation of CD28 in lactacystin-treated (right panel) and untreated (left panel) WT and JM8A-4 cells. As observed before (Figure 8.21), CD28 was downregulated after stimulation in CTLA-4 mutants. This effect was only observed in cells that were not treated with lactacystin (Figure 8.34, left panel). Indeed, the degradation of CD28 in CD3-stimulated CTLA-4 mutants was entirely abrogated in cells that were treated with lactacystin. CD28 expression was not decreased, as seen on the left panel, but elevated after anti-CD3 stimulation in CTLA-4 mutants (right panel). Thus, lactacystin treatment not only inhibited the degradation of CD28, but even led to increased protein

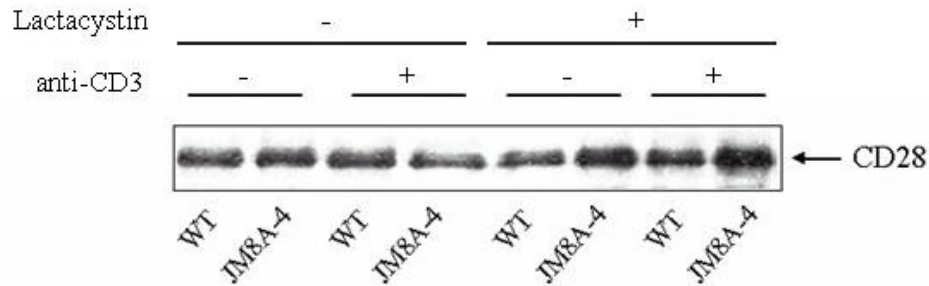


Figure 8.34: **Lactacystin inhibits degradation of CD28 in CTLA-4 mutants.** CD28 was immunoprecipitated from 0.5 mg cell lysate of 24-hour anti-CD3 stimulated or resting WT and CTLA-4 mutants that were left untreated (lanes 1-4) or were treated for 16 hours (during stimulation/ resting) with 2 μ M lactacystin, a proteasome inhibitor (lanes 5-8). The western blot was stained with an anti-CD28 polyclonal antibody. CD28 was downregulated after stimulation in CTLA-4 mutants, if not treated with Lactacystin. The degradation of CD28 in CD3-stimulated CTLA-4 mutants was entirely abrogated in cells, which were treated with lactacystin (right panel), leading to accumulation of CD28 in the cytosol.

expression of CD28 after stimulation. The enhanced expression of CD28 in CTLA-4 mutants might be explained by accumulation of CD28 over time in the cytosol, due to the inhibition of proteasomal degradation during CD3 stimulation. In addition, significant effects of lactacystin treatment were only seen in CTLA-4 mutants and not in WT cells.

Therefore, this experiment indicates that proteasomal degradation plays a more considerable role in the regulation of CD28 expression in CTLA-4 transfectants than in WT cells.

8.13 CD28 associates with E3 ubiquitin ligase cbl

The E3 ubiquitin ligases are responsible for specific substrate recognition and for promoting ubiquitin ligation to the target protein. Therefore, to target CD28 for proteolysis, CD28 has to associate with an E3 ubiquitin ligase. E3-ubiquitin ligase cbl has been described to play an important role in the regulation of T cell activation by ubiquitination of TcR ζ . To investigate, whether cbl associates

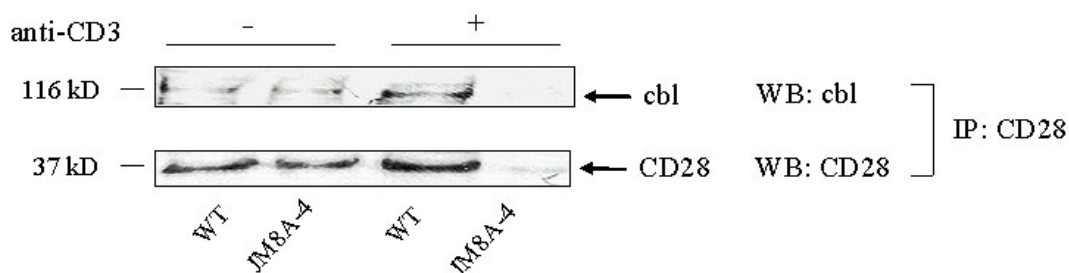


Figure 8.35: **CD28 associates with E3 ubiquitin ligase cbl.** CD28 was immunoprecipitated from resting and stimulated (anti-CD3) WT cells and CTLA-4 mutants. The western blot was stained with anti-CD28 polyclonal antibody and the same membrane reprobated with polyclonal anti-cbl antibody.

with CD28, we attempted to co-immunoprecipitate cbl with CD28. WT cells and CTLA-4 mutants were stimulated and CD28 precipitated from the lysates. The precipitate was analyzed by SDS-PAGE and western blotting and the membrane first stained with an anti-cbl-antibody. After stripping, the same membrane was reprobated with an anti-CD28-antibody. Figure 8.35 shows that cbl was detectable in the CD28 immunoprecipitate, although weak, suggesting that CD28 indeed associates with cbl. Consequently, after stimulation of CTLA-4 mutants, cbl could not be co-precipitated due to the low protein concentration of CD28 after its degradation.

These data suggest that CD28 is ubiquitinated and subsequently degraded by the 26S proteasome, presumably after association with E3-ubiquitin ligase cbl. However, the exact mechanism by which CTLA-4 might influence this process remains unclear.

Here, we identified a novel inhibitory effect of CTLA-4, its negative influence on the expression of CD28 in CD8⁺ T cells. We provide evidence that CD28 internalization and proteolysis are enhanced after CTLA-4 upregulation, leading to reduced cell-surface expression of CD28, which in turn might reduce the ability of T cells to respond to antigen stimulation. This would provide an additional mechanism by which CTLA-4 impedes the activation of CD8⁺ T cells.

CHAPTER 9

DISCUSSION

Thus far, three different levels of interference of CTLA-4 with an ongoing immune response have been described: competition with CD28 for the same ligands on APCs;¹¹⁹ interference with proximal and distal TcR signals;^{90,120} and most recently the so-called "reverse signaling" to dendritic cells (DC).¹²¹ By competing for the same ligand, CTLA-4 could decrease the T cell response by limiting the access of B7 for CD28, thus inhibiting CD28 signaling. In the "reverse signaling" pathway model, CTLA-4 acts as a ligand for B7 on DCs, resulting in signal transduction and onset of suppressive activity of DCs via interference with tryptophan catabolism.¹²² This type of "reverse signaling" has most recently been shown for CD28,¹²³ complicating the interactions of CD28, CTLA-4, B7-1 and B7-2 in T cell costimulation.

In the present study, we have demonstrated that CTLA-4 expression in CD8⁺ T cells leads to abrogation of IL-2 production and a decrease in tyrosine kinase phosphorylation of TcR proximal tyrosine kinases, previously shown by our group as a characteristic of anergic CD8⁺ T cells.⁹⁷ The anergic phenotype was achieved without the ligation of CTLA-4 and no significant difference was observed in tyrosine kinase phosphorylation as well as IL-2 production of activated CTLA-4 mutants that were additionally ligated with an anti-CTLA-4 antibody. The high expression of CTLA-4 molecules on the cell surface of activated CTLA-4 mutants most likely results in a close proximity of CTLA-4 receptors, therefore creating a state similar to that achieved by CTLA-4 ligation. This might explain the insignificant difference in this model between CTLA-4 expressing cells before and after CTLA-4 ligation.

In addition, we describe a new characteristic of CTLA-4, its effect on the expression of its opponent CD28. We demonstrate that CTLA-4 upregulation leads to enhanced internalization of CD28, thereby reducing its cell-surface expression.

It has been shown by Cefai et al. that CD28 undergoes clathrin-dependent endocytosis,¹¹⁸ which is mediated by clathrin and its adaptor proteins, AP-1 and AP-2.¹²⁴ However, whether CD28 is endocytosed in a clathrin-dependent manner or not is controversially discussed.^{105,125} Our data agree with the findings by Cefai et al.¹¹⁸ and demonstrate that CD28 endocytosis is at least partially clathrin-dependent. However, besides the clathrin-dependent endocytosis pathway, the ubiquitination of proteins, in addition to targeting them for degradation, can also enhance the endocytosis of membrane proteins.¹²⁶ Therefore, the clathrin-independent, ubiquitin-mediated internalization might additionally be involved in the process of CD28 endocytosis, as CD28 was clearly ubiquitinated after stimulation in CTLA-4 mutants.

Interestingly, the observed internalization of CD28 is enhanced after expression and internalization of CTLA-4, suggesting that CTLA-4 upregulation influences the endocytosis of CD28. Our current hypothesis is that CTLA-4 facilitates the internalization of CD28 by heterodimerization of CD28 with CTLA-4. Both CD28 and CTLA-4 exist as disulfide-linked homodimeric glycoproteins,^{107,67,127} but it has also been suggested that both molecules can exist as monomeric proteins.^{128,129} Walunas et al. showed the existence of CTLA-4 on murine activated T cells as both disulfide-linked dimer and a non-sulfide-linked monomer.⁹² Both CD28 and CTLA-4 have an unpaired cysteine residue at a position proximal to the transmembrane domain, which is believed to be the site of dimerization for both CD28 and CTLA-4 monomers.¹³⁰ This site could be potentially involved in the formation of heterodimers between CTLA-4 and CD28 glycoproteins, thus leading to internalization of CD28 with CTLA-4. Our data show that CTLA-4 indeed co-localizes with CD28 (Figure 8.23), underscoring our hypothesis. It has also been described that CD28 and CTLA-4 are selectively recruited to the immunological synapse and that they co-localize in lipid rafts.^{131,132}

An alternative explanation for the enhanced internalization of CD28 might be its ubiquitination after stimulation in CTLA-4 mutants, as ubiquitination can also enhance the endocytosis of membrane proteins.¹²⁶

After its internalization, CD28 might be degraded in the cytosol in CTLA-4 transfectants, as its protein concentration is significantly diminished after stimulation. We provide evidence that the observed CD28 degradation in CTLA-4 transfectants can be inhibited with proteasome inhibitors, such as lactacystin and MG-132. This finding suggests that, after its ubiquitination, CD28 is degraded in a proteasome-dependent manner and that this process is enhanced after upregulation of CTLA-4.

After being internalized, CD28 might associate with E3 ubiquitin ligase cbl, as proposed in Figure 8.35. Thus, this E3 ubiquitin ligase might be responsible for the targeting of CD28 for degradation. The importance of E3 ubiquitin ligases in T cell function and the development of T cell anergy have been described previously.^{133,134,135} For example, the identification and characterization of GRAIL as an ubiquitin ligase that is necessary for the induction of anergy in CD4⁺ T cells reiterates the important role of the ubiquitin pathway in controlling T cell tolerance. The involvement of adaptor proteins in the process of targeting proteins for ubiquitination is another possibility that needs to be investigated, as for example ZAP-70 has been described to have an adaptor function in the ubiquitination of TcR ζ .¹³⁶

It is known that the degradation of T cell signaling proteins, tyrosine kinases p56^{lck} and p59^{lyn} represents a mechanism to terminate sustained signaling in T cells,^{137,138,139} and is proposed to be responsible for tolerance induction of self-reactive T cells.¹⁴⁰ Interestingly, the degradation of p56^{lck} is dependent on the activation state of T cells, suggesting that ubiquitination plays a specific role in downregulating Src kinases, which are activated by receptor stimulation.¹³⁷ This phenomenon has also been described for the TcR-CD3 complex in activated T cells.¹⁴¹

Thus, the interference of CTLA-4 with the ubiquitin system might play an important role in the regulation of T cell activation and T cell anergy. However, it is thus far not clear by which means CTLA-4 can facilitate the degradation of CD28.

To verify and show the physiological significance of CD28 downregulation in our CD8⁺CTLA-4⁺ T cell model, we analyzed CD28 expression after CTLA-4 upregulation in primary anergized T cells. Our results agree with observations by Xu et al.,¹¹⁵ which show upregulation of CTLA-4 upon rechallenge of anergized primary T cells using SEA for multiple superantigen stimulation. They also report that the CD28 surface expression of activated T cells is slightly upregulated upon antigen rechallenge, whereas the surface expression of CTLA-4 in activated T cells did not change. However, anergized T cells showed highly increased CTLA-4 surface expression after 60 hours of rechallenge. The CD28 expression level decreased after 60 hours of rechallenge, coinciding with the increase of CTLA-4 surface expression. Our data indicate that downregulation of CD28 during increasing surface expression of CTLA-4 (as observed in CTLA-4 transfectants) also occurs in primary anergized T cells.

Thus, the present results suggest that CTLA-4 induction leads to an anergic phenotype in CD8⁺ T cells and provide evidence that CTLA-4 is involved in the regulation of CD28 expression by augmenting CD28 internalization and degradation, a previously unknown paradigm. The downregulation of CD28 could lead to further impairment of T cells by the reduced T cell response to costimulation.

These findings could prove helpful for further targeting CTLA-4 as molecule for the induction of tolerance or the use of CTLA-4 antibodies in tumor immunotherapy to allow a potent anti-tumor response.

Part III

Conclusions and Summary

CHAPTER 10

CONCLUSIONS

MHC peptides derived from tumor associated antigens (TAA) can serve as the basis for development of immunotherapeutics for treatment of human malignancies. In this study we identified a possible candidate for tumor immunotherapy, peptide p1028 (GLIEKNIEL), which is derived from a broadly expressed tumor-associated enzyme, DNMT-1.

Four candidate peptides eluted from soluble HLA-A2 were selected on the basis of their abundance and association with tumor antigens. Peptide p1028 , derived from DNMT-1, showed the highest affinity to HLA-A2 and was relatively abundant in the sMHC/peptide complexes of all transfected breast, ovarian and prostate cancer cell lines. Peptide p1028 was immunogenic in vitro and p1028 specific CTL were capable of lysing tumor cells. CTL were highly peptide specific and HLA-A2 restricted. Due to the fact that peptide p1028 can induce CTL that are capable of tumor cell lysis and that DNMT-1 is over-expressed in a variety of tumors, this peptide may provide a suitable candidate for a therapeutic cancer vaccine.

However, a major obstacle for the immunotherapy of tumors with tumor antigens is the development of tumor-specific naive T cells that are already tolerant towards the tumor antigens and the development of immune escape mechanisms by tumors. It is also apparent that tumors fail to elicit protective immune response due to the lack of expression of costimulatory ligands, which would obviously strongly impair the potency of a peptide cancer vaccine. Thus, significant effort has been placed in engineering costimulatory molecules into vaccines in an attempt to enhance their activity. One approach is the induction of costimulator ligand expression in tumor cells to induce T cell-mediated rejection of immunogenic tumors. This concept has been proven successful by transfecting tumor cells with B7 in a variety of models.^{142, 143}

Another strategy involves the manipulation of inhibitory receptors, such as CTLA-4. The usefulness of CTLA-4 based therapies has been shown in a variety of studies. For example CTLA-4 blockade can be of use in tumor immunotherapy. Initial studies in mouse models revealed that blockade of B7/CTLA-4 interactions with antibodies resulted in rejection of tumors.¹⁴⁴ The effectiveness of anti-CTLA-4 in inducing tumor rejection was also shown in other transplantable tumor systems in mice,¹⁴⁵ but was not able to prevent the induction of tumor antigen-specific tolerance.⁷² Anti-CTLA-4 as a single agent against poorly immunogenic tumors failed to be effective against tumor growth.⁶⁶

These data suggest that the susceptibility to CTLA-4 blockade strongly depends on the immunogenicity of the tumor, as does the treatment with peptide-based tumor vaccines. It is also still controversial as to whether CTLA-4 plays a role in tolerance induction or maintenance or whether it only modulates the activity of primed T cells. Some studies have suggested that CTLA-4 blockade is most effective in amplifying the activity of primed T cells but not in breaking established tolerance. Thus, if tolerance against cancer is already established at the time of therapeutic intervention, combinations of vaccination might prove most effective. For example the anti-CTLA-4/ GM-CSF tumor cell vaccine treatment against B16 melanoma resulted in effective breakage of peripheral tolerance to the tissue-specific differentiation antigen TRP-2. The breaking of tolerance was not achieved using the GM-CSF tumor cell vaccine alone.⁶⁶ Thus, the importance for costimulation, both positive (CD28) and negative (CTLA-4), in the regulation of T cell responses to tumors has been proven. The understanding of costimulatory mechanisms is therefore crucial for the development of new strategies for the improvement of existing cancer therapies.

Therefore, in the second part of this study we analyzed the inhibitory function of CTLA-4 and its effect on the expression of CD28 in CD8⁺ T cells. These studies revealed that, besides inducing an anergic phenotype in CD8⁺ T cells, CTLA-4 was capable of affecting the expression of CD28. It significantly reduced CD28 expression on the cell surface, by promoting internalization and degradation

of CD28. The reduced surface expression of CD28 may impede T cell response to stimulation or even lead to anergy or tolerance induction.

Our findings, the identification of a potential candidate for a cancer vaccine, and the elucidation of the inhibitory mechanisms of T cell regulation by CTLA-4, might prove helpful for the development of a successful cancer vaccine.

CHAPTER 11

ZUSAMMENFASSUNG

11.1 Teil I

Der antigenspezifische Rezeptor der T-Zellen erkennt einen Komplex aus antigenem Peptid, welches vom MHC präsentiert wird. Peptide von Tumorzellproteinen, sogenannte tumor-assoziierte Antigene (TAA) können für die Immuntherapie von Tumoren genutzt werden. Diese Peptide können von MHC Molekülen isoliert werden. Um neue TAA-assoziierte Peptide zu gewinnen, wurden Tumorzelllinien mit löslichen MHC Molekülen transfiziert. Diese wurden aufgereinigt und die gebundenen Peptide isoliert und mittels Massenspektrometrie analysiert. Auf diese Weise identifizierte Peptide wurden, wenn sie von tumor assoziierten Proteinen stammten, synthetisiert und eine Auswahl von diesen Peptiden hier auf ihre Affinität zu membrangebundenen MHC Molekülen und ihre Immunogenität untersucht.

Peptid p1028, welches aus dem Protein DNMT-1 (DNA-Methyl-transferase I) stammt, zeigte die höchste Affinität zu HLA-A2 und wurde von allen transfizierten Tumor-Zelllinien (Brust,- Ovarial,- und Prostatakrebszelllinien) produziert. DNMT-1 ist ein Enzym, dass in vielen Tumoren aussergewöhnlich hoch exprimiert ist, also ein potentielles Tumorantigen.

Zur Untersuchung der Immunogenität des isolierten Peptides wurden T-Lymphozyten mit Hilfe von p1028-beladenen autologen dendritischen Zellen stimuliert und daraufhin auf ihre Zytotoxizität und Spezifität getestet. p1028 zytotoxische T-Zellen (CTL) waren in der Lage spezifisch sowohl Peptid-beladene Zellen, als auch HLA-A2-positive Brustkrebszelllinien (MCF-7, MDA-231) zu lysieren. Stimulation der CTL mit Peptid p1028 hat ausserdem zur Interferon- γ Produktion geführt. Die Spezifität der CTL konnte anhand von Tetramer-Färbungen gezeigt werden. Da DNMT-1 ein tumor-assoziiertes Enzym ist, könnte

dieses DNMT-1-assoziierte HLA-A2-abhängige Peptid einen Kandidat zur Tumor-Immuntherapie darstellen.

Da der Erfolg der Tumor-Immuntherapie bis heute eher begrenzt ist, ist es notwendig, neben der Identifizierung von neuen TAA, die Effektivität der Therapien zu verbessern. Dies kann zum Beispiel durch Beeinflussung von inhibitorischen oder stimulierenden T-Zell-Corezeptoren, wie CTLA-4 und CD28, erreicht werden. Der zweite Teil dieser Arbeit befasst sich daher mit der Aufklärung des inhibitorischen Mechanismus von CTLA-4, insbesondere dem Einfluss von CTLA-4 auf die Expression von CD28 in CD8⁺ T Zellen.

11.2 Teil II

CD28 und CTLA-4 sind costimulatorische Moleküle auf der Oberfläche von T-Zellen, die zur Aktivierung oder Inaktivierung von T-Zellen beitragen. Es ist bekannt, dass die Bindung von B7 auf antigenpräsentierenden Zellen an CD28 eine Stimulation bewirkt, während Bindung von B7 an CTLA-4 zur Inhibition der T-Zelle führt. Der genaue inhibierende Mechanismus von CTLA-4 ist jedoch noch ungeklärt. In der vorliegenden Studie haben wir anhand von CTLA-4-transfizierten CD8⁺ T-Zellen den Mechanismus der Toleranz-Induktion durch CTLA-4 und den Einfluss von CTLA-4 auf die Expression von CD28 untersucht. Die Hochregulation von CTLA-4 auf CD8⁺ Jurkat T-Zellen führte zu Defiziten in der Produktion des Wachstumsfaktors IL-2 und zur Inhibition der Phosphorylierung von Proteintyrosinkinasen ZAP-70, p56^{lck} und p59^{fyn}.

Neben der Konkurrenz von CTLA-4 und CD28 für den gleichen Liganden zeigt diese Studie, dass CTLA-4 Einfluss auf die Expression von CD28 besitzt. Die Expression von CD28 auf der Zelloberfläche war auf CTLA-4-negativen Zellen nach Aktivierung durch den T-Zell-Rezeptor erhöht, während die Expression von CD28 in CTLA-4-transfizierten Zellen nach Aktivierung reduziert war. Eine Erklärung für dieses Phänomen ist die in CTLA-4 Transfektanten beobachtete induzierte Internalisierung von CD28. Ausserdem war die Proteinexpression von CD28 in

aktivierten CTLA-4 Transfektanten reduziert, was auf ubiquitin-abhängige Degradierung von CD28 zurückgeführt werden konnte.

Die in der vorliegenden Arbeit gesammelten Erkenntnisse zeigen einen neuen Mechanismus der T-Zell-Inaktivierung durch CTLA-4. Durch Verringerung der CD28-Expression auf der Zelloberfläche ist die Restimulation der aktivierten T-Zelle erschwert oder könnte sogar zur Anergisierung der T-Zelle führen.

Das Verstehen der Regulation von T-Zell-Aktivierung ist die Grundlage für die Entwicklung immunbasierter Therapien zur Toleranz-Induktion im Rahmen von Transplantationen oder zum Überwinden der T-Zell-Toleranz gegenüber Tumorzellen.

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Hiermit erkläre ich, dass diese Arbeit bisher von mir weder der Mathematisch-Naturwissenschaftlichen Fakultät der Christian-Albrechts-Universität Kiel noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Quellen benutzt habe.

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- Berg, M., Zavazava, N., Tumor-derived peptides eluted from tumor secreted MHC molecules elicit a T cell-mediated immune response; Prostate Cancer Congress, Iowa City, 2002
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