Tolerance and Immunity to Human Tumor-Associated Antigens

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To my parents

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Abbreviations

A2(.1)	HLA-A*0201
$A2K^b$	$A2.1/K^b$, class I MHC molecule that consists of the α1 and α2 domain of A2.1 and the α3 domain of $H\text{-}2K^b$
aa	Amino acid
Ab	Antibody
Ag	Antigen
AIRE	Autoimmune regulator
APC	Antigen-presenting cell
B-LCL	B-lymphoblastoid cell line
Blimp-1	B lymphocyte-induced maturation protein 1
BSA	Bovine serum albumin
c20S	Constitutive 20S proteasome
$cA2K^b$	Equivalent to $A2K^b$, but expressed by $cA2K^b$ transgenic mice, a different line of $A2K^b$ mice
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
DMSO	Dimethylsulfoxide
EBV	Epstein-Barr virus
ELISPOT	Enzyme-linked immuno spot (assay)
ER	Endoplasmic reticulum
E : T	Effector-to-target cell (ratio)
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FluM1	Influenza virus matrix protein 1
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HBSS	Hanks` balanced salt solution
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus

HLA	Human histocompatibility leukocyte antigen
hTyr	Human tyrosinase
Hu	Human
i20S	Immunoproteasome
IFA	Incomplete Freund`s adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
IRF4	Interferon regulatory factor 4
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MM	Multiple myeloma
mTyr	Mouse tyrosinase
Mu	Murine
MUM1	Multiple myeloma oncogene 1
MVA(-wt)	Modified vaccinia virus Ankara
MVA-hTyr	Modified vaccinia virus Ankara encoding human tyrosinase
MVA-mTyr	Modified vaccinia virus Ankara encoding murine tyrosinase
NK	Natural killer (cell)
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrine
PMSF	Phenylmethylsulfonyl fluoride
PRDI-BF1	Positive regulatory domain I-binding factor 1
pre B-ALL	Pre-B acute leukemia
PVDF	Polyvinylidene difluoride
rh	Recombinant human

RP-HPLC	Reverse phase high-performance liquid chromatography
RT	Reverse transcriptase
s.c.	Subcutaneous
SDS	Sodium dodecyl sulfate
SPI	Serine protease inhibitor
TBS	Tris-buffered saline
TCEP	Tris (2-carboxyethyl) phosphine hydrochloride
TCR	T cell receptor
Tg	Transgenic
TGF	Transforming growth factor
Th	T helper (cell)
Treg	T regulatory cell
Tyr	Tyrosinase
U	Unit
VSV-N	Vesicular stomatitis virus nucleoprotein
wt	Wildtype
XBP-1	X box-binding protein 1

Abbreviations are also used in plural.

Amino Acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1. Introduction

1.1 Tumor-associated antigens: The key to cytotoxic T lymphocyte (CTL)-based immunotherapy of malignant disease

T lymphocytes comprise different subsets of effector cells. The expression of two molecules, CD4 and CD8, allows the classification of at least two major subpopulations: $CD8^+$ cytotoxic T lymphocytes (CTL) that represent effector T cells of the cell-mediated immune response and $CD4^+$ T helper (Th) cells. Th cells can further be divided into at least two main classes, Th1 and Th2 cells that originate from an uncommitted Th cell precursor and arise during an immune response. Th1 cells that secrete interferon (IFN)-γ are responsible for cell-mediated immune responses and help to initiate a CTL attack whereas Th2 cells that secrete interleukin- (IL)-4 are involved in humoral immunity and help to activate B cells.

$CD8^+$ CTL recognize peptides, usually of 8-11 amino acids (aa) in length, presented by class I major histocompatibility complex (MHC) molecules on the cell surface. These peptides are derived predominantly from proteins of intracellular pathogens, commonly viruses. In humans, the genes for MHC molecules are called human (Hu) histocompatibility leukocyte antigen (Ag) (HLA) genes as they were first discovered through antigenic differences between white blood cells from different individuals. In mouse, they are known as the H-2 genes. The MHC-presented peptides are generated from proteolytic processing in the cytosol and nucleus. This step involves a multicatalytic enzyme complex, the proteasome (Rock and Goldberg, 1999). Precursor peptides are subsequently transported into the endoplasmic reticulum (ER) where they are further trimmed at their N-terminus by aminopeptidases (Serwold et al., 2002) before they are loaded onto class I MHC molecules for their transport to the cell surface. CTL detect these peptide Ag in association with class I MHC molecules via their T cell receptor (TCR), a situation termed class I MHC-restriction. The CD8 coreceptor contributes to this TCR-Ag recognition by its binding to a highly conserved domain of the class I MHC molecule, the α3 domain. The peptide-binding site of class I MHC molecules is formed by class I heavy chains which are encoded by genes that are highly polymorphic in various species. Most of the variability between these heavy chain alleles resides in the residues located in the peptide-binding groove. As a consequence, non-binding of pathogen-derived peptides to at least one of the available MHC variants

is an extremely rare event. Each class I allele presents therefore a unique spectrum of peptides to the immune system (Rammensee et al., 1993). Although the rules that govern peptide-binding to different alleles are far from simple, most peptides recovered from a given allele conform to a simple motif dictated largely by the nature of two or three “pockets” present at the bottom of the binding groove (Madden, 1995).

CTL recognition of an infected cell results in target cell destruction through the induction of the programmed cell death, apoptosis, and this occurs via two distinct mechanisms. The first cytotoxic mechanism involves exocytosis of cytotoxic granules. Apoptosis via this pathway is due to the combined actions of the pore-forming molecule perforin and the cytotoxic protease granzyme B (Heusel et al., 1994; Shresta et al., 1995). After secretion by the CTL, this granzyme enters the target cell via receptor-mediated endocytosis (Motyka et al., 2000; Froelich et al., 1996) and, following perforin-dependent release into the cytosol, initiates the apoptotic signalling cascade (Trapani et al., 2000; Heibein et al., 2000). The second pathway used by a CTL to kill its target occurs via cross-linking of death receptors on the target cell and mainly involves Fas ligand (CD95 ligand) which has been shown to induce a caspase cascade that leads to target cell death (Berke, 1995; Peter et al., 1999).

Apart from the ability of CTL to clear the host of intracellular pathogens, CTL were also shown to mediate efficient anti-tumor immune responses *in vivo* (Rosenberg, 1999; Hanson et al., 2000). The CTL-recognized peptide Ag on the tumor cell can be divided into two main categories. The first is known as tumor-specific Ag. Those genes are only expressed in malignant cells and not in nontransformed cells. The second group, called tumor-associated Ag, is present at elevated levels in tumor cells, but is also expressed at lower level in normal cells. Theoretically, tumor-specific Ag are the most desirable target molecules for anticancer treatment as they specifically target the CTL response towards the malignant cells. However, few tumor-specific Ag have been identified so far and this may be related to the fact that such Ag are frequently invisible to CTL because they do not successfully compete against the vast majority of peptides produced from normal cellular proteins. Another problem with tumor-specific Ag, such as mutated proteins, is that they are often restricted to an individual tumor and are therefore not suitable for a broader immunotherapeutic application in many patients. For these reasons, the majority of immunotherapeutic approaches to target malignant cells

by CTL are directed against tumor-associated Ag. Most of the experience in tumor immunotherapy has by far been gained in melanoma patients. The largest category of peptide Ag recognized by patient-derived melanoma-reactive CTL originates from proteins which are also expressed in normal melanocytes and are associated with pigment production (Engelhard et al., 2002), e.g., the tyrosinase (Tyr) molecule. The usage of these melanocyte differentiation Ag for the immunotherapy of malignant melanoma has been at least in part associated with positive clinical responses (Engelhard et al., 2002). These findings were encouraging to extend CTL-based immunotherapies to other malignancies.

Multiple myeloma (MM) is a fatal hematological malignancy characterized by an accumulation of monoclonal malignant plasma cells within the bone marrow (Bataille and Harousseau, 1997). To understand this paradign disease of humoral immunity, it is necessary to point out some features of plasma cell development. Plasma cells are terminally differentiated effectors of the humoral immune response. They produce and secrete high amounts of antibodies (Ab)/immunoglobulins (Ig). Naive B lymphocytes develop into plasma cells after specific Ag encounter. A small subset of B cells located in the marginal zone of the white pulp nodule in the spleen directly proliferates after Ag encounter. They differentiate into short-lived plasmablasts and give rise to the early humoral response characterized by low-affinity Ig. The majority of B cells in primary follicles, however, is selected after Ag recognition in spleen and lymph node germinal centers to produce Ab of high-affinity, a process called somatic hypermutation. The leaving cells either commit to long-lived plasma cells secreting high-affinity Ig or give rise to memory B cells. The non-secreting memory B cells allow a rapid and massive clonal expansion and generation of high numbers of plasmablasts in response to secondary Ag stimulation. MM appears to originate in a postgerminal center cell as malignant plasma cells underwent somatic hypermutation (Bakkus et al., 1994; Vescio et al., 1995). Interestingly, however, as the bone marrow of patients with MM contains B cell populations at different stages of differentiation which are clonally related to the malignant plasma cells, MM may represent a constellation of several subtypes of disease that may reflect differences in the cell of origin (Billadeau et al., 1993; Zhan et al., 2003).

Patients suffering from this disease have currently no curative therapeutic option (Attal et al., 1996). However, growing clinical and experimental evidence indicates that MM is susceptible to CTL-based immune interventions. These clinical data are solely derived from immunotherapy trials based on Ig idiotype vaccination (Stevenson and Anderson, 2000). The idiotype, a unique feature of an individual Ig that determines plasma cell clonality, represents a private tumor-associated Ag. It thus cannot provide shared target Ag for the immunotherapy of various MM patients. Broader MM-associated Ag have only rarely been identified and most of them are expressed in less than 30 % of malignant plasma cells from MM patients (Pellat-Deceunynck, 2003). Obviously, there is an urgent need for identifying MM-associated Ag that are expressed in all types of MM cells in order to allow a more common immunotherapeutic approach.

Recent work has begun to identify the regulatory cascade that iniates and maintains the plasma cell phenotype. Two transcription factors were shown to represent major regulators of plasmacytic differentiation, the X box-binding protein 1 (XBP-1) and the mouse B lymphocyte-induced maturation protein 1 (Blimp-1) and its Hu homologue, the positive regulatory domain I-binding factor 1 (PRDI-BF1) (Calame et al., 2003).

XBP-1 is a 28 kDa nuclear protein consisting of 261 aa that was first discovered based on its ability to bind an X box sequence in the promoter of a class II MHC allele (Liou et al., 1990). Apart from this, it was demonstrated that XBP-1 is absolutely required for the plasma cell phenotype, as in its absence no plasma cells develop (Reimold et al., 2001). The precise function of XBP-1 in plasma cell development has not been determined yet. Very recent data have shown that the production of high amounts of Ig in plasma cells is combined with the presence of unfolded proteins in the ER and induces an unfolded protein response (Iwakoshi et al., 2003). Activation of this response causes the splicing of a small intron from XBP-1 mRNA resulting in a frame shift that leads to the removal of the C-terminal 97 aa from open reading frame 1 and the addition of the 212 aa of open reading frame 2 (Yoshida et al., 2001). Thus, spliced XBP-1 mRNA encodes a protein of 376 aa. Additionally, it was suggested that it is only the spliced XBP-1 protein that is essential for plasma cell differentiation (Iwakoshi et al., 2003).

Blimp-1, a 98 kDa transcriptional repressor (located in the nucleus) has the unique ability to drive plasmacytic differentiation upon enforced expression in a B cell

lymhoma line (Turner, Jr. et al., 1994) or primary splenic B cells (Schliephake and Schimpl, 1996; Piskurich et al., 2000). Two of the five known direct targets of Blimp-1 repression are *c-myc* and *Pax5*. Repression of *c-myc* allows cessation of the cell cycle necessary to undergo differentiation (Lin et al., 2000). Blimp-1-mediated repression of *Pax5* is critical because it relieves repression of XBP-1 (Calame et al., 2003). *In vivo*, Blimp-1/PRDI-BF1 is found in all plasma cells, including those formed in a primary response, those formed from memory cells in a secondary response and in long lived plasma cells in the bone marrow (Angelin-Duclos et al., 2000).

For these reasons, it seemed attractive to investigate whether peptide Ag derived from these two transcription factors may provide abundant and universal MM-associated target molecules for CTL.

1.2 Self tolerance to tumor-associated antigens

One of the major problems governing the use of tumor-associated Ag for immunotherapy is their low-level expression in normal cells that can lead to the inactivation of reactive T cells expressing high-affinity TCR (high-avidity T cells) by immunological tolerance mechanisms. These tolerance mechanisms are operating both, in the thymus during T cell development (central tolerance) and in peripheral lymphoid and non-lymphoid organs (peripheral tolerance). They shape a T cell repertoire that does not respond to self Ag but reacts to a multitude of pathogen-derived Ag.

In brief, T lymphocytes develop in the bone marrow and leave as immature cells to migrate to the thymus where they are tested for recognition of self peptide bound to MHC molecules (Starr et al., 2003). Most of the immature T lymphocytes (thymocytes) lack TCR-specificity for self peptide/MHC complexes and these cells die by neglect as they fail to receive a TCR signal. A small proportion of thymocytes displays intermediate reactivity for MHC ligands and receives a low-level TCR signal that is protective and rescues these cells from death (positive selection). They survive and differentiate into mature T cells. Another subset of thymocytes has strong reactivity for self peptide/MHC complexes and receives a strong TCR signal that drives them to enter apoptosis (negative selection). One constraint on central deletion is the requirement for the relevant self Ag to be present in the thymus. Although recent data indicate that certain tissue-specific Ag are expressed in thymic medullary epithelial cells by virtue of

the autoimmune regulator (AIRE) (Derbinski et al., 2001; Kyewski et al., 2002; Anderson et al., 2002), this seems not to be the case with all tissue-specific proteins and it is unclear whether the amount of Ag that are expressed ectopically in the thymus is sufficient to allow tolerance induction. Nethertheless, negative thymic selection seems limited to T cells bearing high-affinity TCR for self MHC ligands, allowing self-reactive T cells of lower avidity to escape into the periphery.

These latter cells are then held in check by peripheral mechanisms of tolerance and homeostasis (Walker and Abbas, 2002; Jameson, 2002). Peripheral tolerance can act at several levels. The simplest scenario involves T cell ignorance of self Ag, either because the latter are sequestered in sites not easily accessible to the immune system (Alferink et al., 1998; Zinkernagel, 1996) or because the amount of Ag does not reach the threshold required to trigger a T cell response (Kurts et al., 1998). Alternatively, T cell encounter with self Ag leads to functional inactivation, called anergy. The rationale for maintaining anergic T cells is not clear at all although this evidently happens in vivo (Pape et al., 1998). Neither the number of cell-surface receptors involved, nor their precise nature has been determined. The most effective way that prevents autoimmune destruction by a given T cell clone is the deletion of that specificity from the repertoire by activation-induced cell death. A key mechanism underlying this deletion is the ligation of the Fas receptor by its ligand (Green et al., 2003). This is underscored by the finding that defects in the Fas pathway in humans are associated with the autoimmune lymphoproliferative syndrome (Watanabe-Fukunaga et al., 1992; Fisher et al., 1995).

Apart from these tolerance mechanisms that act directly on the responding T cell, other mechanisms have been described that evoke additional subsets of cells, including dendritic cells (DC) and T regulatory cells (Treg). The phenomenon of tissue-derived Ag being transported to local lymphoid tissues and presented to T cells in a tolerogenic manner has been carefully studied using mice that express various transgenes (e.g., hemagglutinin or ovalbumin) as model self Ag. This approach has shown that self proteins are continuously sampled by bone marrow-derived Ag-presenting cells (APC) for presentation to T cells (Kurts et al., 1996; Adler et al., 1998) and it was also observed that DC alone are sufficient to perform this function (Kurts et al., 2001). As DC are also potent APC for the induction of T cell immunity, these data have raised the questions whether a distinct lineage of tolerogenic DC exists or whether the decision of

tolerance versus immunity rather depends on the activation/maturation status of the DC. It is commonly believed that mature DC have the ability to activate T cells and initiate an immune response. Recent works that suggest immature DC to mediate T cell tolerization (Mahnke et al., 2002) seem to support the idea that a distinct tolerogenic DC is not necessary to confer T cell tolerance. However, even these reports do not necessarily preclude the existence of a tolerogenic DC subset.

A significant development in the field of tolerance has been the re-emergence of the concept that T cell reactivity is controlled indirectly by a distinct subset of T cells with a regulatory function. Many types of Treg have been described in a number of systems (Roncarolo et al., 2001). Treg seem to suppress immune responses via production of cytokines, for example IL-10 and transforming growth factor (TGF)-β, and/or cell-to-cell interactions (Roncarolo et al., 2001). Besides type I Treg that are defined by their ability to produce high levels of IL-10 and TGF-β and suppress naive Th type I and II responses (Roncarolo et al., 2001), the $CD4^+$ Treg that constitutively express CD25 are best characterized (Sakaguchi et al., 2001). Thymic-derived $CD4^+CD25^+$ T cells comprise 5-10 % of peripheral $CD4^+$ T cells in normal mice and exhibit potent immunoregulatory functions *in vitro* and *in vivo* (Sakaguchi et al., 2001). These cells require activation via the TCR to exert their regulatory function, once activated their suppressive function is Ag non-specific, seems to require cell-to-cell contact, and is possibly linked to their constitutive expression of CTL-associated Ag 4 (CTLA-4) (Sakaguchi et al., 2001). In contrast to Treg, CTLA-4 is not constitutively expressed on conventional T cells but induced by T cell activation to exert its critical role in inhibiting signalling through the TCR and eventually terminating the T cell response (Egen et al., 2002).

All of the described tolerance mechanisms may limit the available T cell repertoire with specificity for self and nonmutated tumor-associated Ag and may thus account for the limiting responses obtained in immunotherapeutic approaches using melanocyte-differentiation Ag in melanoma patients. Therefore, an important aspect of using these and other tumor-associated Ag is a better understanding of the establishment, maintenance and breaking of their tolerogenic potential.

An attractive and well characterized target molecule that allows these important studies is Tyr. Tyr is the key enzyme involved in pigmentation that is expressed not only by

malignant melanomas and normal melanocytes, but also by nonmutated cells of the eye and central nervous system, and, based on polymerase chain reaction (PCR) amplification of Tyr transcripts, by a broad range of Hu tissues as well (Colella et al., 2000). Limited responses, if any, were observed in anti-self Hu Tyr (hTyr)-based clinical vaccination trials of melanoma patients (Scheibenbogen et al., 2000; Mackensen et al., 2000; Lewis et al., 2000; Mitchell et al., 2002). In contrast, however, strong melanoma-reactive immune responses were revealed by challenging HLA transgenic (Tg) mice with the xenogeneic, non-self hTyr Ag (Drexler et al., 1999). As with other tumor-associated self Ag (Theobald et al., 1997; Stanislawski et al., 2001), these findings could be interpreted as an effect on the repertoire due to Tyr-specific self tolerance (Colella et al., 2000), thereby limiting the frequency and avidity of Tyr-reactive CTL.

By taking advantage of the posttranslationally modified, HLA-A*0201 (A2.1)-presented hTyr (369-377) (YMDGTMSQV) CTL epitope and its naturally processed mouse homologue, mTyr (369-377) (FMDGTMSQV) (Colella et al., 2000; Skipper et al., 1996), it was possible to study the impact of self tolerance on Tyr-specific CTL in A2.1 Tg mice and in man, and its susceptibility to Treg.

1.3 Strategies to circumvent self tolerance

Limitation in efficacy of CTL responses to tumor-associated Ag is the leading force for the development of strategies to overcome self tolerance. One approach is based on the fact that Ag-specific self tolerance of T cells is self MHC-restricted (Matzinger et al., 1984; Rammensee and Bevan, 1984). The nature of TCR-peptide/MHC recognition that allows a TCR to recognize a variety of foreign peptides in association with self MHC leads to a significant number of T cells that have the ability to crossreact to non-self, allogeneic MHC molecules (Geluk et al., 1997). This alloreactivity is well-known as it is responsible for rapid rejection of transplanted organs from MHC-mismatched donors. There are two ways by which a TCR may bind to non-self MHC molecules (Lechler et al., 1990). In some cases, the peptide bound by the non-self MHC molecule interacts strongly with the TCR and the T cells bearing such a receptor are stimulated to respond (peptide-dependent or peptide-dominant alloreactivity). In a second type of crossreactive recognition, known as peptide-independent or MHC-dominated

alloreactivity, T cells respond because of the direct binding of the TCR to distinctive features of the non-self MHC molecule. As alloreactive T cells are not tolerant to allogeneic peptide/MHC complexes and a significant fraction of alloreactive T cells is peptide-specific in its recognition of foreign peptide/MHC molecules, it is possible to bypass self tolerance by inducing allo MHC-restricted T cells specific for self tumor-associated Ag (Sadovnikova and Stauss, 1996). Allorestricted T cells generated from an HLA-mismatched donor are likely to be rejected rapidly by the host unless the recipient is profoundly immunosuppressed. Therefore, the transfer of an allorestricted TCR into patient T cells provides a technology to overcome these limitations.

The feasibility of TCR transfer has been demonstrated recently in a different model that also aims at circumventing tolerance (Stanislawski et al., 2001). This strategy is based on A2.1 Tg mice in order to generate A2.1-restricted CTL specific for peptides derived from Hu self proteins associated with cancer. Both, the Ag-processing machinery and the T cell repertoire of humans and these Tg mice are sufficiently similar to select the same immunodominant Ag (Theobald et al., 1995). A2.1 was selected as it is one of the most frequent class I alleles in humans. However, tolerance to endogenous murine (Mu) peptide epitopes does not affect CTL with specificity for Hu epitopes non-identical in sequence to their Mu counterparts. This approach allowed not only the identification of a number of different Hu tumor-associated Ag but also the generation of high-avidity CTL whereas only low-avidity effector T cells, if any, of the same specificity were found in the Hu T cell repertoire (Theobald et al., 1995; Stanislawski et al., 2001). Subsequently, a Tg mouse-derived high-affinity TCR specific for one tumor-associated Ag was cloned, partially humanized and retrovirally transferred into Hu T cells. Both, avidity and Ag-specificity were reconstituted in the Hu T cells after retroviral transduction of TCR genes (Stanislawski et al., 2001). Thus, this method rescues the Hu T cell repertoire by producing high-affinity TCR that have been lost through the establishment of Ag-specific tolerance.

Alternative approaches of generating high-affinity TCR for tumor-associated peptide epitopes have been described recently. For example, *in vitro* mutagenesis followed by selection with the relevant synthetic MHC-peptide complexes can be used to isolate TCR with improved Ag-specific binding-affinity (Kessels et al., 2000). This technology allows to convert low-affinity TCR specific for tumor-associated self Ag into high-

affinity TCR and thus would improve tumor cell recognition (Kessels et al., 2001a). Phage display of single chain Ab fragments provides another strategy for the isolation of Ab molecules that bind specifically to a given MHC/peptide complex, thus mimicking the binding-specificity of TCR (Chames et al., 2002).
Based on these various developments, it seemed attractive to take advantage of A2.1 Tg mice in order to investigate the concept of universal MM-targeting by CTL specific for peptide epitopes to be identified from PRDI-BF1 and XBP-1 transcription factors.

1.4 Specific aims

The studies presented in this work aimed at:

Part 1

- Analyzing the level of Tyr-specific tolerance in A2.1 Tg mice.

- Investigating the regulation of Tyr-specific tolerance by $CD4^+CD25^+$ Treg and CTLA-4.

- Evaluating Tyr-specific tolerance in man.

Part 2

- The identification of PRDI-BF1- and XBP-1-derived A2.1-presented CTL epitopes.

- Evaluating whether PRDI-BF1- and XBP-1-derived peptide epitopes provide universal MM-associated Ag.

- Characterizing PRDI-BF1- and XBP-1-specific CTL in their response to various tumor entities and nontransformed cells.

- Investigating PRDI-BF1- and XBP-1-specific tolerance in man.

2. Materials and Methods

2.1 Mice

Tg mice (Sherman et al., 1992) were kindly provided by L. A. Sherman (The Scripps Research Institute, La Jolla, CA) (TSRI). All mice were in the C57BL/6 (H-2^b) background. Following Tg mice lines were used:

A2.1/K^b ($A2K^b$) Tg mice were homozygous for both, the H-2^b gene and the chimeric transgene $A2K^b$ which encoded the α1 and α2 domain of the Hu A2.1 and the α3 domain of the Mu H-$2K^b$ molecule to allow the interaction with Mu CD8.

$cA2K^b$ was a homozygous $A2K^b$ Tg line, was derived from a different founder animal and expressed a 3-fold higher level of the transgene as compared to line $A2K^b$.

A2.1 Tg mice were homozygous for the H-2^b gene and heterozygous for the Hu transgene A2.1.

CD8 Tg mice were homozygous for both, the Mu CD8 and the Hu CD8 transgene which encoded the α and β chain of the Hu CD8 coreceptor.

CD8 x A2.1, CD8 x $A2K^b$ and CD8 x $cA2K^b$ were heterozygous for both transgenes and expressed Hu CD8αβ and either A2.1, $A2K^b$ or $cA2K^b$. All mice were homozygous for the H-2^b gene.

C57BL/6 mice were purchased from the breeding colony of the Johannes Gutenberg-University.

Mice were maintained at the animal facility of the Johannes Gutenberg-University. Experimental procedures were performed according to German federal and state regulations and the NIH Guide for the Care and Use of Laboratory Animals.

2.2 Synthetic peptides

All peptides were synthesized by Affina Immuntechnik (Berlin) or Neosystem S.A. (Strasbourg). Purity of synthetic peptides was ascertained by reversed phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry and was > 80%. Purity of PRDI-BF1 (401-410) and (402-410) was > 97%.

Besides peptides shown in table 1 and 2, hTyr (369-377) (YMDGTMSQV) and mTyr (369-377) (FMDGTMSQV), a peptide representing residues 128-140 of the hepatitis B virus (HBV) core protein, HBV core (128-140) (TPPAYRPPNAPIL) (Sette et al., 1994), a peptide representing residues 476-484 of the Hu immunodeficiency virus

(HIV) reverse transcriptase (RT), HIV-RT (476-484) (ILKEPVHGV) (Tsomides et al., 1991), and a peptide representing residues 264-272 of the Hu p53 protein, p53 (264-272) (LLGRNSFEV) (Theobald et al., 1995), were synthesized. Based on their aa sequence, all peptides were dissolved at 10 mg/ml in dimethylsulfoxide (DMSO) (Sigma, Deisenhofen), DMSO/H_2O or H_2O.

2.3 Antibodies

A2.1 expression of various cells, cell lines and A2.1 Tg mice was determined by staining with anti-A2.1 (BB7.2, mouse IgG1) (ATCC HB-82; LGC Promochem, Wesel) hybridoma supernatant and fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (polyclonal $F(ab`)_2$, Jackson Immuno Research, Dianova, Hamburg) Ab.

Peripheral blood mononuclear cells (PBMC) were polyclonally activated with anti-CD3/CD28 magnetic beads (Dynabeads, Dynal, Oslo, Norway).

Western blot analysis of XBP-1 expression was performed with anti-XBP-1 (polyclonal rabbit IgG) Ab ((M-186), Santa Cruz, Heidelberg) and peroxidase-labeled anti-rabbit IgG (polyclonal goat IgG) Ab (Southern Biotech Associates, Birmingham, AL, USA).

PRDI-BF1 expression was evaluated in immunohistochemistry using a crossreactive anti-Blimp-1 antiserum (rabbit) (kindly provided by Dr. K. Calame, Columbia University, New York, USA) (Chang et al., 2000). Pre-immune serum (isolated from a New Zealand rabbit) served as control. Secondary Ab, biotin-labeled anti-rabbit IgG (polyclonal goat IgG) (Southern Biotech Associates), was detected by peroxidase-conjugated streptavidin (Southern Biotech Associates).

Treg were depleted by administration of purified anti-CD25 (PC61, rat IgG) and anti-CTLA-4 (9H10, hamster IgG) monoclonal Ab (mAb). Control rat and hamster IgG were purchased from Jackson ImmunoResearch, Dianova. Depletion was confirmed by staining with FITC-labeled anti-CD25 (7D4, rat IgM) and phycoerythrin (PE)-labeled anti-CD4 (RM4-5, rat IgG2a) mAb, rat IgM-FITC and rat IgG2a-PE served as controls, all were obtained from Becton Dickinson (San Jose, CA, USA). Fc receptors were blocked using anti-CD16/32 mAb (Becton Dickinson).

MHC-immunoprecipitation was performed with anti-HLA-A, -B, -C (W6/32, mouse IgG2a) (ATCC HB-95) purified mAb.

IFN-γ secretion by Hu and Mu CTL was measured in an enzyme-linked immuno spot (ELISPOT) assay. Hu IFN-γ secretion was determined with anti-Hu IFN-γ capture (1-D1K, mouse IgG1) and biotin-labeled detection (7-B6-1, mouse IgG1) (Mabtech AB, Hamburg) mAb followed by avidin-peroxidase (Vectastain Elite Kit, Vector, Burlingames, CA, USA). Mu IFN-γ secretion was examined with anti-mouse IFN-γ capture (RMMG-1, rat IgG1) (Biosource Europe, Solingen) and biotin-labeled detection (XMG 1.2, rat IgG1) (Becton Dickinson) mAb followed by streptavidin-peroxidase (Jackson Immuno Research, Dianova).

2.4 Viruses, cells and cell lines

All cells were maintained in RPMI (Bio Whittaker, Verviers, Belgium) supplemented with 10 % fetal calf serum (FCS) (PAA, Pasching, Austria), 1 % glutamine (Bio Whittaker), 50 µg/ml gentamycin (Gibco BRL, Invitrogen, Karlsruhe) and 5 x 10^{-5} M β-mercaptoethanol (Sigma) (RPMI complete), unless otherwise indicated.

Hu B cell-derived tumor cell lines included the $A2.1^+$ MM cell lines U266 (ATCC TIB 196), DEPU (LB-1696) (kindly provided by N. von Baren, Ludwig Institute for Cancer Research, Brussels, Belgium), L363 and OPM-2 (Stanislawski et al., 2001) as well as the $A2.1^-$ MM cell lines LP-1 (DSMZ, ACC41, Brunswick) and NCI-H929 (ATCC CRL-9068), the $A2.1^+$ Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCL) SY (Drexler et al., 1999), MC-CAR (ATCC CRL-8083), JY and IM9 (Stanislawski et al., 2001), the $A2.1^+$ pre-B acute leukemia cell lines (pre B-ALL) EU3 and UoC-B11 (Stanislawski et al., 2001), and the $A2.1^-$ pre B-ALL EU1 and UoC-B1 (Stanislawski et al., 2001).

$A2.1^+$ solid tumor-derived cell lines were the osteosarcoma U2OS (Stanislawski et al., 2001), the colorectal cancer cell line SW480, the breast cancer cell lines BT549 and MCF7 (Theobald et al., 1995), the cervix carcinoma CaSki (ATCC CRL 1550), the hepatocellular carcinoma HepG2 (ATCC HB-8065), and the melanoma cell lines Malme 3M (ATCC HTB-64), NA8-Mel and the same cells transfected with the gene for hTyr (NA8-Mel-Tyr) (Drexler et al., 1999), kindly provided by T. Wölfel (Johannes Gutenberg-University).

The TAP-deficient cell line T2 and the same cells transfected with the gene for chimeric $A2K^b$ (T2 $A2K^b$), Jurkat-A2 (JA2) and the mouse thymoma EL4 have been described

(Theobald et al., 1995). Natural Killer (NK)-sensitive K562 cells (ATCC CCL-243) were used as cold and hot targets.

The Mu melanoma cell line B16-F1 (ATCC CRL-6323) was transfected with plasmid pZeoSV2+ (Invitrogen) containing the genes for $A2K^b$ and zeocin (B16-$A2K^b$-zeocin) (kindly provided by I. Drexler, Institute for Molecular Virology, GSF, and Institute for Virology, Technical University, Munich). One transfectant with zeocin resistance only served as negative control (B16-zeocin). B16 transfectants were pretreated with Mu IFN-γ (R&D Systems, Wiesbaden) at 20 ng/ml for 20 h when used as targets in ^{51}Cr-release assays.

For Western blot analysis, whole cell lysates were obtained from U266 cells that were treated with or without recombinant Hu (rh) IL-6 (PBH Strathmann Biotech, Hanover) at 2275 units/ml (U/ml) for 15 h as reported (Wen et al., 1999).

SY cells were infected for 7 h with wildtype (wt) modified vaccinia virus Ankara (MVA) (isolate F6) (MVA-wt), MVA-hTyr and MVA-mTyr at 10 plaque forming units per cell as reported (Drexler et al., 1999) and subsequently used as targets in ^{51}Cr-release assay. MVA-hTyr and MVA-mTyr expressing Hu and Mu Tyr under control of the strong early-late synthetic promoter and MVA-wt were provided by I. Drexler.

Monocyte-derived mature DC were generated under serum-free conditions as described (Stanislawski et al., 2001). Briefly, PBMC were isolated by Ficoll (Seromed, Biochrom, Berlin) density centrifugation and washed in phosphate-buffered saline (PBS) (Bio Whittaker) to remove platelets. Cells were resuspended in serum-free X-VIVO 15 (Bio Whittaker) and seeded for 45 min at 37 °C and 5 % CO_2 in 6-well plates (Costar/Corning, Wiesbaden) at 1 x 10^7 cells/well. Non-adherent cells were removed by washing the wells with medium. Adherent cells were cultured in serum-free X-VIVO 15 containing rh IL-4 at 1000 U/ml (PBH Strathmann Biotech), rh granulocyte/macrophage colony-stimulating factor (GM-CSF) at 800 U/ml (Leukomax 300, Sandoz, Nuremberg) and 1.5 % autologous heat-inactivated plasma. On days 3 and 5, 1 ml/well of fresh medium supplemented with rh IL-4 at 1000 U/ml and rh GM-CSF at 1600 U/ml was added. On day 7, immature DC were harvested, washed and seeded in fresh serum-free X-VIVO 15 1.5 % autologous heat-inactivated plasma at 1 x 10^6 cells/well, and the following cytokines were added for 2-3 days to induce maturation: rh IL-4 at 500 U/ml, rh GM-CSF at 800 U/ml, rhIL-1β at 10 ng/ml, rh IL-6 at 1000 U/ml (PBH Strathmann

Biotech), tumor necrosis factor-α at 10 ng/ml (R&D Systems), and prostaglandin E_2 at 1 µg/ml (Minprostin E_2, Pharmacia & Upjohn, Fribourg). Mature DC were used as targets in ^{51}Cr-release assay or as autologous stimulator cells for Hu T cells. The maturation state of DC was ascertained by flow cytometry including anti-CD83 staining (data not shown).

Isolated PBMC were also used as targets in ^{51}Cr-release assay, were polyclonally activated in the presence of anti-CD3/CD28 Dynabeads (1 µl beads/1 x 10^6 cells) in RPMI complete containing 40 U/ml IL2 (Proleukin, Chiron), or were further separated to obtain purified T and B cell populations. T and B cells resulted from negative selection of PBMC using Dynabeads (Dynal) labelled with anti-CD19 plus anti-CD14 or anti-CD2 plus anti-CD14 mAb, respectively, and was performed according to the manufacturer`s advice.

A2.1$^+$ hepatocytes were kindly provided by S. Strand, I. Med Klinik, Johannes Gutenberg-University. Tubular epithelial cells were provided by the group of A. Schwarting**,** I. Med Klinik, Johannes Gutenberg-University. A2.1$^+$ lung fibroblasts (MRC-5, ATCC CCL-171) were cultured in DMEM supplemented with 10 % FCS, 1 % glutamine, 50 µg/ml gentamycin and 5 x 10^{-5} M β-mercaptoethanol.

Primary MM cells were obtained from bone marrow of MM patients and analyzed by fluorescence activated cell sorter (FACS) for the content of clonal plasma cells. Cell samples were stored in liquid nitrogen until they were used as targets in ELISPOT.

Lipopolysaccharide (LPS)-activated spleen cells were obtained by incubating spleen cells for 3 days at 1 x 10^6 cells/ml with 25 µg/ml LPS (Salmonella typhosa) (Sigma) and 7 µg/ml dextran sulfate (Pharmacia Biotech, Fribourg).

2.5 Flow cytometric analysis

Depletion of Treg was monitored by FACS analysis of blood lymphocytes from Ab-treated animals. Briefly, blood was collected in heparin-coated tubes (Brand, Wertheim) and erythrocytes were lysed using standard laboratory protocols. Cells were washed in PBS 0.1 % bovine serum albumin (BSA) (Sigma). Prior to staining, Fc receptors were blocked using anti-CD16/32 mAb to avoid non-specific binding of Ab via their Fc domain. After being washed with PBS 0.1 % BSA, cells were stained with FITC-labeled anti-CD25 (7D4) and PE-labeled anti-CD4 (RM4-5) mAb or rat IgM-FITC and

rat IgG2a-PE controls for 15 min at room temperature. Cells were again washed with PBS 0.1 % BSA, fixed in PBS containing 1 % formaldehyde and analyzed with Cellquest Pro 2.0 on a FACSort instrument (Becton Dickinson).

A2.1 expression was analyzed in the same way except for a 2-step instead of a 1-step staining procedure. Cells were first stained with primary Ab BB7.2 (30 µl supernatant) for 1 h at 4 °C and after being washed in PBS 0.1 % BSA, cells were incubated with secondary Ab anti-mouse IgG (polyclonal $F(ab`)_2$) (diluted 1 : 30 in PBS) for 30 min on ice.

2.6 Western blot analysis

Approximately 2 x 10^7 cells were washed twice in PBS at room temperature. After centrifugation (1500 rpm, 5 min, room temperature) supernatant was carefully removed, cells were resuspended in 400 µl of ice cold RIPA buffer (PBS, 1 % Nonidet P-40 (Roche, Mannheim), 0.5 % sodium deoxycholate (Sigma), 0.1 % sodium dodecyl sulfate (SDS) (Roth, Karlsruhe)) in the presence of freshly added proteinase inhibitors (100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 50 µg/ml Aprotinin, 1 mM sodium orthovanadate, 10 µg/ml Leupeptin, 7.5 µg/ml Pepstatin A, and 1 mM dithiothreitol (all obtained from Sigma)) and incubated on ice for 30 min. Cells were further disrupted and homogenized by passage through a 21 gauge needle. After adding 4 µl of 10 mg/ml PMSF stock, lysates were incubated for further 30 min on ice and centrifuged (13000 rpm, 10 min, 4 °C). The supernatant that represents the total cell lysate was analyzed for protein content by the Bradford protein quantitation method. Accordingly, 60 µg of whole cell proteins was resolved by electrophoresis on 12 % SDS-polyacrylamid gels according to standard protocols and electroblotted overnight onto a polyvinylidene difluoride (PVDF) membrane (Roche) in buffer containing 25 mM Tris (Sigma), 190 mM glycine (Roth), 0.1 % SDS (Roth), and 20 % methanol. Blots were washed in PBS, non-specific binding was blocked with diluted (1 : 10 in PBS) blocking solution (BM chemiluminescence blotting substrate kit, Roche) for 60 min and labeled with diluted (1 : 200 in PBS 10 % blocking solution) anti-XBP-1 (polyclonal rabbit IgG) Ab for 2 h. After being washed with PBS 0.1 % Tween 20, blots were treated with diluted blocking solution (1 : 20 in PBS) for 20 min, and incubated with peroxidase-conjugated diluted (1 : 10000 in PBS 5 % blocking solution) anti-rabbit IgG Ab for 2 h. Blots were washed

with PBS 0.1 % Tween 20 and then in PBS alone. Finally, blots were developed and labeled XBP-1 protein was visualized by chemiluminescence using ECL (western blotting analysis system, Amersham Biosciences, Buckinghamshire, England).

2.7 Immunohistochemistry

For PRDI-BF1 expression analysis, 2 x 10^5 cells were washed twice in PBS and spun down on microscope slides (4 x 10^4 cells/spot). All steps were performed at room temperature, unless otherwise indicated. Slides were air-dried for 1 h and cell spots were encircled using a pap pen (Immunotech, Marseille, France). Slides were fixed for 20 h in 4 % formalin (Department of Pathology, Johannes Gutenberg-University) and for 10 min in methanol. To eliminate endogenous peroxidase activity, slides were pretreated with PBS 0.3 % H_2O_2 (Central Pharmacy, Johannes Gutenberg-University) for 10 min and washed twice with tris-buffered saline (TBS: 20 mM tris base, 140 mM sodium chloride, pH 7.6). Slides were incubated in TBS 10 % goat serum (PAA Laboratories) 1% BSA for 30 min. Blocking solution was removed and cells were stained with diluted (1:1000 in TBS 1 % BSA) primary Ab, anti-Blimp-1 antiserum (rabbit), for at least 14 h at 6-12 °C (Chang et al., 2000). Pre-immune serum (rabbit) was used as negative control for each cell type to ensure that the staining observed was not due to background. Slides were washed in TBS 0.1 % Tween 20 (Sigma). Biotinylated secondary Ab, goat anti-rabbit IgG, was diluted 1 : 200 in TBS 1 % BSA and applied to the slides. After a 30 min incubation, slides were again washed with TBS 0.1 % Tween 20. Finally, slides were treated for 30 min in diluted (1 : 200 in TBS 1 % BSA) peroxidase-streptavidin, washed four times with TBS 0.1 % Tween 20 and once with TBS. For color development slides were treated with aminoethyl carbazole (Sigma) for 2-3 min. AEC tablets were dissolved according to manufacturer`s instructions. The reaction was stopped by washing the slides in tap water. Slides were lightly counterstained with hematoxylin (Sigma) for a few seconds. After washing in tap water, slides were sealed with aquaeous mounting medium (Aquatex, Merck, Darmstadt) and subsequently examined under a laser scan microscope (Zeiss, Jena) (with the assistance of D. and S. Strand, I Med Klinik, Johannes Gutenberg-University).

2.8 Peptide-binding to HLA-A*0201 (A2.1)

A competition assay (Theobald et al., 1995) was used to assess binding of hTyr (369-377) and mTyr (369-377) and PRDI-BF1/XBP-1 peptides to A2.1. T2 cells were pulsed with 0.01 µg of the A2.1-binding peptide Hu p53 (264-272) and either 3 or 10 µg test peptide. The A2.1-restricted p53 (264-272)-specific CTL clone, A2 clone 46, was assayed at various effector-to-target (E : T) ratios for lytic activity against the peptide- and nonpeptide-pulsed T2 targets in a 4 h ^{51}Cr-release assay. Percent inhibition of clone 46-mediated lysis of p53 (264-272)-pulsed T2 cells by the indicated peptides was calculated at an E : T ratio of 3 : 1.

The A2.1-binding peptide representing residues 58-66 of the influenza virus matrix protein 1 (FluM1), FluM1 (58-66) (Morrison et al., 1992), served as positive control. The H-2K^b-binding peptide of the vesicular stomatitis virus nucleoprotein (VSV-N), VSV-N (52-59), served as negative control (Theobald et al., 1995).

2.9 Peptide priming of A2.1 transgenic mice, propagation of CTL and depletion of T regulatory cells

Depletion of Treg was accomplished by intraperitoneal (i.p.) injection of 400 µg of anti-CD25 (PC61) mAb at day -4 of Ag priming, as described previously (Sutmuller et al., 2001; Onizuka et al., 1999). Depletion was confirmed by flow cytometry using the noncrossreactive mAb, anti-CD25 (7D4), in combination with anti-CD4-PE on day 0. Mice were injected i.p. with 200 µg of anti-CTLA-4 (9H10) mAb on days -1, 0 and +1 of Ag priming (Hernandez et al., 2001).

For immunization, mice were injected subcutaneously (s.c.) at the base of the tail with 100 µg of the indicated peptide and 120 µg of the I-A^b-binding synthetic Th peptide HBV core (128-140) emulsified in 100 µl of incomplete Freund`s adjuvant (IFA) (DIFCO, Detroit, USA). After 10 days, spleen cells of primed mice were cultured at 7 x 10^6 cells/well with irradiated A2K^b or A2.1 Tg LPS-activated spleen cell stimulators (3 x 10^6 cells/well) that had been pulsed with the priming peptide (unless otherwise indicated) (Theobald et al., 1995). After 6 days of *in vitro* stimulation, the resultant effector cells were assayed in a 4-6 h standard ^{51}Cr-release assay.

Polyclonal and peptide-specific CTL lines were established by weekly restimulation of primary CTL with irradiated peptide-pulsed JA2 (0.5 x 10^6 cells/well), irradiated

C57BL/6 spleen filler cells (6 x 10^6 cells/well) and 2-10 % (vol/vol) rat concanavalin A supernatant (Theobald et al., 1995). Unless otherwise indicated, stimulator cells were pulsed with peptide at 5 µg/ml in the presence of 5 µg/ml of Hu β_2-microglobulin (Sigma) in RPMI and unbound peptide was removed by washing the cells twice in RPMI complete.

The derivation of an A2.1-restricted CTL line specific for FluM1 (58-66) (CTL CD8 x $A2K^b$ FluM1) has been described (Drexler et al., 1999). An alloreactive A2-specific CTL line (CTL CD8 Allo A2) was established as reported (Theobald et al., 1995). The CTL clone, A2 p53 (264-272) clone 46, was established by limiting dilution of a polyclonal CTL line A2 p53 (264-272) (Theobald et al., 1995). CTL lines were used as effector cells in 4-6 h ^{51}Cr-release assays.

2.10 Inhibiting 20S proteasomal antigen production

OPM-2 cells were washed in Hanks` balanced salt solution (HBSS) (Bio Whittaker) and exposed for 1 min to 0.13 M citric acid/0.061 M $Na_2HPO_4$ (pH 3.0). Cells were washed in RPMI and cultured for 5 h with or without lactacystin (Boston Biochem, Cambridge, MA) at 10 and 30 µM. Lactacystin was dissolved according to manufacturer`s instruction in DMSO. Cells were used as targets in a 5.5 h ^{51}Cr-release assay in the continuous presence or absence of lactacystin at 10 and 30 µM.

2.11 Extracting natural peptides by MHC-immunoprecipitation and RP-HPLC separation

Pellets of approximately 4 x 10^{10} OPM-2 and U266 cells were collected and treated with lysis buffer (PBS, 0.6 % CHAPS (Roche), + 3 complete protease inhibitor tablets/200 ml (Roche)). Lysates were homogenized, ultracentrifuged (1h at 40 000 rpm) and filtrated, before incubating with anti-HLA-A, -B, -C (W6/32) mAb for 1 h at 4 °C. Lysates/Ab mixtures were circulated over a protein A column (Pharmacia Biotech) overnight. MHC-peptide/Ab complexes were eluted with citrate buffer (50 mM sodium citrate, 0.02 % sodium azide; pH 3.0). The eluates were treated with trifluoroacetic acid and filtered through a Centricon filter (Amicon, Beverly, MA). The centricon filtrates were desalted and concentrated with a RP-C18 column (Agilent Technologies, Waldbronn) under standard conditions. The desalted samples were separated by a RP-

HPLC SMART system (accomplished by S. Stevanovich, Institute for Cell Biology, Tübingen).

2.12 ^{51}Cr-release and ELISPOT assay

Lytic activity of Hu and Mu CTL was determined in a standard ^{51}Cr-release assay. In brief, 1 x 10^6 target cells were labeled with 100 µCi ^{51}Cr (Perkin Elmer, Zaventem, Belgium) in PBS 10 % FCS. In case of peptide-pulsed target cells, 2 µl of the relevant peptide dilution was added. To test the antigenicity of HPLC fractions, T2 target cells were pulsed with 2/3 of each fraction in serum-free RPMI containing 5 % BSA and Hu β_2-microglobulin at 10 µg/ml. Target cells were incubated for 1.5 h and excess radioactivity was removed by subsequent washing in RPMI 2 % FCS. Target cells were adjusted to 1 x 10^5 cells/ml in RPMI complete. Effector cells were prepared in RPMI complete according to the respective E : T ratio and added to 96-well microtiter plates (Corning/Costar, Wiesbaden) as 3-fold serial dilutions with replicates of two wells for each effector cell concentration. Target cells were added (1 x 10^4 cells/well) and cocultures were incubated for 4-6 h at 37 °C and 5 % CO_2. For measuring lytic activity under reducing conditions, tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was freshly dissolved in H_2O and used at 200 µM, both, at peptide-pulsing and at coculturing of targets with effector T cells (Chen et al., 1999). Plates were centrifuged, supernatant was harvested and measured for ^{51}Cr in a γ-counter (Cobra II, Packard Bioscience, Dreieich). Percent specific lysis was calculated using the mean cpm for each replicate of wells:

$$\% \text{ specific lysis} = 100 \times \frac{\text{test } ^{51}\text{Cr-release} - \text{spontaneous } ^{51}\text{Cr-release}}{\text{maximum } ^{51}\text{Cr-release} - \text{spontaneous } ^{51}\text{Cr-release}}$$

Spontaneous ^{51}Cr-release corresponds to ^{51}Cr released from targets incubated in medium alone, maximum ^{51}Cr-release corresponds to the whole ^{51}Cr content of target cells.

IFN-γ secretion by Hu and Mu effector T cells was measured by ELISPOT. For detection of Mu IFN-γ, 96 w screen plates (NUNC, Wiesbaden) were coated with 50

µl/well of purified anti-mouse IFN-γ capture (RMMG-1) mAb which had been diluted to 10 µg/ml in coating buffer. Coating buffer was freshly prepared: 1 ml solution A + 1 ml solution B + 8 ml H_2O; pH was adjusted to 9.6 by adding solution B. Stocksolution A: 1.59 g Na_2CO_3 adjusted to 100 ml with H_2O; stocksolution B: 2.93 g $NaHCO_3$ adjusted to 100 ml with H_2O. Plates were incubated overnight at 6-12 °C. After plates had been washed with H_2O and blocked with RPMI complete for 1 h at 37 °C, effector cells were added at 1.5 x 10^4 cells/well. Bone marrow cells from MM patients served as target cells and were seeded at 1 x 10^5 MM cells/well. As normal marrow samples from the same patients were not available, marrow cells of a healthy donor were quantitatively adjusted to control for unspecific IFN-γ secretion in response to non-MM cells. Effector and target cells were cocultured for 18-22 h at 37 °C and 5 % CO_2. Cell suspensions were removed and plates were washed with PBS 0.25 % Tween 20 (Sigma). Biotinylated anti-mouse IFN-γ detection (XMG1.2) mAb was diluted to 1 µg/ml in PBS 0.2 % BSA and applied to the plates for a 2 h incubation at room temperature. After being washed with PBS 0.25 % Tween 20, plates were treated for 2 h at room temperature with peroxidase-conjugated streptavidin that had been diluted (1 : 250) in PBS 0.25 % Tween 20 5 % FCS. Unbound streptavidin was removed by washing the plates with PBS 0.25 % Tween 20. For color development, plates were treated with aminoethyl carbazole (Sigma) for 5-10 min. The reaction was stopped by washing the plates in tap water. The number of IFN-γ spots was determined by an automated and computer-assisted video-image analyser (Zeiss-Kontron, Jena).

The Hu IFN-γ differed from the Mu IFN-γ ELISPOT assay as follows: The anti-Hu IFN-γ capture (1-D1K) mAb was diluted to 10µg/ml in PBS and added to Nitrocellulose plates (MAHA S4510, Millipore, Schwalbach). Plates were washed with PBS and blocked for at least 1 h at 37 °C with X-VIVO 20 medium. Hu effector T cells were added at 0.3 or 1 x 10^5 cells/well and incubated with 0.5 x 10^5 target cells in X-VIVO 20 medium for 20 h at 37 °C and 5 % CO_2. Washing buffer was PBS 0.05 % Tween 20. The biotin-conjugated anti-Hu IFN-γ detection (7-B6-1) mAb was added at a final concentration of 2 µg/ml in PBS 0.5 % BSA. Avidin-peroxidase (Vectastain Elite Kit, Vector) was applied for 1 h. Ag-specificity of Hu T cells was evaluated by adding test or control peptides at a final concentration of 10^{-5} M. Phytohemagglutinin (Gibco BRL, Invitrogen) (2 µg/ml) supplemented cocultures served as additional positive controls.

2.13 Inducing A2.1-restricted and peptide-specific human CTL

$A2^+$ PBMC were selected for $CD8^+$ T lymphocytes by a MACS device (Miltenyi Biotec). $CD8^+$ T cells in serum-free X-Vivo 20 (Bio Whittaker) and in the presence of rh IL-7 at 5 ng/ml were stimulated at 1.5 x 10^6/well in 24-well plates with 5 x 10^4/well of irradiated autologous mature DC pulsed with either XBP-1, PRDI-BF1 or Tyr peptides at 50 µg/ml (unless otherwise indicated) and Hu β_2-microglobulin at 10 µg/ml. Responder cells at 1.5 x 10^6/well were restimulated weekly in X-Vivo 20 containing rh IL-2 at 10 U/ml with 5 x 10^4/well of peptide- and Hu β_2-microglobulin-loaded mature DC. The resultant A2-restricted and peptide-specific $CD8^+$ Hu CTL were used as effector cells in 6 h ^{51}Cr-release assays. In one experiment, peptide-specific $CD8^+$ Hu CTL served as targets for recognition by PRDI-BF1- and XBP-1-specific CTL in ^{51}Cr-release assay.

3. Results and Discussion

Part 1 Partial tyrosinase-specific self tolerance by A2.1-restricted CTL in mice and man

3.1 Partial self tolerance to murine tyrosinase in A2K^b and CD8 x A2 transgenic mice

After having confirmed that mTyr and hTyr (369-377) are naturally processed and bound by A2.1 with equivalent efficacy in Mu and Hu APC (data not shown) (Colella et al., 2000), cA2K^b mice were immunized with synthetic m/hTyr peptides. Spleen cells were propagated with the primary stimulating Ag *in vitro* and tested for recognition of T2 targets pulsed with either Tyr peptide. CTL of low avidity for mTyr (369-377) and with almost no crossresponse to hTyr were obtained in 1 out of 3 mice primed with the mTyr self epitope (**Fig. 1A**). In contrast, CTL of higher avidity for hTyr (369-377) were induced in all mice challenged with the xenogeneic hTyr peptide (**Fig. 1B**). These hTyr-specific CTL were not able to sufficiently crossrecognize mTyr-pulsed T2 cells (**Fig. 1B**). The Ag-specificity and A2.1-restriction of responding CTL were ascertained based on their failure to lyse T2 cells labeled with no peptide (**Fig. 1**) or the A2.1-binding FluM1 (58-66) epitope, and their lack of recognition of A2.1-negative EL4 targets that had been coated with either stimulatory Tyr peptide (data not shown). Generating Tyr-specific CTL required the *in vivo* priming with m/hTyr as opposed to immunizing mice with only the I-A^b-binding HBV core (128-140) helper peptide in IFA (data not shown). This result precludes the possibility of a primary *in vitro* induction of effector cells by peptide-pulsed APC. Limiting the quantity of peptide pulsed onto APC during the 6 d period of *in vitro* restimulation from 5 and 0.5 to 0.05 nM had no effect on the number and avidity of CTL generated in response to both, mTyr and hTyr (369-377) (data not shown).

CTL from an A2K^b Tg background are usually at disadvantage in responding to Hu (T2) targets that express A2.1 as opposed to chimeric A2K^b (Sherman et al., 1992; Theobald et al., 1995). Sufficient interaction of CD8 with the α3 domain of Hu A2.1 would facilitate T cell recognition of A2.1-presented m/hTyr and would also resemble Hu CTL-target interactions more closely. To test this hypothesis, m/hTyr-specific CTL responses were studied in Hu CD8 x A2 Tg mice. After challenge with mTyr (369-377), self epitope-reactive CTL were again observed in 1 out of 3 mice, albeit with a

more robust lytic activity and hTyr-specific crossrecognition as compared to cA2K^b mice (**Fig. 1C**). In response to hTyr (369-377), CD8 x A2 mouse-derived CTL with profoundly higher avidity as compared to both, mTyr and hTyr primed double and single Tg mice, respectively, were induced in all animals (**Fig. 1D**). Although hTyr-induced CTL from all CD8 x A2 Tg mice crossrecognized the mTyr self peptide, a 10-fold higher amount of Ag was required to obtain equivalent half-maximum lysis of mTyr-coated T2 targets (**Fig. 1D**). Responding CD8 x A2 Tg CTL induced with either self mTyr or hTyr demonstrated an almost identical lower avidity for mTyr (369-377) (**Fig. 1C, D**). Consistent with these findings, primary hTyr-specific CTL were capable of killing A2.1$^+$ and hTyr$^+$ melanoma targets, such as Malme 3M, whereas mTyr$^+$ B16-A2K^b transfectants were ignored by mTyr-reactive CTL (data not shown). Although the addition of the HBV core helper peptide to the IFA-based Tyr-vaccine facilitated the induction of Ag-specific T cell responses, it had no substantial qualitative effect on the differential avidity of m/hTyr-reactive CTL (data not shown).

To test the possibility that mTyr-specific CTL could be raised more efficiently by taking advantage of the xenogeneic hTyr epitope, spleen cell aliquots of m/hTyr-immunized mice were restimulated *in vitro* with both, the primary Ag and the due Tyr peptide homologue. Ag-specific CTL were generated after *in vivo* priming with mTyr in 1 out of 3 CD8 x A2 Tg mice (**Fig. 2A**). Responding CTL recognized mTyr (369-377) pulsed onto T2 targets regardless of the Tyr peptide species used for *in vitro* restimulation (**Fig. 2A**) and at a concentration of less than 100 nM (data not shown). CTL could only be derived from hTyr-vaccinated animals provided that spleen cells had been restimulated with hTyr as opposed to mTyr-loaded APC (**Fig. 2B**). While hTyr-responding CTL were induced in all animals (data not shown), 2 out of 3 hTyr-specific CTL populations crossrecognized mTyr-coated targets above background (**Fig. 2B**) and at peptide quantities higher than 1 nM (data not shown).

These experiments suggested that the mTyr-specific T cell repertoire is governed by partial self tolerance in cA2K^b and CD8 x A2 Tg mice. While the overall response rate to mTyr (369-377) could obviously be increased by immunizing mice with the hTyr homologue, neither xenogeneic vaccination nor hTyr-based *in vitro* restimulation had a substantial effect on the avidity of mTyr-specific CTL.

Fig. 1

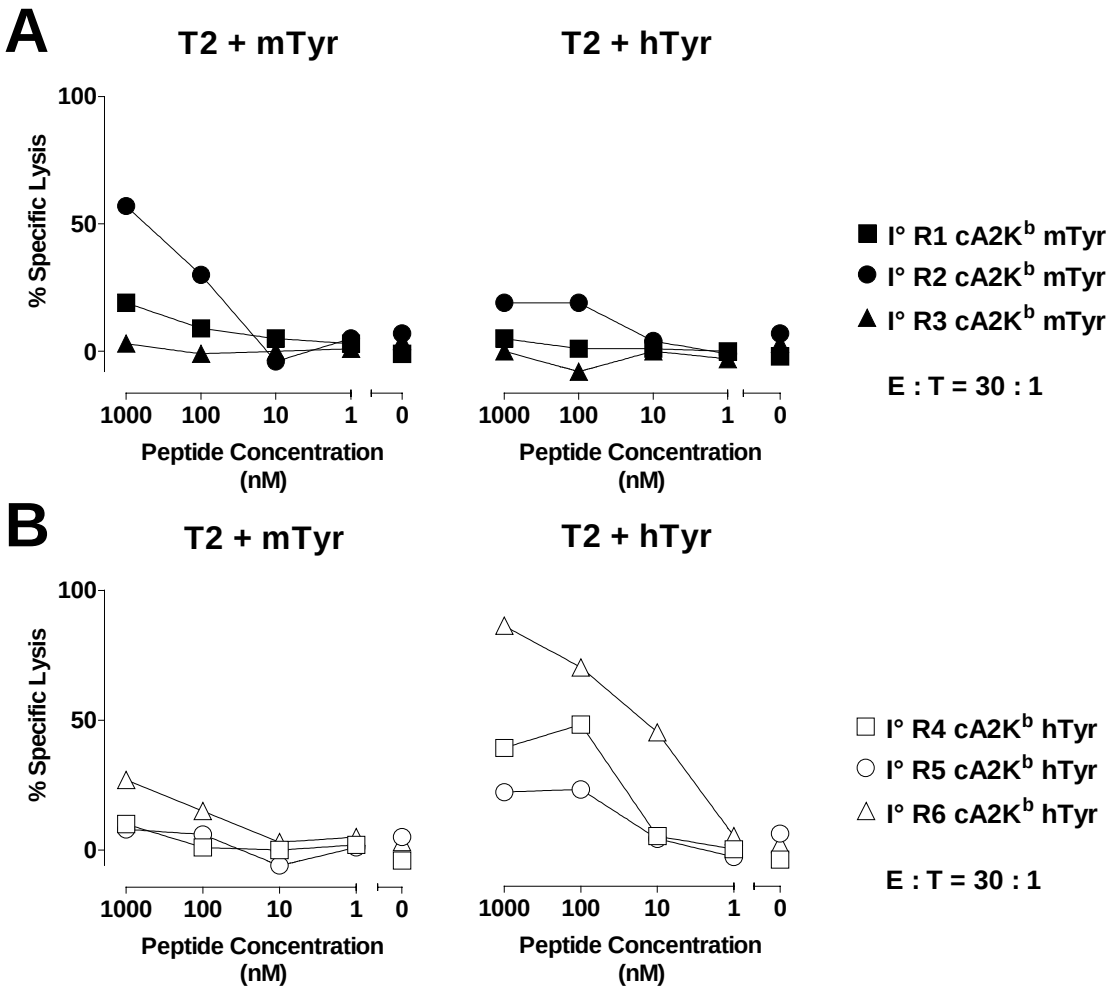


Fig. 1: Efficiency of peptide recognition by mTyr and hTyr (369-377)-specific CTL derived from cA2K^b and CD8 x A2 Tg mice. Responder spleen cells from mTyr- (■●▲) and hTyr-primed (□○△) cA2K^b mice (A, B) or CD8 x A2 mice (C, D) were restimulated *in vitro* with the priming peptide. After 6 days, effector cells were tested in a 4 h (A, B) or 5.5 h (C, D) ^{51}Cr-release assay at an E : T ratio of 30 : 1 for cytolytic activity to T2 cells and the same targets loaded with mTyr (369-377) (A-D, left panel) and hTyr (369-377) (A-D, right panel) at the indicated peptide concentrations.

Fig. 1

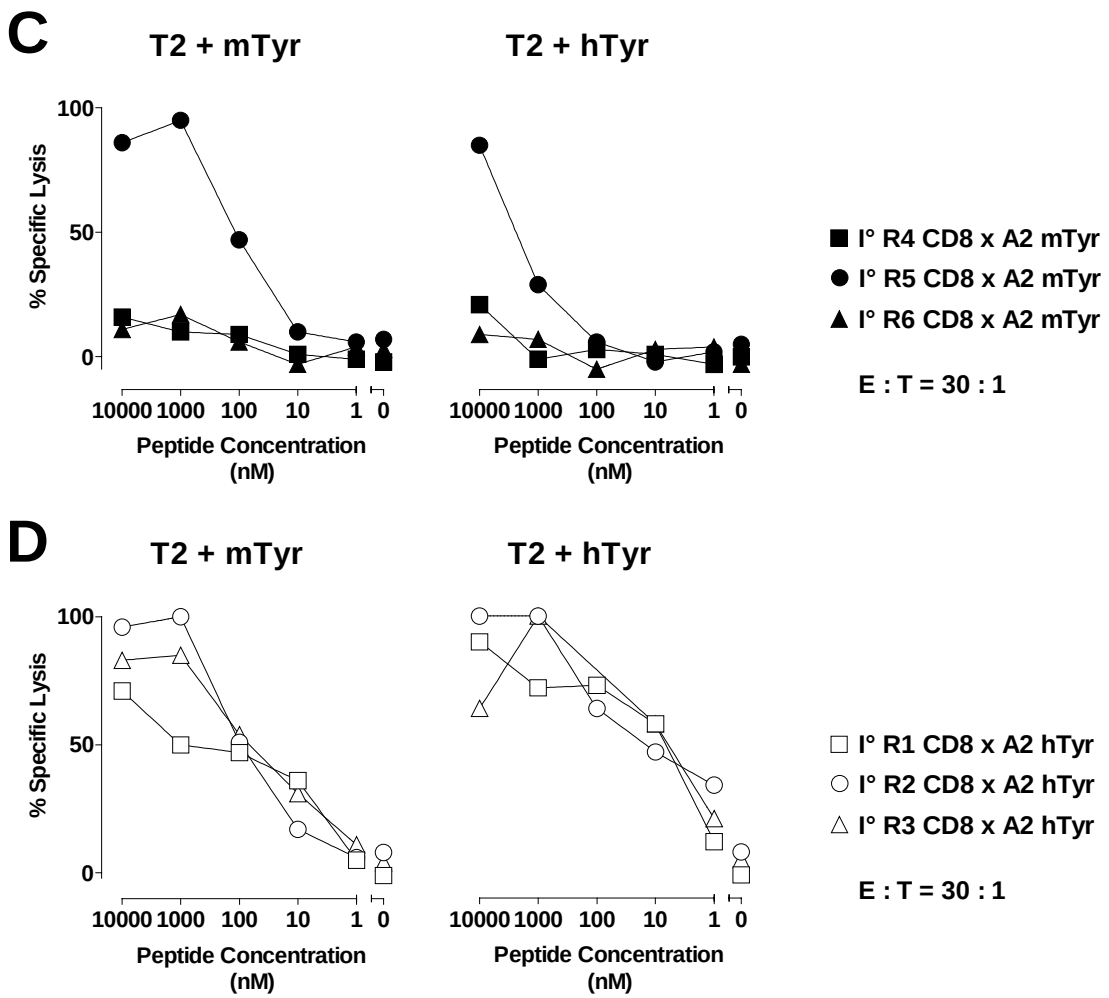

C
T2 + mTyr
T2 + hTyr
% Specific Lysis
100
50
0
10000 1000 100 10 1 0
Peptide Concentration (nM)
I° R4 CD8 x A2 mTyr
I° R5 CD8 x A2 mTyr
I° R6 CD8 x A2 mTyr
E : T = 30 : 1
D
T2 + mTyr
T2 + hTyr
% Specific Lysis
100
50
0
10000 1000 100 10 1 0
Peptide Concentration (nM)
I° R1 CD8 x A2 hTyr
I° R2 CD8 x A2 hTyr
I° R3 CD8 x A2 hTyr
E : T = 30 : 1

Fig. 2

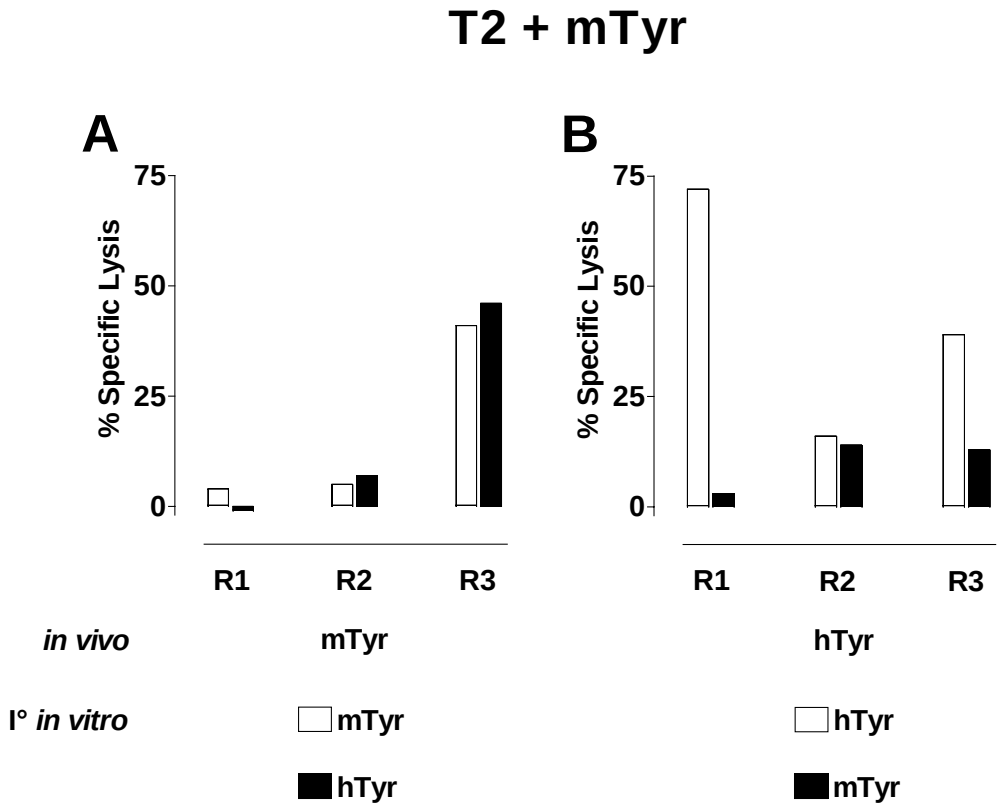


Fig. 2: mTyr-reactive CTL responses after simultaneous stimulation of Tyr-primed splenocytes with mTyr and hTyr (369-377) peptides. Splenocytes from 3 CD8 x A2 Tg responder mice immunized with the mTyr (A) or hTyr peptide (B) were stimulated for 6 days with the priming Ag and its homologue in parallel cultures. Responder cells (R1 - R3) propagated *in vitro* with the priming peptide (open bars) or the peptide homologue (closed bars) were evaluated at an E : T ratio of 30 : 1 for recognition of T2 targets pulsed with mTyr at 10^{-6} M in a 5.5 h ^{51}Cr-release assay. Nonspecific background lysis was 15 %.

3.2 Residual murine tyrosinase-specific CTL of higher avidity are able to kill melanoma targets

As T cells of lower avidity for mTyr clearly escaped tolerance induction, it was important to explore whether or not at least a residual repertoire of high-avidity T cells specific for self mTyr could be expanded. To potentially select these residual mTyr-specific CTL of higher efficiency, responding effector T cells (**Fig. 1C**) were exposed to the self peptide over several cycles of restimulation and compared functionally with a CTL line stimulated with hTyr and derived from primary culture (**Fig. 1D**). Allo A2.1-reactive and FluM1 (58-66)-specific Tg mouse CTL served as positive and negative control, respectively (**Fig. 3E, F**). The selection process resulted in a 100-fold increase in avidity for both m/hTyr-reactive CTL lines (**Fig. 3A, C**). Higher avidity mTyr CTL gained the ability to specifically kill Mu B16 melanoma cells transfected with $A2K^b$ and even responded to Hu Malme 3M melanoma targets (**Fig. 3B**), consistent with an equivalent crossrecognition of hTyr (**Fig. 3A**). Lysis of Malme 3M was more profound with hTyr effector CTL (**Fig. 3D**) that were at least 1-log more sensitive to the hTyr peptide (**Fig. 3C**). Their impaired crossrecognition of mTyr (**Fig. 3C**), however, corresponded with a lower response to the Mu melanoma transfectant (**Fig. 3D**). Along this finding, the mTyr crossrecognition potential of hTyr CTL appears to vanish upon continuous hTyr restimulation.

However, the experiments confirm that not only low-avidity but also residual T cells of higher avidity for self mTyr may escape tolerance induction.

3.3 Partial self tolerance to murine tyrosinase in transgenic mice is not affected by $CD4^+CD25^+$ T regulatory cells

As Tyr-specific tolerance appears not to be affected by thymic selection (Engelhard et al., 2002), the possibility was explored whether the self Tyr-reactive T cell repertoire is governed by $CD4^+CD25^+$ Treg that have been reported to suppress autoreactive anti-melanoma immune responses (Sutmuller et al., 2001). At day -4 of Ag priming, CD8 x $cA2K^b$ mice were depleted of $CD25^+$ cells by i.p. administration of anti-CD25 mAb, PC61. Control mice received the corresponding rat IgG isotype. Depletion of $CD4^+CD25^+$ Treg was monitored on day 0 by staining PBMC of treated animals with the noncrossreactive anti-CD25 mAb, 7D4, and an anti-CD4 mAb.

Fig. 3

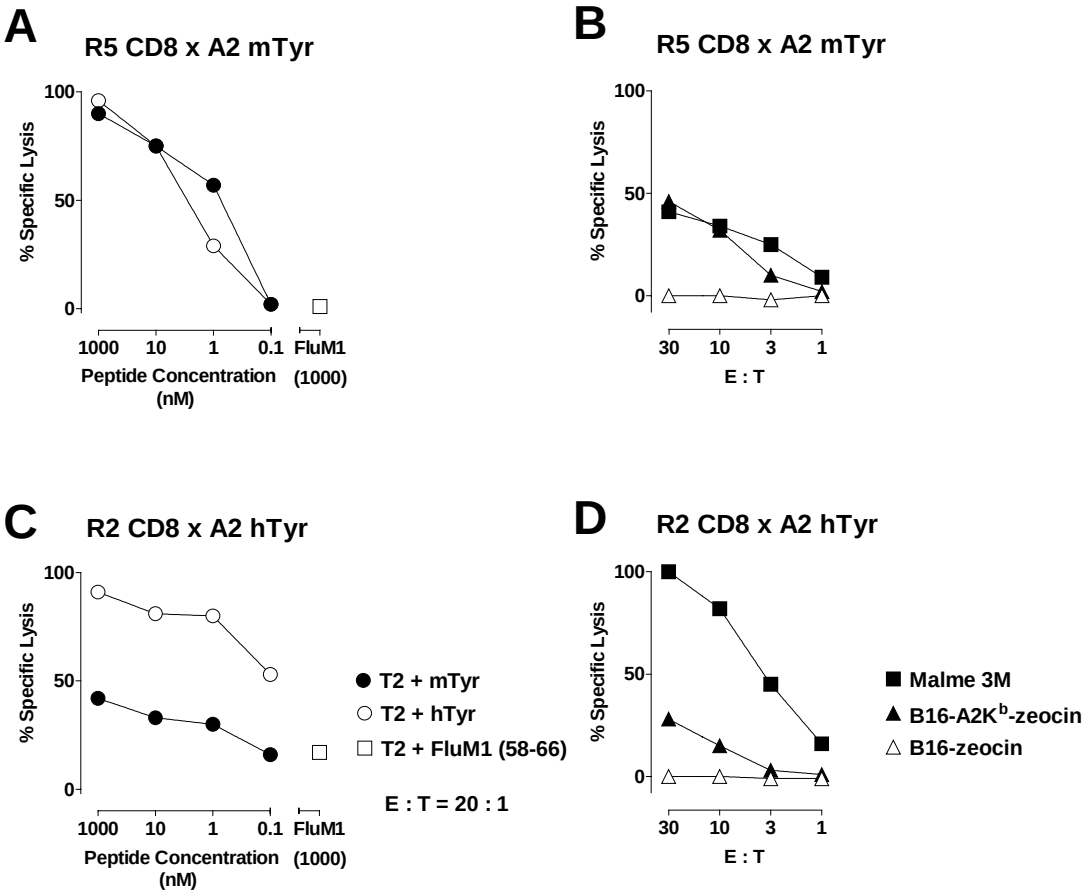
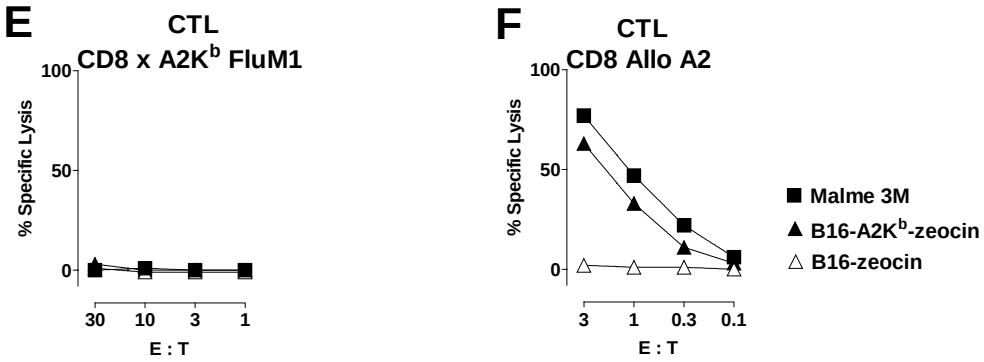


Fig. 3: Recognition of melanoma cells by mTyr and hTyr (369-377)-specific CTL lines derived from CD8 x A2 mice. (A, C) CTL lines established from primary cultures of mTyr- and hTyr-induced effector T cells were tested at an E : T ratio of 20 : 1 for recognition of T2 targets coated with mTyr (●) and hTyr (○) at the indicated concentrations, or FluM1 (58-66) (□) at 10^{-6} M. (B, D) Specific lysis of Malme 3M (■), IFN-γ-treated B16-A2K^b-zeocin (▲) or B16-zeocin (△) by hTyr and mTyr (369-377)-specific CTL lines as well as (E, F) CD8 x A2K^b FluM1 and CD8 Allo A2 effector controls in a 5.5 h ^{51}Cr-release assay at the indicated E : T ratios.

Fig. 3

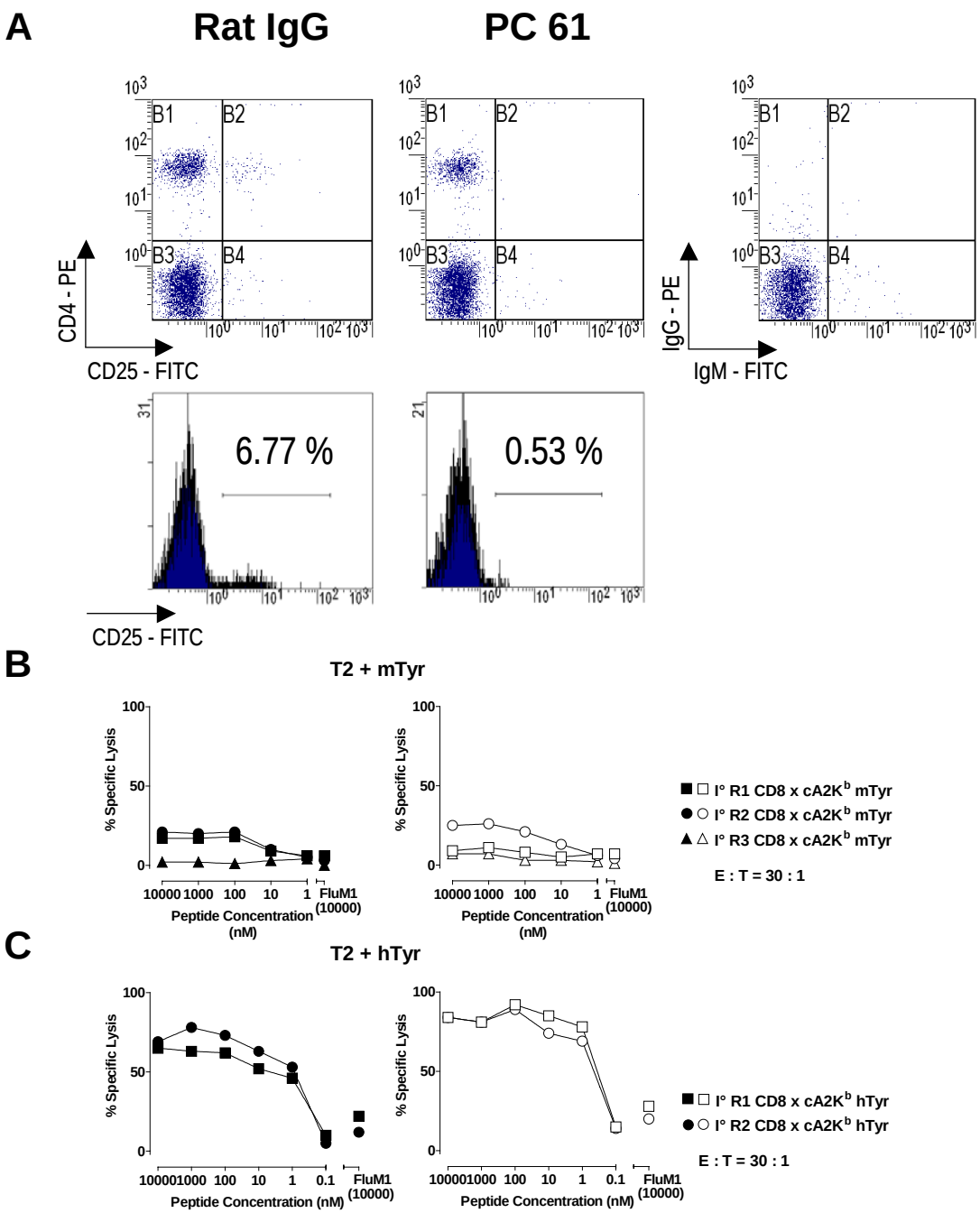


Although depletion of $CD4^+CD25^+$ cells (6.77 % to 0.53 %) was almost complete (**Fig. 4A**), no effect on mTyr-specific CTL responses after immunizing treated animals with the Tyr self Ag was observed (**Fig. 4B**). The possibility that the lack of an effect was due to the depletion of $CD25^+$ effector T cells activated by the vaccination protocol was precluded by the finding that Tyr-reactive CTL responses following hTyr antigenic challenge were not affected by the PC61 treatment (**Fig. 4C**). Inhibition of CTLA-4 alone or in combination with anti-CD25 mAb treatment has been recently reported to promote CTL activity to self tumor Ag (Sutmuller et al., 2001; Hernandez et al., 2001; van Elsas et al., 1999). However, even $CD25^+$ cell depletion along with anti-CTLA-4 mAb (9H10) treatment on days -1, 0 and +1 after Ag priming did not augment the quantity and quality of mTyr-reactive CTL (data not shown).

These results indicated that the $CD4^+CD25^+$ Treg subset and counterregulation via CTLA-4 are not involved in shaping Tyr-specific tolerance.

Fig. 4: Influence of Treg on Tyr-specific tolerance. (A) PBMC of rat IgG-treated control (left panel) and PC61-treated (anti-CD25) (mid panel) CD8 x cA2K^b mice were stained after 4 days with anti-CD4-PE and anti-CD25-FITC or the appropriate isotype controls, IgG-PE and IgM-FITC (right panel). Corresponding numbers of $CD25^+$ cells gated on $CD4^+$ T cells are shown in the lower panel (A). Identical results were obtained in all treated animals. Rat IgG- (■●▲) and PC61-treated (□○△) mice were primed with mTyr (B) or hTyr (369-377) (C) at day +4 after mAb treatment. At day +14, splenocytes were harvested and restimulated for 6 days with the priming peptide *in vitro*. Responder cells were tested at an E : T ratio of 30 : 1 for recognition of T2 cells loaded with the indicated amounts of mTyr (B), hTyr (C) or the FluM1 (58-66) control peptide (B, C).

Fig. 4


A
Rat IgG
PC 61
B1
B2
B3
B4
CD4 - PE
CD25 - FITC
IgG - PE
IgM - FITC
6.77 %
0.53 %
CD25 - FITC
B
T2 + mTyr
% Specific Lysis
Peptide Concentration (nM)
FluM1 (10000)
I° R1 CD8 x cA2K^b mTyr
I° R2 CD8 x cA2K^b mTyr
I° R3 CD8 x cA2K^b mTyr
E : T = 30 : 1
C
T2 + hTyr
I° R1 CD8 x cA2K^b hTyr
I° R2 CD8 x cA2K^b hTyr
E : T = 30 : 1

3.4 Partial self tolerance to human tyrosinase in man

To study whether or not the A2.1-restricted Hu T cell repertoire is affected by Tyr-specific self tolerance too, Hu $CD8^+$ T lymphocytes underwent primary *in vitro* induction with mature $A2^+$ autologous DC pulsed with either mTyr or hTyr peptides and were tested for specific lytic activity after 3 to 4 rounds of DC/Ag-based restimulation. Allo A2.1-reactive and FluM1 (58-66)-specific Tg mouse CTL served as positive and negative control, respectively (data not shown). While Ag-specific $CD8^+$ effector CTL could be raised in 3 out of 3 donors, as shown for donor 1 in **Fig. 5A**, hTyr- as compared to mTyr-stimulated CTL required about 5-fold more peptide to mediate equivalent lysis of T2 targets loaded with the primary Ag (**Fig. 5B, C**). However, there was no advantage to the use of the more efficient mTyr CTL in crossrecognizing the hTyr peptide (**Fig. 5B**). Consistent with this finding, $A2.1^+$ SY targets infected with MVA-mTyr, as opposed to SY MVA-hTyr, were killed by mTyr CTL only (**Fig. 5D, F**). Regardless of the higher versus intermediate avidity of mTyr and hTyr CTL, respectively, both CTL were sufficiently effective to lyse $A2.1^+$ Hu melanoma targets that express hTyr protein at high level, such as Malme 3M and NA8-Mel-Tyr transfectants (**Fig. 5D, E, F, G**). Both, lack of NK-activity and exclusive A2.1/Ag complex-specificity by Tyr-specific CTL was demonstrated by their ignorance of K562, NA8-Mel controls (**Fig. 5D, E, F, G**), and Tyr^+ $A2.1^-$ SK28 tumor targets (data not shown). These studies demonstrate for the first time that the hTyr-specific T cell repertoire in man is also governed by partial self tolerance that allows a window of opportunity for residual higher avidity self Tyr-specific T cells to escape tolerance induction. Although mTyr-reactive CTL of increased efficiency could be raised from Hu T lymphocytes, the employment of the xenogeneic mTyr peptide homologue did not rescue hTyr-specific partial tolerance.

The amount of peptide used for CTL induction and maintenance has substantial impact on the quality of T cell responses, including Tyr-specific CTL in Tg mice (Alexander-Miller et al., 1996a; Alexander-Miller et al., 1996b; Bullock et al., 2000). Therefore various doses of peptide were used during induction and restimulation of primary hTyr- and mTyr-specific Hu T cells. Resulting effector cells were tested for their functional avidity.

Fig. 5

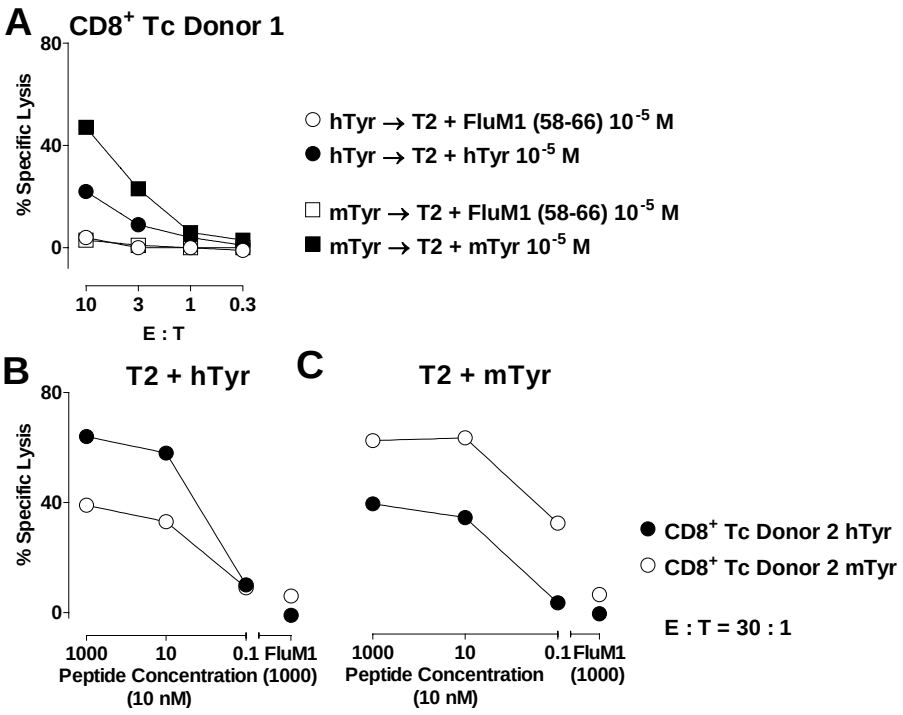
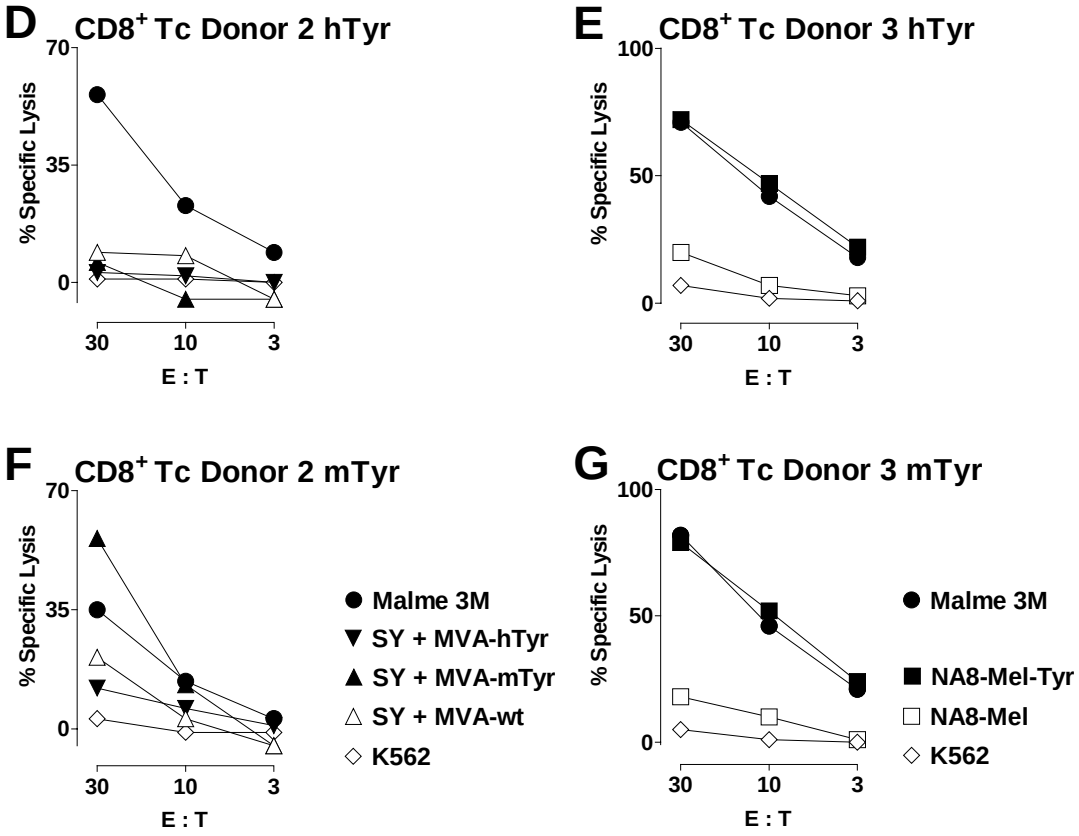


Fig. 5: Efficiency of peptide and melanoma cell recognition by hTyr and mTyr (369-377)-specific CTL derived from Hu $CD8^+$ T cells. Hu $CD8^+$ T lymphocytes were isolated from 3 $A2^+$ healthy donors, stimulated in parallel for 5 cycles with hTyr and mTyr peptide-loaded autologous mature DC and tested for cytolytic activity in a 5.5 h ^{51}Cr-release assay. (A) $CD8^+$ T cells (donor 1) stimulated with either hTyr (●○) or mTyr 369-377 (■□) were tested at the indicated E : T ratios for recognition of T2 targets coated with hTyr (●) and mTyr (■) or FluM1 (58-66) at 10^{-5} M (○□). (B, C) Donor 2-derived $CD8^+$ effector CTL stimulated with either hTyr (●) or mTyr (○) were tested at an E : T ratio of 30 : 1 for cytolytic activity to T2 targets pulsed with the indicated concentrations of (B) hTyr, (C) mTyr and (B, C) FluM1. $CD8^+$ T cells from donors 2 and 3 stimulated either with (D, E) hTyr or (F, G) mTyr were tested at the indicated E : T ratios for recognition of (D-G) Malme 3M (●) and K562 targets (◇), (D, F) SY infected with MVA-hTyr (▼), MVA-mTyr (▲) and MVA-wt (△), (E, G) NA8-Mel-Tyr (■) and NA8-Mel (□). Cold K562 cells were added at a cold : hot target ratio of 10 : 1 throughout.

Fig. 5



Upon both, hTyr peptide titration (**Fig. 6A**) and Hu melanoma recognition (**Fig. 6B**), it became obvious that only low-to-intermediate as opposed to high doses of the non-self mTyr peptide homologue favored the avidity of hTyr-crossreactive CTL. This, however, contrasted with the finding that increasing amounts of the stimulatory self peptide were required for the generation of higher avidity hTyr-specific CTL.

It may be concluded from these experiments that it is more difficult to obtain a response with the hTyr self epitope versus the mTyr non-self peptide, consistent with an effect on the repertoire due to partial self tolerance.

Fig. 6

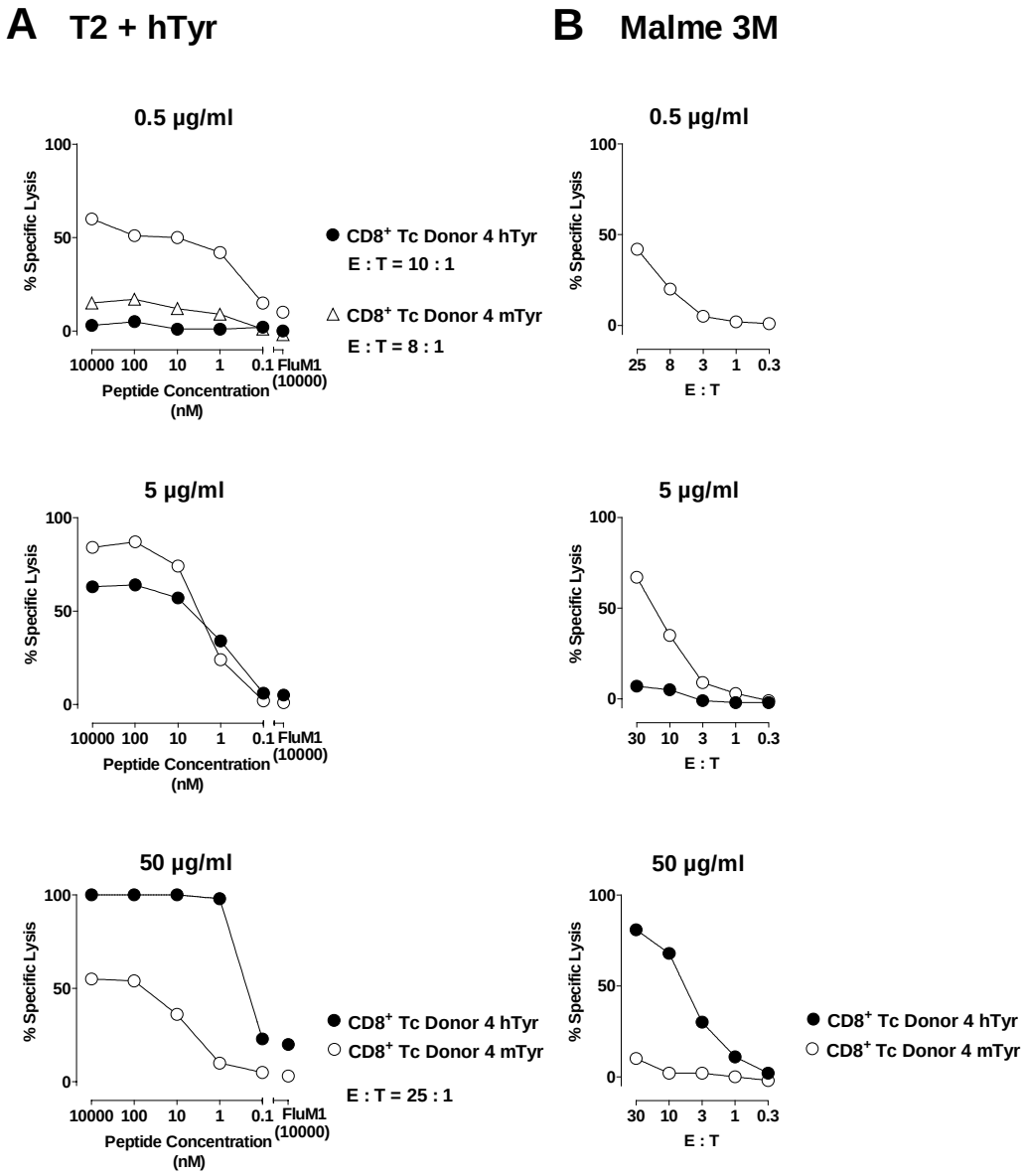


Fig. 6: Influence of peptide dose on the avidity of Tyr-specific Hu CTL during in vitro stimulation. $CD8^+$ T cells isolated from 2 healthy donors were induced with Ag-loaded autologous mature DC. CTL were tested for target recognition after 4 cycles of restimulation with the indicated amounts of the priming hTyr (●) or mTyr (369-377) (○) peptides. (A) Representative results of effector CTL obtained from 1 donor in response to T2 cells labeled with the hTyr peptide at the indicated concentrations and at E : T ratios of 25 : 1 are shown. Cytolytic activity of responders stimulated with 0.5 µg/ml of hTyr and an additional mTyr-stimulated equivalent (△) are plotted at E : T ratios of 10 : 1 and 8 : 1, respectively. (B) The same effector cells were tested for their cytolytic response to Malme 3M targets at the indicated E : T ratios. Cold K562 cells were added at a cold : hot target ratio of 10 : 1.

Part 2 Transcription factors associated with B cell terminal differentiation as targets for multiple myeloma-reactive CTL

3.5 Identifying A2.1-presented immunogenic peptides derived from PRDI-BF1 and XBP-1

To test the concept that peptide epitopes derived from PRDI-BF1 and XBP-1 provide MM-associated Ag, both proteins were screened for potential peptide sequences representing A2.1-bound CTL epitopes. A total number of 61 PRDI-BF1 (8-11-mer) and 16 XBP-1 (9-10-mer) peptide sequences were selected (**Table 1** and **2**). Based on the known consensus motifs for A2.1-binding peptides, PRDI-BF1 peptides of 8 and 11 aa length had at their N-terminal position 2 and at their C terminus either one of the classical anchor aa for A2.1, leucine (L) or valine (V) (Rammensee et al., 1993). The 9- and 10-mer peptides representing sequences within both proteins were selected according to computational scoring models for A2.1-presented peptides, such as the Parker program (Parker et al., 1994) and the SYFPEITHI algorithm (Rammensee et al., 1999). Criteria for PRDI-BF1 peptide selection were a minimum score of 10 predicted by at least one of the two models (**Table 1**). For XBP-1 peptides, rules were modified such that the SYFPEITHI score had to be above 20 (**Table 2**). As a baseline, score prediction for A2.1-binding of positive and negative control peptides, such as the A2.1-presented FluM1 (58-66) and H-2K^b-bound VSV-N (52-59) epitopes, is shown in **Table 2**. XBP-1 also differed from PRDI-BF1 peptide selection as it was based additionally on data derived from a newly developed computational program, PAProC II (Toes et al., 2001; and unpublished data). This program allows the prediction of the intracellular generation of peptides due to cleavages by two Hu proteasome species: the constitutive proteasome (c20S) and the immunoproteasome (i20S) (**Table 2**). One peptide corresponding to XBP-1 sequence 200-209 was synthesized despite of the negative PAProC result because of its extremely high A2.1-binding score as revealed by the Parker program (**Table 2**). The PAProC II algorithm is unfinished and not accessible via the internet yet. As a result, the presented data, generated and kindly provided by H. J. Schild (Institute for Cell Biology, Tübingen) have a reliability of about 60 % in retrieving naturally processed peptide epitopes (H. J. Schild, personal communications). Synthesized PRDI-BF1 peptides were later analyzed by PAProC II too (**Table 1**).

Table 1: Selected PRDI-BF1 peptides

Position	Sequence	Homology [a]	Score KP [b]	Score SYFPEITHI [b]	PAProC II [c]	
					c20S	i20S
402-410	G L F P R L C P V		592	28	no	no
747-755	G L L S S G C S L	homologous	79	25	no	yes
681-690	G L P L E D L T R I	homologous	24	23	no	no
718-726	V V E K E I L A V		2	20	yes	no
754-763	S L Y E S S D L P L		281	26	no	no
717-726	S V V E K E I L A V		57	24	yes	no
660-668	Y I H L C S L K V	homologous	44	23	no	yes
377-386	F L L P P Y G M N C		85	15	yes	yes
401-410	F G L F P R L C P V		18	16	no	no
56-65	A T N S E E V I G V		2	22	yes	yes
501-510	A M A A P S S D E A		2	18	no	no
261-269	V V Y P I R A P L	homologous	10	20	no	no
406-415	R L C P V Y S N L L		21	23	no	yes
340-350	Y L N A S Y G T E G L		n.d.	n.d.	no	no
273-282	F L K A S L A Y G I		2	20	yes	yes
765-775	K L P P S N P L P L V		n.d.	n.d.	no	no
765-774	K L P P S N P L P L		75	24	no	no
383-392	G M N C N G L S A V		116	25	yes	no
394-403	S M N G I N N F G L		89	24	yes	no
424-434	M L N P T S L P S S L		n.d.	n.d.	no	no
715-723	V I S V V E K E I		2	18	no	no
358-367	P L P H L P P A F I		1	17	yes	yes
383-391	G M N C N G L S A		4	17	no	no
442-451	L L Q P E H P R E V		24	26	no	no
419-428	S L P H P M L N P T		17	21	no	no
349-359	G L G S Y P G Y A P L		n.d.	n.d.	no	no
739-748	S L Q R N M G N G L	homologous	21	24	no	no
451-459	V L V P A P H S A		8	20	yes	yes

Table 1: Selected PRDI-BF1 peptides

Position	Sequence	Homology [a]	Score KP [b]	Score SYFPEITHI [b]	PAProC II [c]	
					c20S	i20S
441-451	R L L Q P E H P R E V		n.d.	n.d.	no	no
277-286	S L A Y G I E R P T		1	17	no	no
406-414	**R L C P V Y S N L**		**32**	**22**	**yes**	**no**
161-169	L V W Y C R D F A	homologous	17	10	no	yes
145-154	N I Y F Y T I K P I	homologous	11	19	no	no
442-452	L L Q P E H P R E V L		n.d.	n.d.	no	no
180-188	L T M M N L T Q T		1	15	no	no
160-169	L L V W Y C R D F A	homologous	23	16	no	yes
765-772	K L P P S N P L		n.d.	<9	no	no
705-713	R L E D V E D D I		1	18	no	no
184-192	N L T Q T Q S S L		21	21	no	no
433-442	S L P S D G A R R L		4	22	yes	yes
237-244	P L T S E K D L		n.d.	<9	no	no
16-25	A E F E E K C T Y I	homologous	17	13	yes	yes
206-214	N V P K R E Y S V		6	17	no	no
714-723	S V I S V V E K E I		1	17	no	no
762-772	P L M K L P P S N P L		n.d.	n.d.	no	no
763-772	L M K L P P S N P L		1	20	no	no
143-151	G M N I Y F Y T I	homologous	14	22	no	yes
156-165	A N Q E L L V W Y C	homologous	13	8	no	no
715-724	V I S V V E K E I L		1	19	no	no
433-443	S L P S D G A R R L L		n.d.	n.d.	no	yes
627-636	V C P A K F T Q F V	homologous	16	11	yes	yes
708-718	D V E D D I S V I S V		n.d.	n.d.	yes	no
413-420	N L L G G G S L		n.d.	<9	no	no
179-188	E L T M M N L T Q T		1	14	no	no

Table 1: Selected PRDI-BF1 peptides

Position	Sequence	Homology [a]	Score KP [b]	Score SYFPEITHI [b]	PAProC II [c]	
					c20S	i20S
714-724	S V I S V V E K E I L		n.d.	n.d.	no	no
80-89	L I G E I Y T N D T		7	17	no	no
62-71	V I G V M S K E Y I		7	17	no	no
667-675	K V H L K G N C A		2	11	yes	no
726-736	V V R K E K E E T G L		n.d.	n.d.	yes	yes
667-676	K V H L K G N C A A		2	10	no	no
229-238	D L Y R S N I S P L		12	24	no	no

Table 2: Selected XBP-1 peptides

Position	Sequence	Homology [a]	Score KP [b]	Score SYFPEITHI [b]	PAProC II [c]	
					c20S	i20S
19-27	**L L S G Q P A S A**		**8**	**22**	**yes**	**no**
18-27	**L L L S G Q P A S A**		**31**	**22**	**yes**	**no**
111-119	K L L L E N Q L L		277	23	no	yes
185-194	N I S P W I L A V L		3	26	yes	yes
125-133	G L V V E N Q E L	homologous	21	23	yes	yes
141-150	A L V A E E E A E A		5	20	yes	yes
94-102	R M S E L E Q Q V	homologous	206	21	yes	no
168-176	R L R A P L Q Q V		12	25	yes	yes
190-198	I L A V L T L Q I		18	26	yes	no
189-198	W I L A V L T L Q I		24	19	yes	no
96-105	S E L E Q Q V V D L	homologous	15	22	yes	yes
183-191	L Q N I S P W I L		11	13	no	yes
200-209	S L I S C W A F W T		238	18	no	no
201-210	L I S C W A F W T T		25	16	no	yes

Table 2: Selected XBP-1 peptides

Position	Sequence	Homology [a]	Score KP [b]	Score SYFPEITHI [b]	PAProC II [c]	
					c20S	i20S
187-196	S P W I L A V L T L		3	20	yes	yes
118-127	L L R E K T H G L V	homologous	5	24	yes	no
controls [d]:						
FluM1 58-66	G I L G F V F T L		551	30	n.d.	n.d.
VSV-N 52-59	R G Y V Y Q G L		n.d.	not detectable	n.d.	n.d.

Table 1 and 2:

Selected PRDI-BF1 and XBP-1 peptides were synthesized. Immunogenic peptides are in color.

[a] Homologous peptides are identical between PRDI-BF1 and Blimp-1 or Hu and Mu XBP-1.

[b] Peptides were predicted and selected according to consensus motifs and computational score models for potentially A2.1-binding peptides:

Score KP: The program is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Applied Biosystems (Parker et al., 1994) (access via: http://bimas.dcrt.nih.gov/molbio/hla_bind/). Prediction is only available for 9- and 10-mer peptides.

Score SYFPEITHI: The algorithm used is based on Rammensee et al., 1999 (access via : http://www.uni-tuebingen.de/uni/kxi/). The maximum score for a A2.1-binding peptide is 36. Prediction is available for 8-, 9- and 10-mers.

[c] **PAProC II**: PAProC II predicts cleavages by two Hu proteasome species: **c20S** and **i20S**. The predictions of PAProC II are based on data published recently (Toes et al., 2001). The algorithm will be available publicly in the near future. The presented data were generated with the immature algorithm kindly provided by the laboratory of H. J. Schild.

[d] A2.1-binding scores of the positive control FluM1 (58-66) and the negative control VSV-N (52-59) are indicated.

Peptides that met all the described criteria and were nonhomologous to Blimp-1 and Mu XBP-1 sequences were favored for synthesis. As few as 12 out of 61 and 4 out of 16 homologous PRDI-BF1- and XBP-1-derived peptides, respectively, were eventually synthesized (**Table 1** and **2**).

The A2.1-binding activity of chosen PRDI-BF1- and XBP-1-derived peptides was determined by a competition assay that assessed the ability of each peptide to inhibit binding of p53 (264-272) to A2.1 on T2 target cells (Theobald et al., 1995) (**Fig. 7A, B**). Inhibition of p53 (264-272)-binding was monitored as a decrease in target cell lysis by a p53 (264-272)-specific and A2.1-restricted CTL clone, clone 46. FluM1 (58-66) and VSV-N (52-59) served as positive and negative control, respectively (**Fig. 7A, B**).

Fig. 7

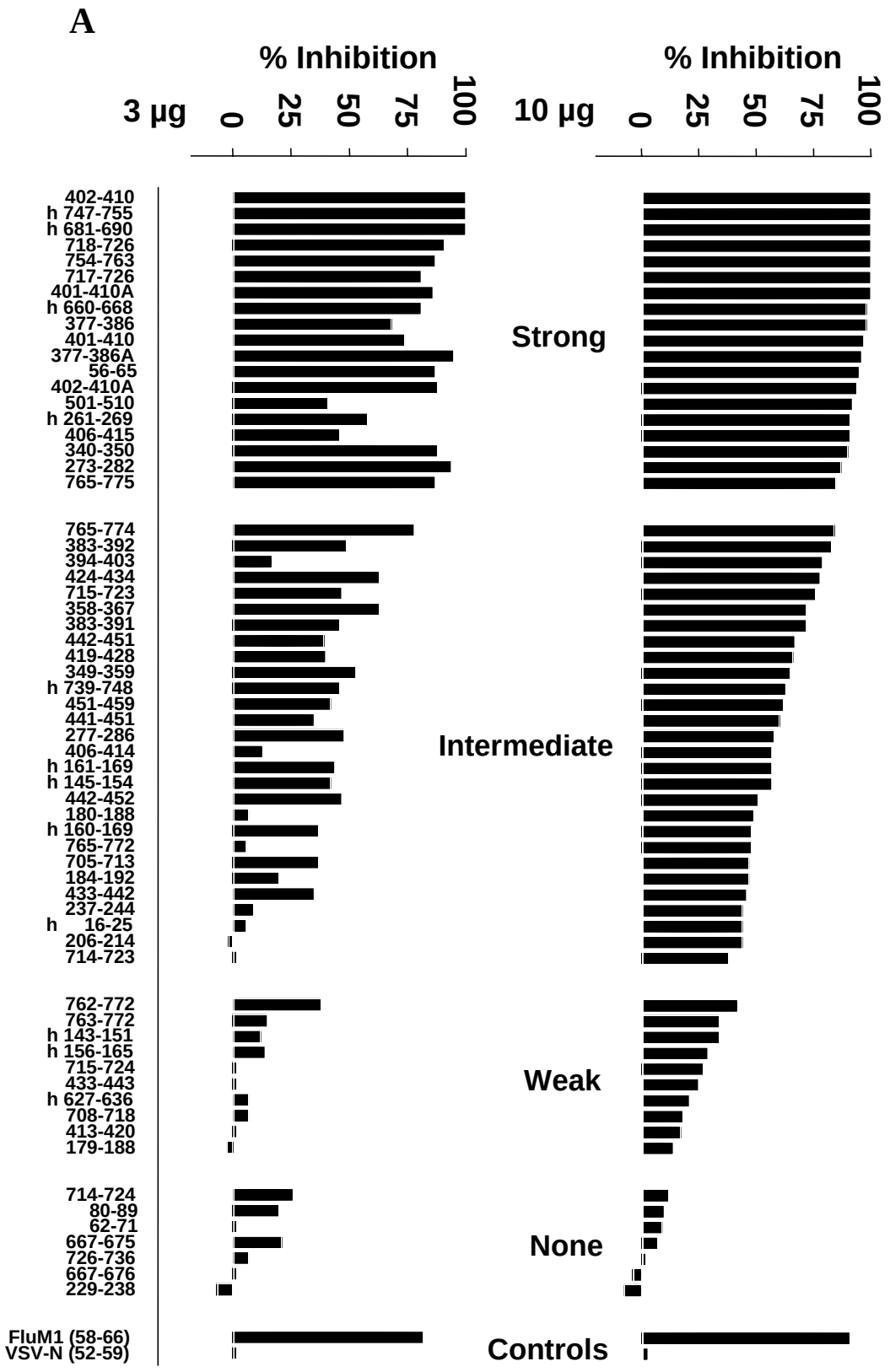

A
% Inhibition
3 µg
0
25
50
75
100
% Inhibition
10 µg
0
25
50
75
100
402-410
h 747-755
h 681-690
718-726
754-763
717-726
401-410A
h 660-668
377-386
401-410
377-386A
56-65
402-410A
501-510
h 261-269
406-415
340-350
273-282
765-775
Strong
765-774
383-392
394-403
424-434
715-723
358-367
383-391
442-451
419-428
349-359
h 739-748
451-459
441-451
277-286
406-414
h 161-169
h 145-154
442-452
180-188
h 160-169
765-772
705-713
184-192
433-442
237-244
h 16-25
206-214
714-723
Intermediate
762-772
763-772
h 143-151
h 156-165
715-724
433-443
h 627-636
708-718
413-420
179-188
Weak
714-724
80-89
62-71
667-675
726-736
667-676
229-238
None
FluM1 (58-66)
VSV-N (52-59)
Controls

Fig. 7

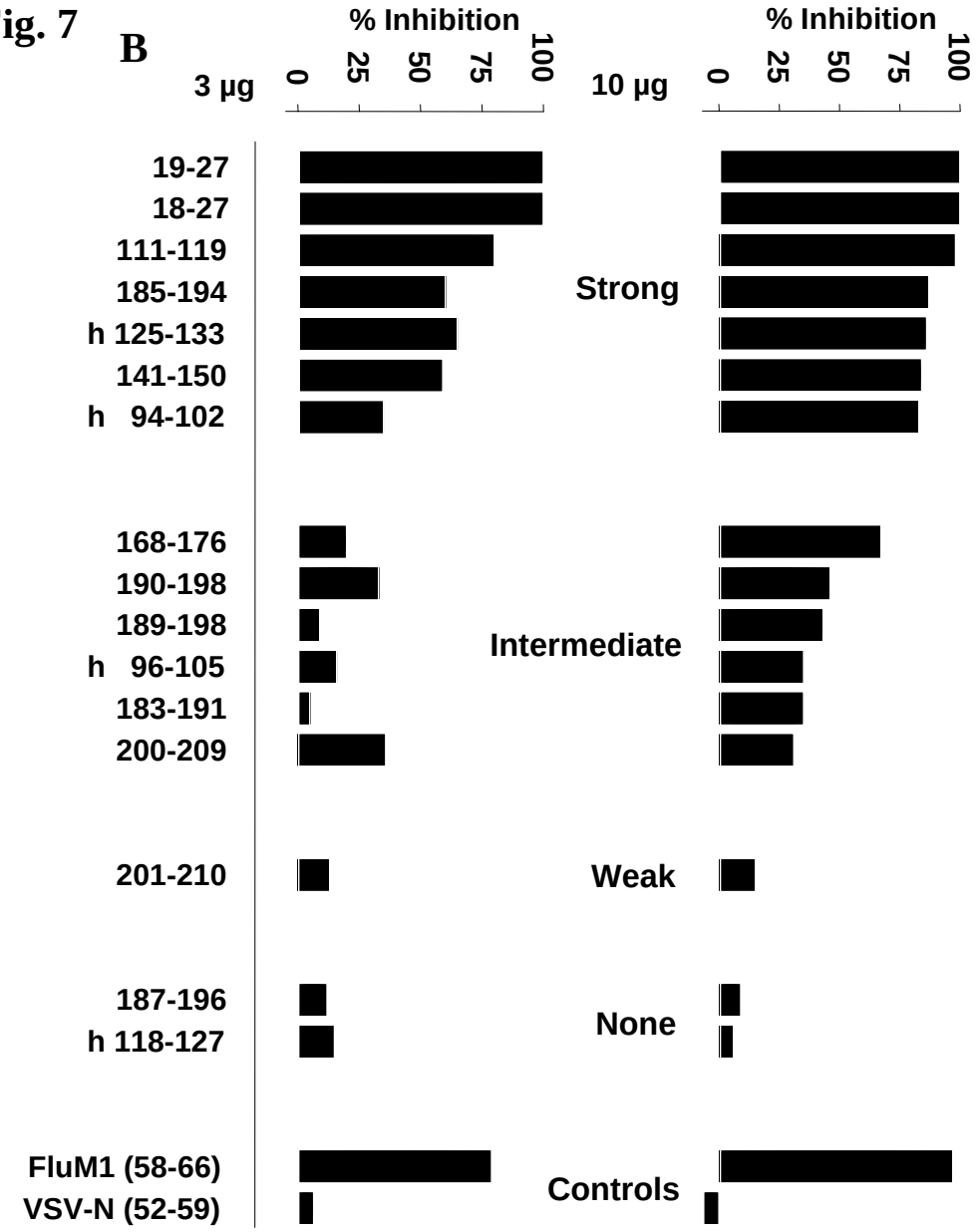


Fig. 7: A2.1-binding activity of selected PRDI-BF1 and XBP-1 peptides. The A2.1-binding activity, shown as percent inhibition, was determined by the ability of the indicated synthetic PRDI-BF1 (A) and XBP-1 (B) peptides to inhibit the A2.1-binding of the p53 (264-272) peptide. This was measured as inhibition of lysis by p53 (264-272)-specific CTL of T2 cells loaded with the corresponding p53 peptide and different concentrations of indicated PRDI-BF1 (A) and XBP-1 (B) peptides. Representative results were shown for the controls, FluM1 (58-66) and VSV-N (52-59). Homologous peptides are marked by "h".

A strong-to-intermediate A2.1-binding activity (> 40 % inhibition of A2.1-binding of p53 (264-272)) was obtained for 44 out of 61 PRDI-BF1 and 13 out of 16 XBP-1 peptides. 17 PRDI-BF1 and 3 XBP-1 peptides, however, showed only weak or no A2.1-binding at all (< 40 % inhibition) (**Fig. 7A, B**).

Almost all of the PRDI-BF1 and XBP-1 peptides with strong-to-intermediate A2.1-binding capacity were tested for their immunogenicity in A2.1 Tg mice. For that purpose, cA2K^b or CD8 x cA2K^b mice were primed with peptide and 10 days later, spleen cells from these mice were restimulated with peptide *in vitro* and tested for an A2K^b- or A2.1-restricted, peptide-specific primary CTL response, (**Fig. 8A, B**). The FluM1 (58-66) peptide served as positive control in all experiments (**Fig. 8A, B**). A2.1/A2K^b-restricted CTL responses specific for nonhomologous PRDI-BF1 (402-410), (401-410), (377-386), (406-415), (406-414) (**Fig. 8A**), and nonhomologous XBP-1 (18-27), and (19-27) peptides (**Fig. 8B**) were observed. The peptide-specificity of the CTL responses was evidenced by the ability of the CTL to lyse target cells pulsed with the immunizing peptide, but not other A2.1-binding peptides (**Fig. 8A, B**). A2.1/A2K^b-restriction was ascertained by CTL ignorance of A2.1-negative EL4 cells coated with the relevant peptide (data not shown). One exception was the PRDI-BF1 (406-415)-specific CTL response that seemed to be dominated by H-2^b-restricted CTL. This suggested the predominant binding of the peptide to either one of the class I H-2^b molecules instead of A2.1 (data not shown).

The results on peptide immunogenicity were consistent with the hypothesis that the majority of functional TCR epitopes are produced by peptides with high affinity for the presenting class I MHC molecule (Theobald et al., 1995; Stanislawski et al., 2001). The lack of CTL responsiveness to some of the nonhomologous PRDI-BF1 and XBP-1 peptides with strong A2.1-binding activity suggested that gaps in the functional T cell repertoire may exist. For this reason, it remained unresolved whether the absence of a CTL response to the homologous PRDI-BF1 (747-755), (660-668) (**Fig. 8A**) and the homologous XBP-1 (125-133), (94-102), (96-105) peptides (**Fig. 8B**) is also due to this latter phenomenon or is a result of PRDI-BF1- and XBP-1-specific self tolerance.

Fig. 8

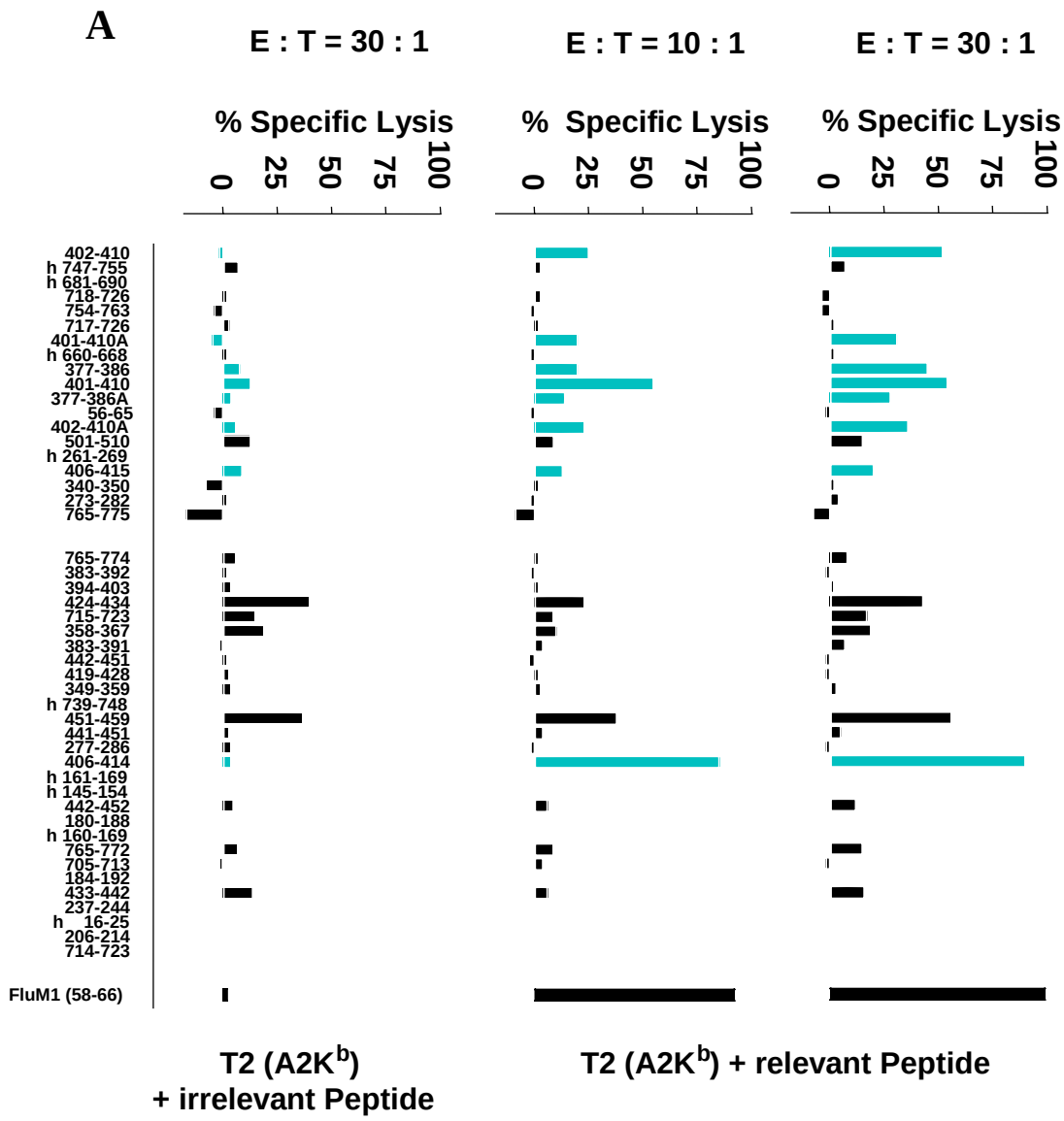

A
E : T = 30 : 1
E : T = 10 : 1
E : T = 30 : 1
% Specific Lysis
% Specific Lysis
% Specific Lysis
0
25
50
75
100
402-410
h 747-755
h 681-690
718-726
754-763
717-726
401-410A
h 660-668
377-386
401-410
377-386A
56-65
402-410A
501-510
h 261-269
406-415
340-350
273-282
765-775
765-774
383-392
394-403
424-434
715-723
358-367
383-391
442-451
419-428
349-359
h 739-748
451-459
441-451
277-286
406-414
h 161-169
h 145-154
442-452
180-188
h 160-169
765-772
705-713
184-192
433-442
237-244
h 16-25
206-214
714-723
FluM1 (58-66)
T2 (A2K^b)
+ irrelevant Peptide
T2 (A2K^b) + relevant Peptide

Fig. 8

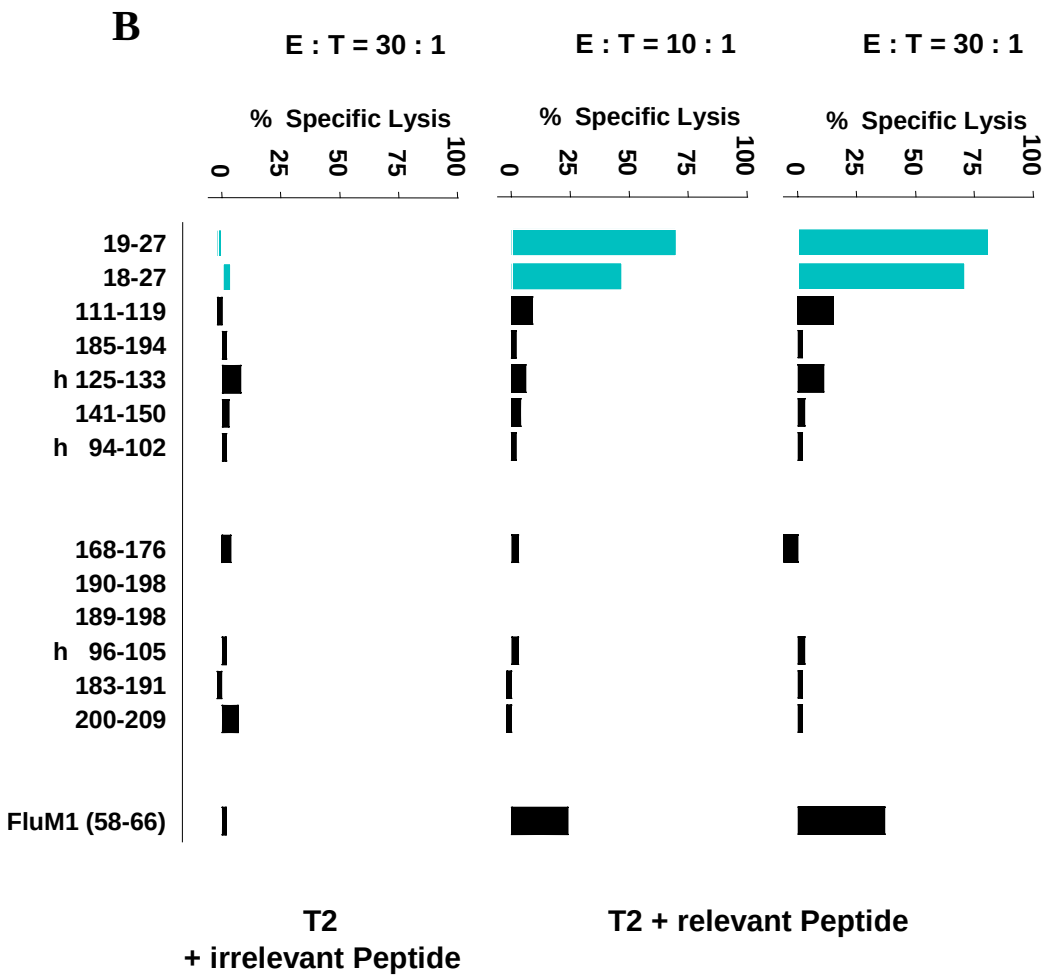


Fig. 8: A2.1-restricted immunogenicity of PRDI-BF1 and XBP-1 peptides in A2.1 Tg mice. Splenocytes from cA2K^b or CD8 x cA2K^b Tg mice primed with the indicated PRDI-BF1 (A) or XBP-1 (B) peptides were stimulated *in vitro* with the priming Ag. After 6 days, effector cells were tested in 4 or 5.5 h ^{51}Cr-release assays at indicated E : T ratios for cytolytic activity to T2A2K^b targets (for cA2K^b responder mice) or T2 targets (for CD8 x cA2K^b responder mice) loaded with the immunizing PRDI-BF1 peptide at 10^{-6} M (A), XBP-1 peptide at 10^{-5} M (B) or an unrelated A2.1-binding peptide at corresponding concentrations (A, B left). Data represent the highest amount of lytic activity obtained after peptide priming of 2-3 mice. Homologous peptides are indicated by “h”. Immunogenic peptides are colored.

3.6 PRDI-BF1 and XBP-1 peptide-specific CTL

To facilitate CTL avidity in order to determine whether the immunogenic PRDI-BF1 and XBP-1 peptides were naturally processed and presented by A2.1 on Hu APC, polyclonal CTL lines were established by repetitive restimulation of primary cultures derived from peptide-primed CD8 and A2.1/(c)A2K^b double Tg mice. The CTL lines that were obtained for PRDI-BF1 (406-414) and (406-415) required as much as 0.1 µM of Ag to induce lysis of peptide-pulsed T2 cells (data not shown). Provided that these Ag would have been endogenously processed, the CTL were likely of too low avidity to allow detection of natural Ag and were therefore not further investigated. In contrast, CTL lines established by stimulation with PRDI-BF1 (377-386), (402-410) and (401-410) were of higher avidity as they required less than 10 nM of either stimulatory Ag to induce killing of peptide-pulsed T2 cells (**Fig. 9A-C**). Again, peptide-specificity and A2.1-restriction of responding CTL were demonstrated by their failure to lyse T2 cells loaded with FluM1 (58-66) and EL4 cells coated with either stimulatory peptide (**Fig. 9A-C**).

A common property of all PRDI-BF1-derived and immunogenic peptides was the presence of a cysteine (**Table 1**). Due to its free S-H group, cysteine is the most chemically reactive aa within the 20 common aa under physiological conditions. Accordingly, multiple modifications have been described *in vitro* and *in vivo* for cysteine-containing peptides that are based on oxidization to disulfide bonds, e.g., through peptide-dimerization or the attachment of a second cysteine (cysteinylation) (Chen et al., 1999; Meadows et al., 1997). These reports suggested to explore the possibility whether or not such modifications are relevant for PRDI-BF1-specific CTL recognition. Upon peptide titration in the presence of the reducing agent TCEP that causes reduction of disulfide bonds, a 10-fold increase in peptide sensitivity was observed for PRDI-BF1 (401-410)- and (402-410)-specific CTL (data not shown). However, no effect was seen with CTL specific for PRDI-BF1 (377-386) or a noncysteine-containing Ag (data not shown). These results indicated that disulfide formation can indeed affect antigenicity of PRDI-BF1 peptides and may lead to false interpretation of CTL avidity. To avoid these problems, the properties of synthetic peptides in which cysteine was replaced by alanine (-SH substituted by -H) (Chen et al., 1999) or substituted by α-amino-butyric-acid (-SH substituted by an isosteric -CH_3

group) (Dr. T. Schumacher, The Netherlands Cancer Institute, Amsterdam, The Netherlands, personal communication) were studied. These substitutions that convert the cysteine side chain into a nonreactive form had neither a significant effect on peptide-binding to A2.1 (**Fig. 7A** and data not shown), nor did they affect immunogenicity in Tg mice (**Fig. 8A** and data not shown). The alanine-substituted PRDI-BF1 (377-386) peptide, PRDI-BF1 (377-386A), demonstrated a slightly increased A2.1-binding activity (**Fig. 7A**). By loading limiting quantities of these substitutes onto T2 cells for CTL recognition, it was found that the PRDI-BF1 (377-386)- and (402-410)-stimulated CTL, but not the CTL propagated with PRDI-BF1 (401-410), were at about 1-log more sensitive in recognizing the alanine substitutions as compared to the wt peptides (**Fig. 9A, B** and data not shown). Comparable results were obtained for the α-amino-butyric-acid substitutes (data not shown). It remains unclear whether the increase in antigenicity of PRDI-BF1 (377-386A) was due to its enhanced A2.1-binding, a higher TCR affinity for this peptide variant, or a combination of both. The alanine residue in PRDI-BF1 (377-386A) occupied the C-terminal anchor position and was likely to be hidden in the A2.1-binding pocket distant from TCR contact sides, thus favoring A2.1-binding. However, experiments using the alanine or α-amino-butyric-acid substitutes for *in vivo* priming and subsequent *in vitro* stimulation did not improve the functional avidity of responding effector T cells (data not shown). Employing the substituted peptides for the *in vitro* propagation of PRDI-BF1 (377-386)- and (402-410)-specific CTL lines seemed to be of advantage as these variants stimulated these T cell lines most efficiently and did not allow undesired *in vitro* modifications. Therefore, both CTL lines were maintained with the alanine-substituted peptides. CTL with specificity for PRDI-BF1 (401-410) that did not substantially crossrecognize peptide substitutes but were triggered predominantly by the unmodified peptide as suggested by experiments under reducing conditions, were propagated with the wt peptide in cystine-free RPMI. All these efforts to increase the avidity of PRDI-BF1-reactive CTL were undertaken not only to test recognition of peptide-pulsed target cells, but also of targets that naturally presented processed PRDI-BF1 peptides.

In case of XBP-1, A2.1-restricted CTL lines with specificity for XBP-1 (18-27) and (19-27) were obtained after repetitive Ag-dependent restimulation (**Fig. 9D,E**).

Compared to PRDI-BF1-specific CTL lines, these effector T cells seemed to be of higher avidity as both T cell lines required even less than 0.1 nM peptide for lysis of peptide-coated T2 cells (**Fig. 9D,E**).

Fig. 9

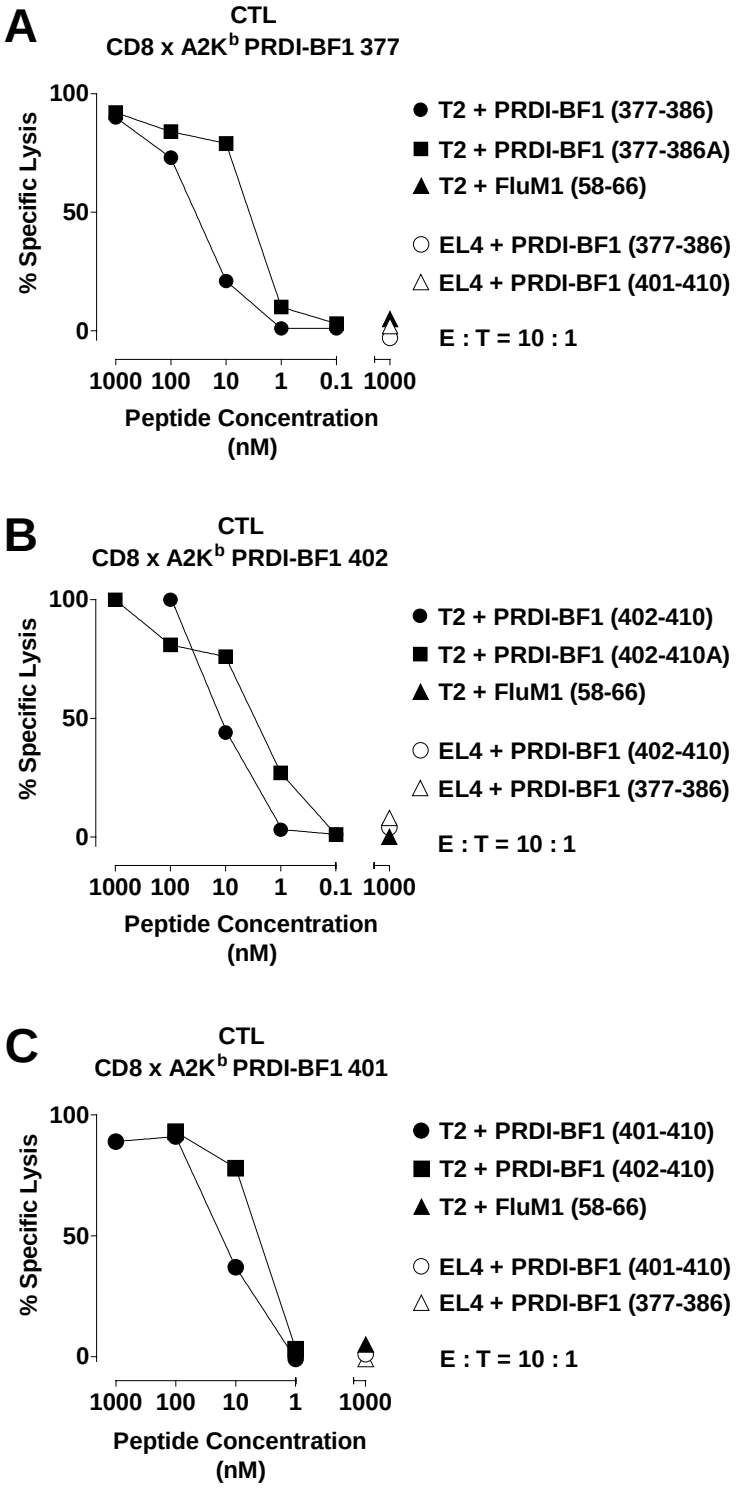


Fig. 9

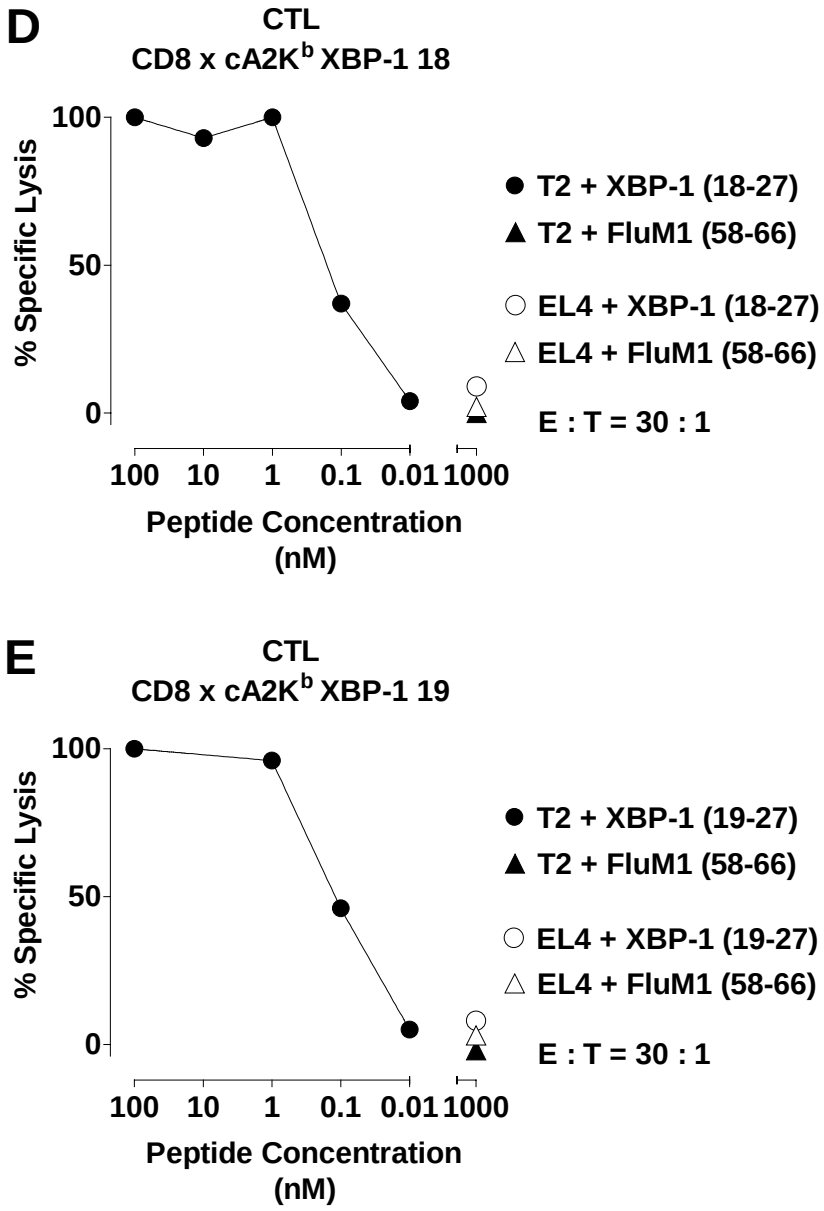


Fig. 9: Efficiency of peptide recognition, specificity and A2.1-restriction of PRDI-BF1 and XBP-1 peptide-reactive CTL lines. CTL lines established from primary cultures of (A) PRDI-BF1 (377-386)-, (B) (402-410)-, (C) (401-410)- and (D) XBP-1 (18-27)-, (E) (19-27)-induced effector T cells were tested at an E : T ratio of 10 : 1 (A-C) or 30 : 1 (D, E) for recognition of T2 targets pulsed with the stimulating peptide Ag (●), the indicated peptide variant (■) or FluM1 (58-66) (▲) and EL4 target cells coated with the stimulating peptide Ag (○) or an unrelated A2.1-binding peptide (△) at indicated concentrations in a 5.5 h ^{51}Cr-release assay.

3.7 Targeting of multiple myeloma by PRDI-BF1- and XBP-1-specific CTL

To assess whether the antigenic PRDI-BF1- and XBP-1-derived peptides represent naturally processed A2.1-bound CTL epitopes, targets for CTL recognition were explored that were likely to express high amounts of both proteins. As PRDI-BF1 and XBP-1 are known to be crucial for the terminal differentiation of B cells into malignant and non-malignant plasma cells, MM cells seemed to be suitable targets, although it had to be confirmed that these cells express both proteins in substantial amounts.

By using a polyclonal and PRDI-BF1 cross-recognizing anti-Blimp-1 antiserum for immunohistochemistry (provided by Dr. K. Calame, Columbia University, New York, USA), a strong PRDI-BF1 protein expression (red staining) was revealed in the nuclei of U266, DEPU, and NCI-H929, but not in OPM-2 and L363 cells (**Fig. 10A**). EL4 thymoma cells served as negative control (**Fig. 10A**).

XBP-1 expression, as studied by western blot analysis, was also detected in a subset of MM cell lines including U266, the same cells treated with IL-6, LP-1, and NCI-H929 (**Fig. 10B**). The PRDI-BF1-negative MM cell lines OPM-2 and L363 did not either appear to express detectable amounts of the XBP-1 protein (**Fig. 10B**). All XBP-1-postitive MM cells expressed the unspliced XBP-1 protein of 28 kDa. In contrast, spleen cells which appeared negative for XBP-1 accumulated the spliced XBP-1 protein of 54 kDa after 3 days of LPS treatment (**Fig. 10B**).

A very recent report that correlated XBP-1 protein splicing with plasmacytic differentiation suggested the occurence of the spliced form of XBP-1 in late stages of plasma cell differentiation (Iwakoshi et al., 2003). These results indicate, that the MM cell lines U266, LP-1 and NCI-H929 may have either originated from early-stage plasma cells or do express spliced XBP-1 in amounts below the detection threshold.

Despite their differential PRDI-BF1 protein expression, the A2.1-positive MM cell lines U266, DEPU, OPM-2, and L363 as opposed to A2.1-negative NCI-H929 were killed by PRDI-BF1 (402-410)-specific CTL (**Fig. 11A**). An even more efficient recognition of $A2.1^+$ MM cells was obtained for CTL with specificity for PRDI-BF1 (377-386) (**Fig. 14A**). These findings clearly indicate that PRDI-BF1 (377-386) and (402-410) represent naturally processed CTL epitopes. CTL responses to all MM cell lines were controlled by allo A2.1-reactive and FluM1 (58-66)-specific T cell lines (**Fig. 11C,D** and **14E, F**).

Fig. 10

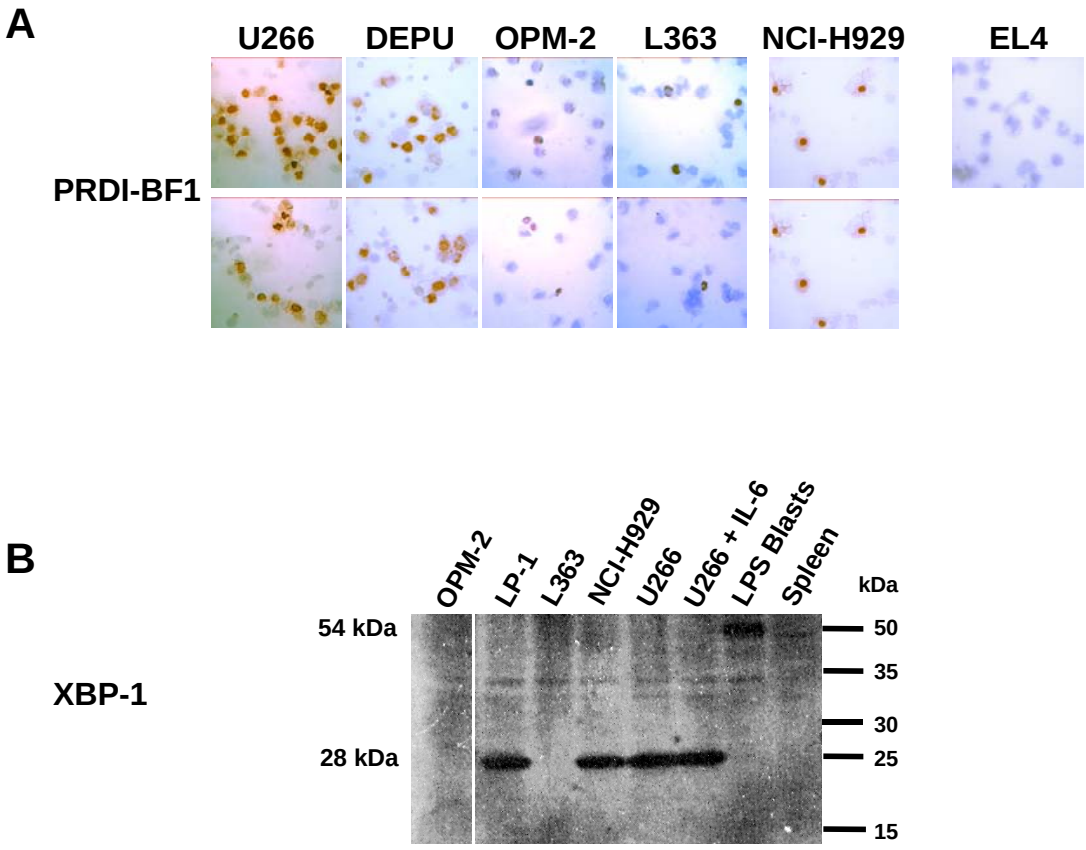


Fig. 10: PRDI-BF1 and XBP-1 protein expression of various MM cell lines. (A) Immunohistochemical staining for expression of PRDI-BF1 (red) of the indicated MM cell lines and EL4 control cells prepared by cytospin. Cells were counterstained with hematoxilin (blue). (B) Western blot analysis for XBP-1 protein in whole cell extracts prepared from the indicated cell lines, normal spleen cells and the same cells treated for 3 days with LPS. The 28 kD unspliced and the 54 kD spliced XBP-1 protein variants are indicated.

Fig. 11

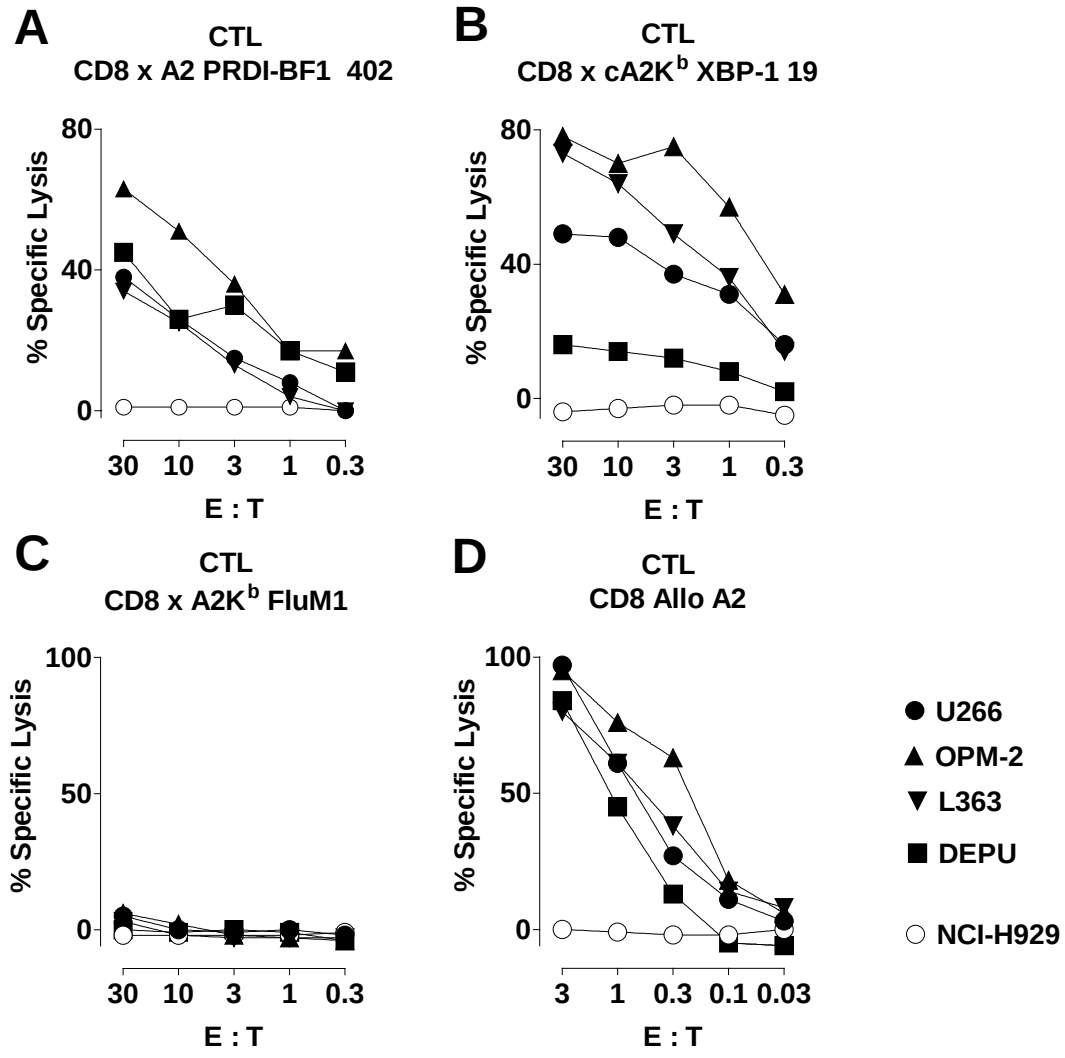


Fig. 11: Recognition of MM cells by PRDI-BF1- and XBP-1-reactive CTL. CTL CD8 x A2 PRDI-BF1 402 (A) and CD8 x cA2K^b XBP-1 19 (B) as well as the effector controls CD8 x A2K^b FluM1 and CD8 Allo A2 (C, D) were tested for cytolytic activity to U266 (●), OPM-2 (▲), L363 (▼), DEPU (■), and NCI-H929 (○) at the indicated E : T ratios in a 5.5 h ^{51}Cr-release assay.

Effector T cells propagated with PRDI-BF1 (401-410) exhibited a more profound lytic activity in response to the 9-mer PRDI-BF1 (402-410) than to the 10-mer (401-410) (**Fig. 9C**). The observation that these CTL were also able to selectively kill $A2.1^+$ MM cells (**Fig. 14B**), simply suggested that the 9-mer is the natural Ag recognized by this T cell line. However, the differential recognition of MM cell lines by PRDI-BF1 (402-410)- versus (401-410)-stimulated CTL (**Fig 11A** and **14 B, C**) seemed not to be consistent with this suggestion. However, not every cysteine in a given peptide is modified in the same way. This leads to different modifications of the very same peptide epitope in one cell type (Meadows et al., 1997) which, along with the distinct recognition pattern of peptide substitutes by both CTL (as discussed in **3.6**), could be one key to explain this inconsistence. It is tempting to speculate that the corresponding MM cell lines may present more than one modified form of PRDI-BF1 (402-410) in different amounts. This conceivable scenario is potentially superimposed by the possibility of an unefficient N-terminal trimming of precursor peptides in the ER (Saric et al., 2002; York et al., 2002; Serwold et al., 2002), resulting in the generation of different quantities of both, 9-mer and 10-mer peptides accessible for various cysteine-modifications. In this context, it has to be mentioned that modifications of cysteine may also be relevant for the CTL epitope, PRDI-BF1 (377-386).

To attempt to gain insight into the underlying modification type and to identify the form of the natural antigenic epitopes presented by A2.1 and recognized by PRDI-BF1-specific effector cells, class I MHC molecules from OPM-2 and U266 cells were immunoprecipitated. The bound peptides were eluted, concentrated, and separated by RP-HPLC (in collaboration with S. Stevanovich, Institute for Cell Biology, Tübingen). Aliquots of the resulting HPLC-fractions were subsequently loaded onto T2 cells to serve as targets for PRDI-BF1-specific CTL. However, as neither of these natural HPLC-fractions reconstituted antigenic activity for PRDI-BF1-specific CTL (data not shown), this effort did not allow to define the natural PRDI-BF1-derived Ag. Possible explanations for this negative result could be that the input cell number of OPM-2 and U266 (amount 4×10^{10} versus 10×10^{10} (Meadows et al., 1997)) was too low to allow Ag detection by this technology, or that cysteine-modifications during the complex experiment resulted in the disappearance of the relevant Ag.

In case of XBP-1-reactive CTL, more profound responses to all A2.1$^+$ MM cell lines except DEPU were obtained with effector T cells specific for XBP-1 (19-27) and (18-27) (**Fig. 11B** and data not shown). Ignorance of A2.1$^-$ XBP-1$^+$ NCI-H929 cells confirmed the A2.1-restriction of both CTL lines (**Fig. 11B** and data not shown). Importantly, the XBP-1 (18-27)- versus the (19-27)-specific CTL line was able to efficiently crossrecognize the shorter (19-27) peptide (data not shown), but this ability vanished upon long term of *in vitro* maintenance and was accompanied by a reduced recognition of natural Ag presented on MM cells (data not shown). As their avidity in response to the stimulatory Ag XBP-1 (18-27) itself was not affected, this indicated that XBP-1 (19-27) and not the XBP-1 (18-27) peptide is the naturally processed CTL epitope. For this reason, further experimental results were exclusively shown for XBP-1 (19-27)-specific CTL. Interestingly, this epitope is common to both, unspliced and spliced forms of XBP-1. Natural peptide fractions obtained from class I MHC-immunoprecipitation and RP-HPLC of U266 and OPM-2 cells were also tested for recognition by XBP-1 (19-27)- and (18-27)-specific effector T cells. However, neither of these fractions did reconstitute antigenic activity for both CTL lines. This observation along with the lack of recognition observed with PRDI-BF1-specific CTL suggested that the input cell number was indeed insufficient to allow Ag detection by this approach.

Although OPM-2 and L363 lacked detectable amounts of PRDI-BF1 and XBP-1 protein expression by immunohistochemistry and western blot analysis (**Fig. 10A, B**), their efficient killing by PRDI-BF1- and XBP-1-specific CTL, suggested at least a high turnover of both proteins to allow the generation of sufficient amounts of Ag for A2.1-restricted CTL recognition. In case of PRDI-BF1, this hypothesis was supported by RT-PCR data demonstrating high quantities of PRDI-BF1 transcripts in both cell lines (Nagy et al., 2002; and data not shown).

To verify whether the epitopes recognized by PRDI-BF1- and XBP-1-specific CTL had been generated by the proteasome, 20S proteasomal Ag production by OPM-2 was inhibited using lactacystin (Rock et al., 1994). OPM-2 were acid-treated to strip natural peptides off their cell surface class I MHC molecules to ensure that only new peptides generated by proteolytic degradation in the presence or absence of lactacystin would be presented for CTL recognition. It was found that exposure of these acid-pretreated cells

to lactacystin inhibited production of the antigenic epitopes, as shown here for PRDI-BF1 (402-410) and XBP-1 (19-27) (**Fig. 12A,B**). Lysis of targets by peptide-dependent allo A2.1-reactive effector T cells was also affected by lactacystin while no effect was observed with CTL CD8 x $A2K^b$ FluM1 (58-66) used as negative control (**Fig. 12C,D**). These findings demonstrated that the natural peptides recognized by PRDI-BF1- and XBP-1-specific CTL were endogenously processed by the 20S proteasome. In conclusion, these data indicate that PRDI-BF1- and XBP-1-derived peptides provide universal MM-associated tumor Ag.

To extend the analysis of PRDI-BF1- and XBP-1-specific CTL to primary MM cells, bone marrow samples from MM patients had been preserved and the percentage of monoclonal plasma cells was determined by FACS analysis (data not shown). As access to these primary cells was limited and MM cell infiltration in the majority of samples was low (about 30 %), specific T cell recognition was assessed by IFN-γ secretion in ELISPOT as opposed to ^{51}Cr-release assays. Normal bone marrow from the same patients was not available. Therefore, marrow cells isolated from a healthy donor served as control. MM-infiltrating marrow cells from 3 out of 7 $A2.1^+$ patients were specifically recognized by XBP-1 (19-27)-reactive CTL (**Fig. 13A, D**). These results are consistent with recent reports demonstrating enhanced XBP-1 expression in different types of non-malignant and malignant plasma cells (Zhan et al., 2003). A2.1-positivity of patients was confirmed by target cell recognition with allo A2.1-reactive CTL (**Fig. 13C, F**). FluM1 (58-66)-specific CTL served as negative control (**Fig. 13B, E**). Measurable IFN-γ secretion was not detected for PRDI-BF1-specific CTL (data not shown), suggesting that corresponding A2.1/peptide complexes were not produced in sufficient amounts to allow recognition by these effector T cells. The possibility that all $A2.1^+$ primary MM cells did not express PRDI-BF1 seemed unlikely because several reports demonstrated PRDI-BF1 expression by cDNA microarrays, quantitative RT-PCR or western blot analysis in all types of non-malignant and malignant plasma cells (Borson et al., 2002; Nagy et al., 2002; Ghosh et al., 2001; Angelin-Duclos et al., 2000; Zhan et al., 2003).

These data implied that primary MM cells can indeed be targeted by PRDI-BF1-specific CTL provided that they are of sufficiently high avidity. Obviously, high-avidity XBP-1-specific CTL target not only long term cultured MM cell lines, but also

primary MM cells freshly isolated from A2.1^{+} patients. These observations provide the rationale basis for a CTL-mediated immunotherapy of MM.

Fig. 12

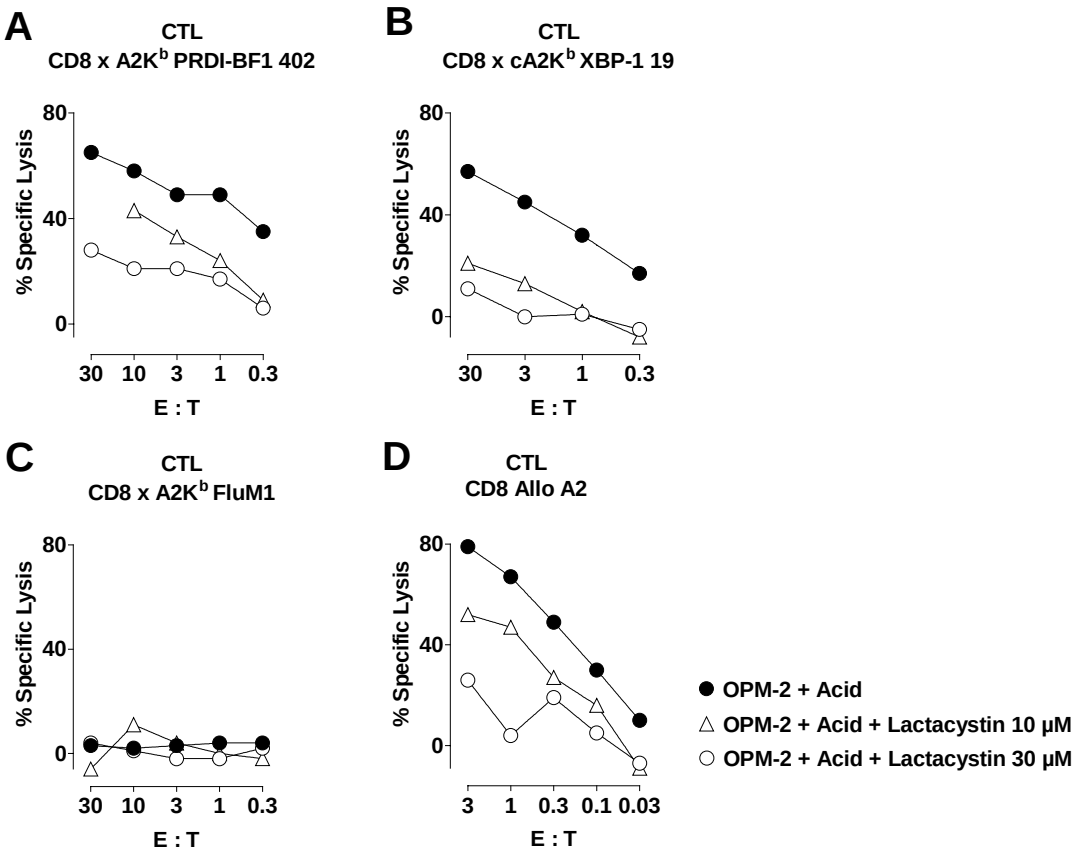


Fig. 12: Recognition of MM cells by PRDI-BF1- and XBP-1-reactive CTL in the presence of lactacystin. Acid-treated OPM-2 cells were not exposed (●) or exposed to lactacystin at 10 µM (△) and 30 µM (○) and tested in the continuous presence or absence of lactacystin for recognition by CTL CD8 x A2K^b PRDI-BF1 402 (A), CD8 x cA2K^b XBP-1 19 (B), CD8 x A2K^b FluM1 (C) and peptide-dependent CD8 Allo A2 (D) at indicated E : T ratios in a 5.5 h ^{51}Cr-release assay.

Fig. 13

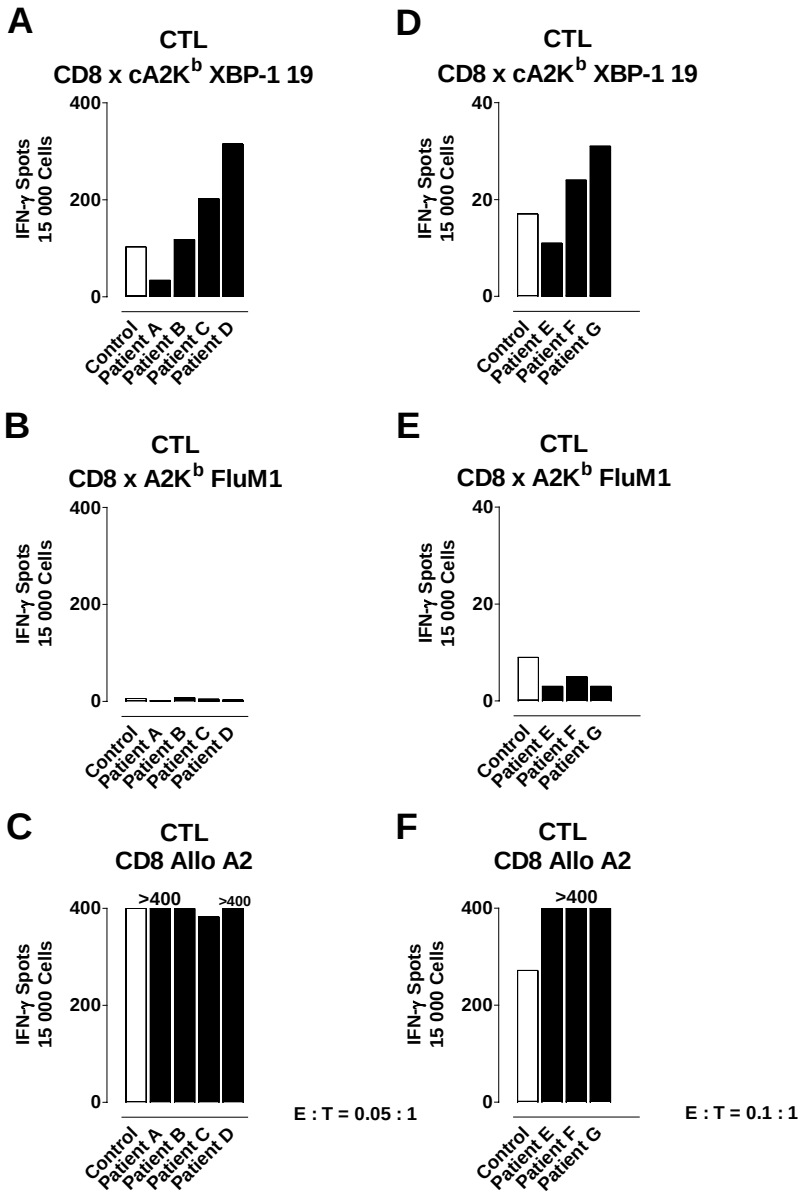


Fig. 13: Recognition of primary MM cells by XBP-1-specific CTL. Bone marrow cells isolated from $A2^+$ MM patients A-G were analyzed for presence and number of malignant plasma cells by FACS analysis. Samples from patient A-D containing 30% and from patient E-G containing 60% malignant plasma cells were used as targets and adjusted to 1 x 10^5 plasma cells per well in IFN-γ ELISPOT assay. Bone marrow cells isolated from a healthy donor and used in corresponding cell numbers served as control target. Effector T cells were CD8 x cA2K^b XBP-1 19 (A, D), CD8 x A2K^b FluM1 (B, E) and CD8 Allo A2 (C, F) and were used at 1.5 x 10^4 cells per well resulting in the indicated E : T ratios.

3.8 PRDI-BF1- and XBP-1-specific CTL activity is not limited to malignant plasma cells

Targeted immunotherapy of MM would ideally be associated with selective killing of transformed cells while preserving nontransformed tissues. Recent data demonstrated a more abundant expression pattern of PRDI-BF1 as had previously been anticipated, arguing for its involvement in differentiation processes distinct from the B cell lineage (Chang et al., 2000). Apart from its expression in plasma cells, XBP-1 is even found in various adult tissues and, predominantly, in the liver (Kokura et al., 2000; Reimold et al., 2001). These findings led to the investigation of PRDI-BF1- and XBP-1- specific CTL in response to other (MM-unrelated) B cell malignancies, different solid tumor entities and nontransformed cells (**Fig. 14A-D**). All experiments were controlled by FluM1 (58-66)-specific and allo A2.1-reactive effector T cells (**Fig. 14E, F**).

Despite the preference for killing of MM cells and EBV-transformed B-LCL among B cell malignancies by all PRDI-BF1-specific CTL, an increase in recognition of different solid tumor cell lines and normal cells was observed and seemed to correlate with T cell avidity. In brief, the PRDI-BF1-reactive CTL responded efficiently to $A2.1^+$ MM cells and EBV-transformed B-LCL while pre B-ALL were ignored (**Fig. 14A-C**). These observations were in accordance with expression analyses of the mouse homologue, Blimp-1 (Turner, Jr. et al., 1994). Among the solid tumor cell lines, PRDI-BF1-specific cytolytic activity was only observed in response to the PRDI-$BF1^+$ osteosarcoma cell line U2OS (Keller and Maniatis, 1991) and the melanoma cell lines Malme 3M and NA8-Mel and increased from PRDI-BF1 (377-386)- and (401-410)- to PRDI-BF1 (402-410)-specific CTL (**Fig. 14A-C**). Overall, these findings corresponded to detectable Blimp-1 expression in skin (Turner, Jr. et al., 1994). Equivalent results were obtained with CTL responding to nontransformed cells. In more detail, whereas all normal cell targets were ignored by PRDI-BF1 (377-386)-reactive effector T cells (**Fig. 14A**), CTL propagated with PRDI-BF1 (402-410) revealed a cytolytic activity of about 25 % to renal tubular epithelial cells and of about 20 % to PBMC containing a substantial number of the specific B cell target population. In fact, PRDI-BF1 (402-410)-stimulated CTL gave rise to about 45 % specific lysis of purified B cells (**Fig. 14C**).

Fig. 14

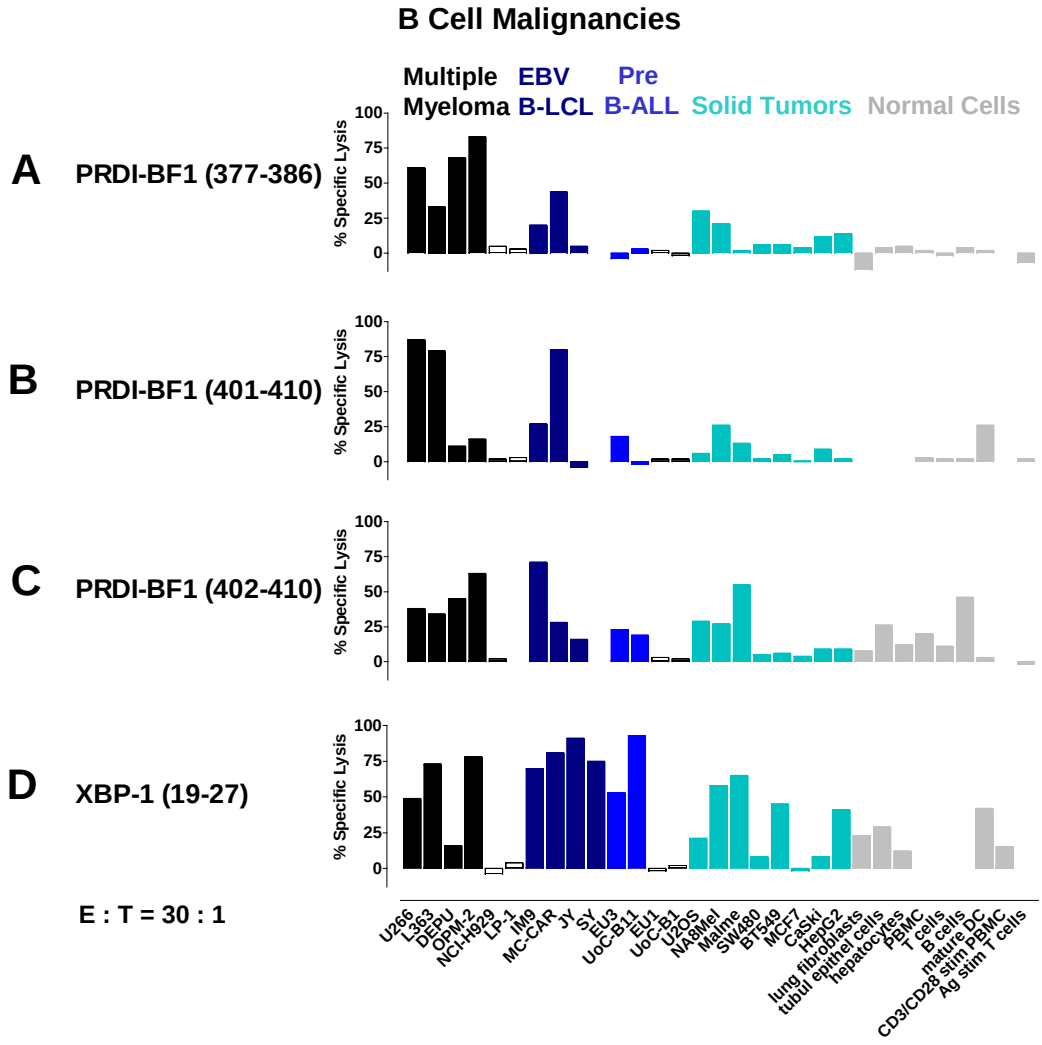
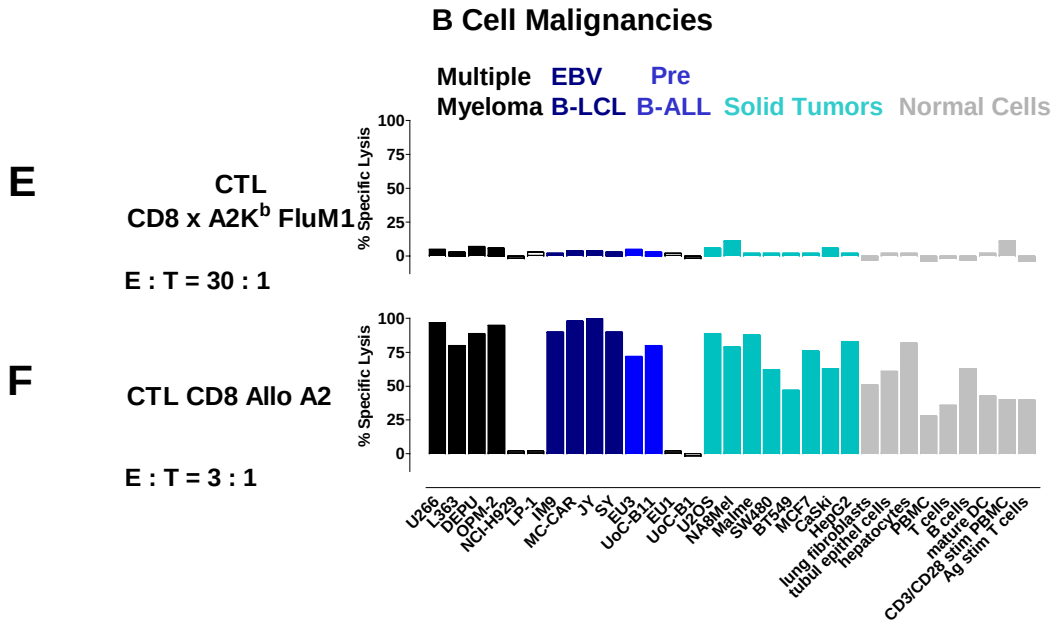


Fig. 14: Recognition of various tumor cell lines and nontransformed cells by PRDI-BF1- and XBP-1-reactive CTL. CTL lines with specificity for PRDI-BF1 (377-386) (A), (401-410) (B), (402-410) (C) and XBP-1 (19-27) (D) as well as the CTL controls CD8 x $A2K^b$ FluM1 (E) and CD8 Allo A2 (F) were tested at an E : T ratio of 30 :1 (A-E) or 3 : 1 (F) for recognition of the indicated B cell malignancies including MM, EBV B-LCL and pre B-ALL, solid tumor cell lines and nontransformed cells in a 5.5 h ^{51}Cr-release assay. Representative results on CTL recognition of nontransformed cells obtained from 2 different donors are shown.

Fig. 14



In comparison, effector T cells with specificity for XBP-1 (19-27) did not distinguish between different B cell malignancies and efficiently lysed all B cell-derived tumor targets, even the majority of solid tumor cell lines were recognized moderately (**Fig 14D**). Among the tested nontransformed cells, specific lysis by XBP-1-reactive CTL was less than 20 % except for renal tubular epithelial cells and mature DC which seemed to be killed with about 30 % and 40 % cytolytic activity, respectively (**Fig. 14D**).

The recognition of mature DC by XBP-1 (19-27)- and PRDI-BF1 (401-410)-propagated CTL seemed to be in conflict with recent findings that mature DC effectively protect themselves against CTL-induced apoptosis by expression of the granzyme inhibitor, serine protease inhibitor (SPI)-6/PI-9 (Medema et al., 2001). Despite the result that both, Th1 and Th2 subsets induce DC maturation, only Th1 cells induce SPI-6 expression and subsequent DC resistance. The observation of DC killing by CTL in this study might be due to the fact that CTL action was measured by the classical ^{51}Cr-release assay as opposed to other assays, such as JAM, that are based on DNA-fragmentation. Th2 cytokine-based DC maturation may also reflect an somewhat unphysiological situation that could have led to false-positive results (Medema et al., 2001).

XBP-1 was also found to be expressed in hepatocellular carcinoma (Kishimoto et al., 1998) consistent with substantial recognition of HepG2 cells (**Fig. 14D**). Although some XBP-1 protein was found in adult Hu liver (Kishimoto et al., 1998), its level of expression is obviously not sufficient to allow XBP-1-specific killing as observed with $A2.1^+$ hepatocytes isolated from two different donors (**Fig. 14D**).

In summary, PRDI-BF1 and XBP-1 expression is not entirely limited to MM-cells and thus correlates with a more broad-spectrum tumor cell recognition by the relevant CTL. In case of XBP-1- and PRDI-BF1 (402-410)-reactive effector T cells, this finding seems to be associated with low-level killing of a few types of nontransformed cells.

3.9 PRDI-BF1- and XBP-1-specific self tolerance in man

The more abundant expression pattern of PRDI-BF1 and XBP-1 suggested that the self A2.1-restricted Hu T cell repertoire is affected by PRDI-BF1- and XBP-1-specific self tolerance. To study this hypothesis, naive $CD8^+$ T lymphocytes from at least 2 different $A2^+$ healthy donors underwent *in vitro* stimulation with mature $A2^+$ autologous DC that had been pulsed with either of the 3 relevant PRDI-BF1 peptides, XBP-1 (19-27) or hTyr (369-377). By using PRDI-BF1 (377-386) and (402-410) for CTL induction, the generated $CD8^+$ T cell cultures were neither cytolytic nor did they secrete IFN-γ in response to peptide-coated T2 targets (data not shown). Despite that the amount of PRDI-BF1 (377-386) in nontransformed cells was insufficient for CTL induction (**Fig. 14A**), it seems to allow the induction of complete self tolerance. In contrast, CTL with specificity for PRDI-BF1 (401-410) and the ability to kill peptide-loaded T2 targets were induced in at least 1 out of 4 different donors (**Fig. 15A**). Interestingly, the responding CTL did not distinguish between PRDI-BF1 (401-410) and the corresponding 9-mer PRDI-BF1 (402-410) peptide (**Fig. 15A**). Responses, although weaker in strength, were also obtained for XBP-1 (19-27)-propagated $CD8^+$ T cells isolated from 1 out of 2 donors (**Fig. 15B**). Absence of detectable CTL activity in non-responding donors was due to the use of PRDI-BF1 and XBP-1 peptides as hTyr (369-377)-reactive CTL were induced in all donors (**Fig. 15C** and data not shown, **Fig. 16C**).

Fig. 15

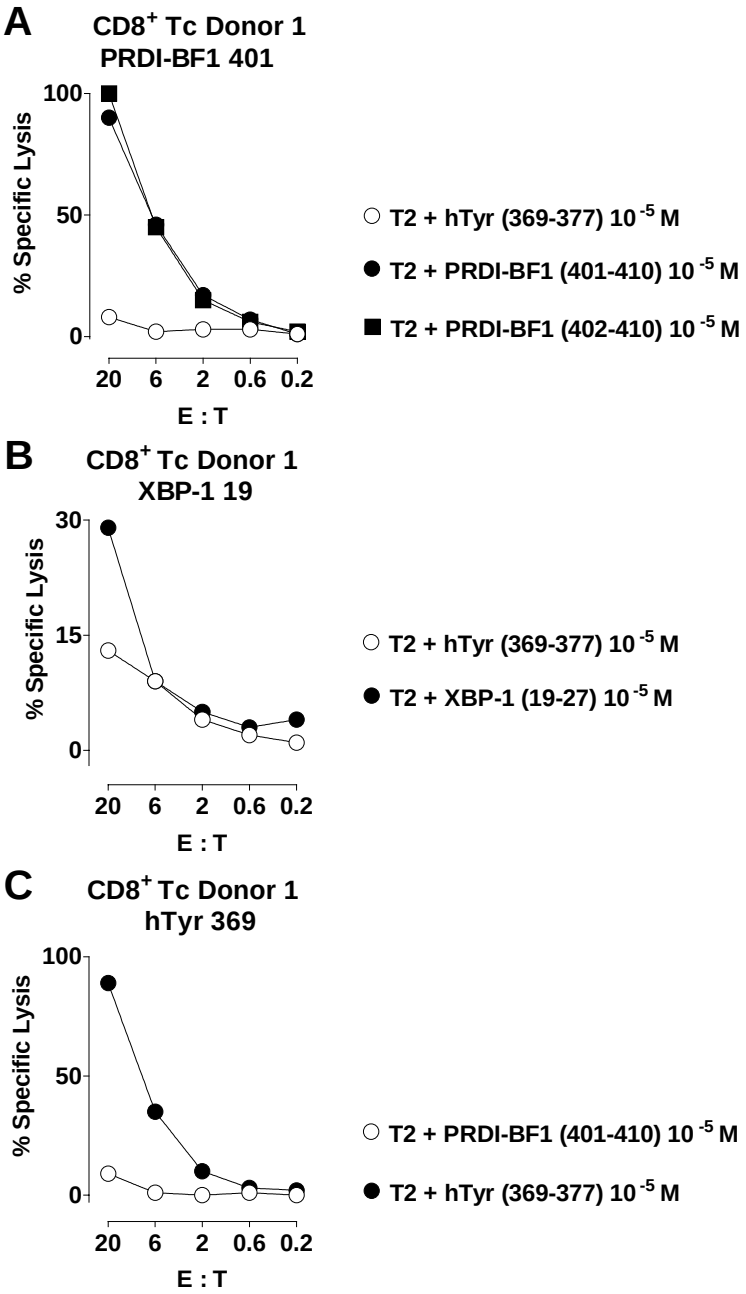


Fig. 15: Ag-specific cytolytic activity of Hu $CD8^+$ T cells induced with PRDI-BF1- and XBP-1-derived peptide epitopes. Hu $CD8^+$ T lymphocytes were isolated from 2 $A2^+$ healthy donors, stimulated in parallel for 5 cycles with (A) PRDI-BF1 (401-410)-, (B) XBP-1 (19-27)- and (C) hTyr (369-377)-loaded autologous mature DC and tested at the indicated E : T ratios for cytolytic activity to T2 target cells coated with the stimulating peptide Ag (●), a peptide variant (■) and an unrelated A2.1-binding peptide (○) at 10^{-5} M in a 5.5 h ^{51}Cr-release assay. Effector cells were plotted for donor 1. Donor 2 was a non-responder to both, indicated PRDI-BF1 and XBP-1 Ag.

Fig. 16

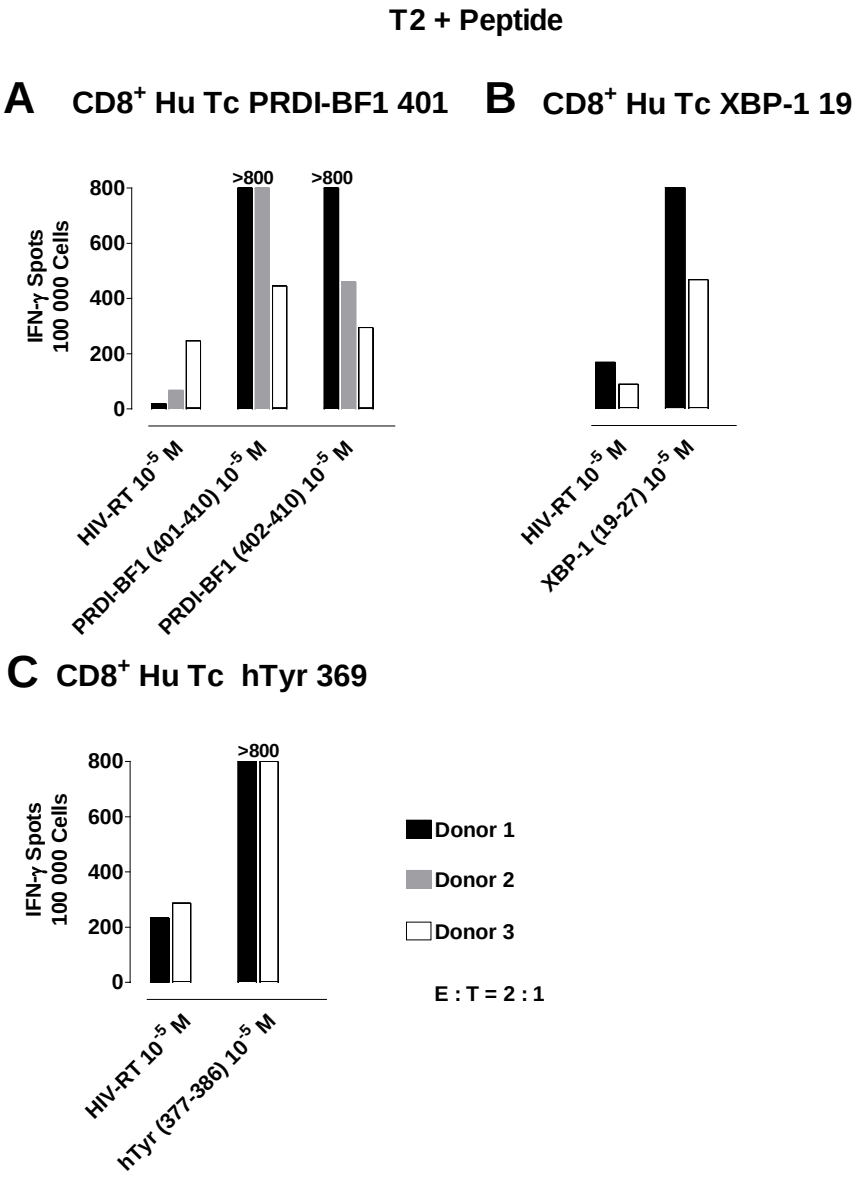


Fig. 16: Ag-specific IFN-γ secretion by Hu $CD8^+$ T cells induced with PRDI-BF1- and XBP-1-derived peptide epitopes. Hu $CD8^+$ T lymphocytes isolated from at least 2 $A2^+$ healthy donors and stimulated in parallel for 5 cycles with PRDI-BF1 (401-410)-, XBP-1 (19-27)- and hTyr (369-377)-pulsed autologous mature DC were tested at an E : T ratio of 2 : 1 for IFN-γ secretion in ELISPOT assay. $CD8^+$ T cells PRDI-BF1 401 (A), $CD8^+$ T cells XBP-1 19 (B) and $CD8^+$ T cells hTyr 369 (C) were evaluated in response to T2 cells coated with the indicated peptides at 10^{-5} M. Effector cells generated from an additional donor (donor 2) are plotted for $CD8^+$ T cells PRDI-BF1 401.

Despite the observation that some of the T cell cultures stimulated with PRDI-BF1 (401-410) and XBP-1 (19-27) did not reveal any cytolytic activity as measured in ^{51}Cr-release assays (data not shown), all of them were able to specifically secrete IFN-γ in response to peptide-coated T2 cells when tested in ELISPOT assays (**Fig. 16A, B**). However, IFN-γ secretion in response to the A2.1^{+} MM cell lines L363 and U266 was only observed with those PRDI-BF1 (401-410)- and XBP-1 (19-27)-specific effector T cells that did show cytolytic potential (**Fig. 15A,B** and **Fig. 17 A, C**). MM cell-recognition was A2.1-restricted and not affected by NK-activity as no substantial recognition of A2.1^{-} NCI-H929 and K562 targets was detected (**Fig. 17A, C**). Corresponding effector T cells stimulated with hTyr (369-377) served as negative control (**Fig. 17B, D**).

Apart from cysteine-modification effects, the most evident explanation for the discrepancy of Hu CTL induction observed with PRDI-BF1 (401-410) versus (402-410) is the endogenous processing and presentation of the 9-mer PRDI-BF1 (402-410) in a quantity that leads to the tolerization of the PRDI-BF1 (402-410) T cells while preserving Ag-specific CTL which are unable to proliferate in response to PRDI-BF1 (402-410). However, the naturally processed PRDI-BF1 (401-410) epitope might be presented at low level by Hu APC sparing residual T cells that are inducible with the 10-mer (401-410) as opposed to the 9-mer (402-410) but, when differentiated to effector CTL, are capable of crossrecognizing 9-mer-labeled target cells. Another possibility might be that PRDI-BF1 (401-410) is not a natural CTL epitope and its employment only rescues the residual tolerance-prone PRDI-BF1 (402-410)-specific T cell repertoire. Both explanations would suggest PRDI-BF1 (402-410) to function as a partial agonist that fails to induce proliferation and differentiation but does give rise to cytolysis or at least the secretion of cytokines (Colella et al., 2000).

Based on these findings, it may be concluded that the peripheral Hu T cell repertoire with specificity for PRDI-BF1 and XBP-1 is governed by partial tolerance. In comparison to self Tyr-reactive Hu CTL, however, the broader expression pattern of PRDI-BF1 and XBP-1 seems to result in a more profound tolerization of responding T cells, allowing only low-avidity and potentially even non-cytotoxic effector cells, if any, to escape into the periphery.

Fig. 17

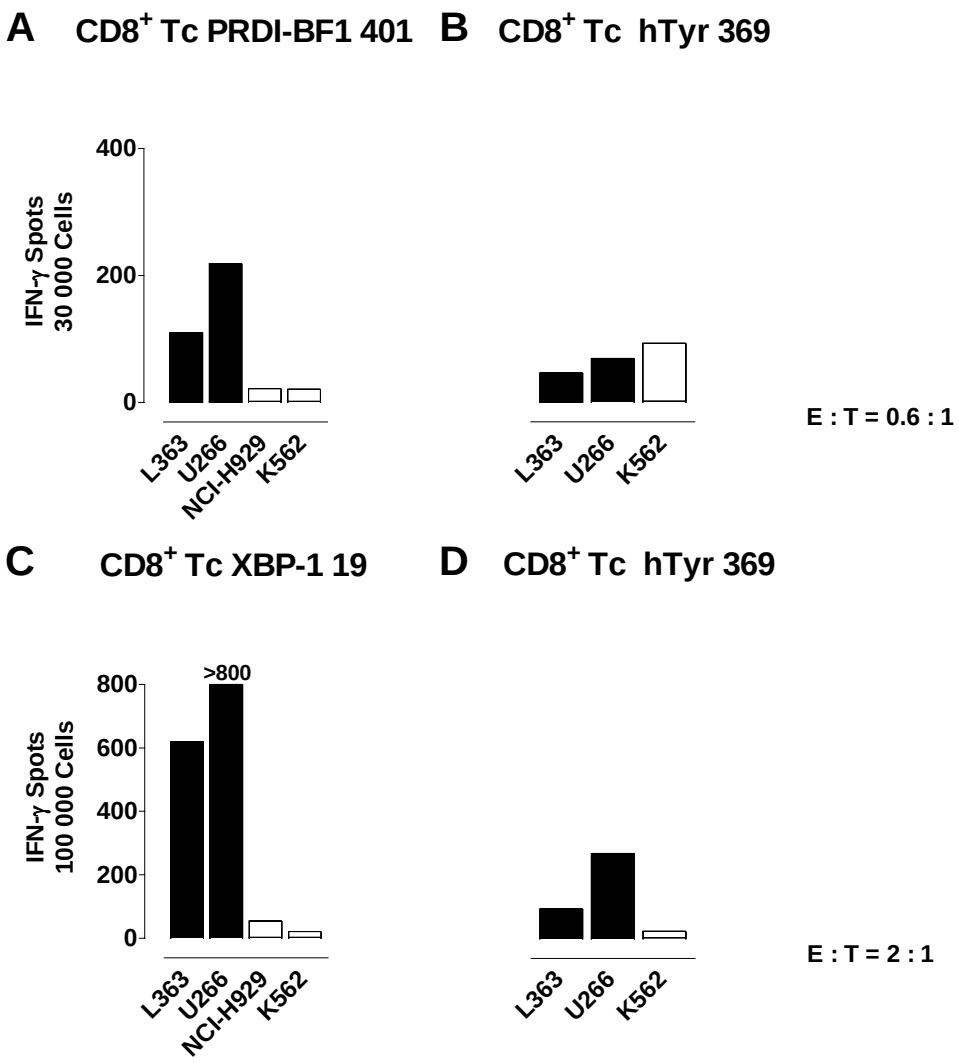


Fig. 17: Recognition of MM cells by Hu $CD8^+$ T cells specific for PRDI-BF1 and XBP-1. (A) PRDI-BF1 (401-410)- and (C) XBP-1 (19-27)-specific $CD8^+$ T cells obtained after 5 rounds of stimulation with peptide-pulsed autologous mature DC from donor 1 and the corresponding control effectors, $CD8^+$ T cells hTyr (369-377) (B, D), were tested at the indicated E : T ratios for IFN-γ secretion in response to the $A2.1^+$ MM cells L363 and U266, the $A2.1^-$ MM NCI-H929 and the NK-sensitive target K562 in ELISPOT assay.

4. Concluding Remarks

Part 1 Partial tyrosinase-specific self tolerance in mice and man

The presented studies on Tyr-specific tolerance in Tg animals and, particularly, in man, extend previous work in mice (Colella et al., 2000; Bullock et al., 2001; Mullins et al., 2001). Although differences in vaccination procedures (peptide versus vaccinia virus recombinants or Ag-loaded DC) as well as the use of CD8 x A2 (or x cA2K^b) versus cA2K^b and A2D^d (AAD) mice appear to have a substantial impact on the ability to promote self mTyr-reactive T cells (Colella et al., 2000; Bullock et al., 2001), they do not affect the fundamental observations of partial Tyr-specific tolerance and the presence of a residual repertoire of T cells with higher avidity for self mTyr. However, these differences may well account for the finding presented here that despite the employment of the xenogeneic hTyr epitope facilitated the frequency of mTyr-reactive CTL inductions, it did not improve the quality of responding self Tyr-specific T cells.

An equivalent result was obtained with Hu T cells. As with CD8 x A2 mice, selection by several cycles of restimulation allowed the expansion of Hu CD8$^+$ CTL with sufficiently high avidity to respond to naturally processed Ag presented by melanoma targets. Nevertheless, the novel finding of differential functional avidity and differential Ag dose-dependency associated with self hTyr- versus non-self mTyr-specific Hu CD8$^+$ CTL clearly demonstrates that the Hu Tyr-reactive immune repertoire is subject of partial tolerance too. As these results have obviously profound consequences for the design of clinical Tyr-based vaccination trials in melanoma patients, it is important to attempt to gain insight into the mechanisms underlying Tyr-specific tolerance induction. It has been recently reported that partial Tyr-specific self tolerance does not result from thymic Ag exposure to immature T cells (Engelhard et al., 2002), although medullary epithelium in the thymus has been demonstrated to express „tissue-specific“ peripheral Ag by virtue of the transcription factor AIRE (Derbinski et al., 2001; Kyewski et al., 2002; Anderson et al., 2002). This was the reason as to why the possibility was explored that partial tolerance to Tyr might be governed by Treg. However, the Tyr-reactive T cell repertoire was not affected by CD4$^+$CD25$^+$ Treg or treatment with anti-CTLA-4. As opposed to other melanoma-associated Ag (Sutmuller et al., 2001; van Elsas et al., 1999; van Elsas et al., 2001), it is therefore unlikely that Tyr-specific tolerance can be bypassed by interfering with CD4$^+$CD25$^+$ Treg or by inhibiting tolerogenic signals, such

as CTLA-4. It is yet possible that limited access of Tyr-reactive T cells to peripheral sites of Tyr expression, such as skin melanocytes, chorioid and retinal pigment epithelium (Kluppel et al., 1991; Tief et al., 1996), contributes to the formation of tolerance (Engelhard et al., 2002).

Part 2 A novel approach: Key transcription factors of B cell terminal differentiation provide universal multiple myeloma-associated tumor antigens

The results presented for PRDI-BF1 and XBP-1 in Tg mice confirm that key transcription factors of B cell terminal differentiation into malignant and non-malignant plasma cells can indeed be exploited to serve as universal MM-associated target molecules. Provided that CTL were of sufficiently high avidity, as demonstrated for effector T cells with specificity for an identified XBP-1-derived peptide epitope, it was found that recognition was not restricted to MM cell lines but was even extended to freshly isolated primary MM cells. TCR susceptibility to cysteine-modifications may account for the lower avidity of CTL with specificity for the cysteine-containing PRDI-BF1 Ag and their ignorance of primary bone marrow myeloma cells despite their recognition of established MM cell lines. However, this assumption could not be proved as all efforts to improve CTL avidity, e.g., by the employment of different cysteine-substituted peptides, failed. The technology of class I MHC-immunoprecipitation followed by peptide extraction and analysis did likewise not allow to determine the occurence of posttranslational alterations involving the cysteine residue in these PRDI-BF1 peptide epitopes *in vivo*. Nethertheless, the results on XBP-1- and, with a few limitations, on PRDI-BF1-specific CTL along with the important function of both proteins in all plasma cell types (Calame, 2001) suggest that this novel concept not only creates the opportunity to target all types of MM, but in addition may also prevent the risk of malignant plasma cell escape from immune recognition by the selection of PRDI-BF1- or XBP-1-loss variants.

The characterization of PRDI-BF1- and XBP-1-specific effector T cells and their responses to non-B cell-derived malignancies revealed recognition of different tumor entities accompanied by low-level cytotoxicity to a few nontransformed cell types. These results appeared to be consistent with the more abundant expression pattern of

both proteins (Chang et al., 2000; Reimold et al., 2001). Using the A2.1 Tg mouse model, it was not possible to study whether the susceptibility of some of the normal tissues to lysis by XBP-1- and PRDI-BF1-reactive CTL observed *in vitro* is actually also relevant *in vivo*: substantial CTL responses to mouse XBP-1- and Blimp-1-derived peptides, as studied so far, were not obtained. Furthermore, effectors specific for the identified PRDI-BF1 or XBP-1 peptide epitopes did either not or unefficiently crossrecognize the corresponding mouse homologues. A feasible approach that allows to address the potential problem of autoimmnuity would be the identification of a mouse XBP-1/Blimp-1 CTL epitope along with the induction of high-avidity T cells. Considering the results on the PRDI-BF1/XBP-1-responsive Hu T cell repertoire, it seems unlikely that this could be achieved in Tg mice. Therefore, the induction of CTL specific for Mu epitopes would require the selection of high-affinity TCR in man to be transferred in mouse T cells for their evaluation in Tg mice.

In case of the Hu T cell repertoire specific for PRDI-BF1- and XBP-1-derived peptide epitopes, it was observed that the number of PRDI-BF1 and XBP-1 Ag displayed by nontransformed cells was sufficient to induce self tolerance. This does not necessarily result in recognition of normal cells by high-avidity PRDI-BF1- and XBP-1-specific CTL derived from Tg mice because the amount of Ag required to establish tolerance is less than that required for effector cell signaling (Yagi and Janeway, Jr., 1990; Pircher et al., 1991). In comparison to Tyr, the PRDI-BF1- and XBP-1-specific Hu T cell repertoire was more profoundly affected by self tolerance as only low-avidity CTL, if any, were promoted by DC/Ag-based stimulation and occasionally even lacked any cytolytic activity towards APC pulsed with high amounts of Ag. It has not yet been evaluated whether the employment of peptide variants, as has been studied with Tyr, would allow to shape the avidity and frequency of the tolerance-prone PRDI-BF1- and XBP-1-specific Hu T cell repertoire. However, the findings reported here along with the mouse results on PRDI-BF1 and XBP-1 suggest that Hu T lymphocytes may be turned into efficient MM-reactive CTL by adoptive transfer of PRDI-BF1- and XBP-1-specific TCR derived from Tg mouse CTL. To prevent the risk of potential autoimmunity by the resultant high-avidity and MM-reactive PRDI-BF1- and XBP-1-reactive Hu CTL, gene-therapeutic “switch-off” techniques, such as the delivery of suicide genes, can be employed (Uckert et al., 1998). Provided that autoimmunity is controlled or limited to

defined tissues as has been observed in vaccination trials of melanoma patients or B cell-specific Ab treatment of malignant lymphomas (Engelhard et al., 2002; Dearden, 2002), this approach would open a novel and promising field for immunotherapy of MM.

The very recent demonstration of the post-transcriptional processing of XBP-1 that seems specifically associated with the unfolded protein response (Yoshida et al., 2001) and plasmacytic differentiation (Iwakoshi et al., 2003), suggests that the identification of CTL epitopes corresponding to sequences that were unique to the spliced XBP-1 protein would allow efficient T cell-targeting of MM with a decreased risk of autoimmunity.

Finally, the presented experiments provide the basis to consider a new class of proteins for the universal targeting of MM. Apart from PRDI-BF1 and XBP-1, another transcription factor, IFN regulatory factor 4 (IRF4), also known as MM oncogene 1 (MUM1), was reported to be inherently involved in plasmacytic differentiation (Mittrucker et al., 1997). Recent studies even report a recurrent genetic abberation in MM that juxtaposes the Ig heavy chain locus to the IRF4 gene resulting in IRF4 overexpression, an event that may contribute to tumorigenesis as IRF4 has oncogenic activity *in vitro* (Iida et al., 1997). According to these findings and the results presented here, it is proposed that IRF4 is likely to serve as another attractive candidate MM-associated tumor Ag. Ultimately, new technologies like oligonucleotide microarray analyses used for gene expression profiling in plasma cells revealed that these cells display a highly specialized genetic program including the expression of unique sets of known and novel transcription factors (Zhan et al., 2003; Underhill et al., 2003) that may also provide novel target Ag associated with MM.

Part 1 and 2 Tolerance and immunity to tumor-associated antigens

The studies presented in this work are focused on the T cell-targeting of 3 proteins, PRDI-BF1, XBP-1 and Tyr, shown to be expressed at elevated levels in tumor cells but also to be present in normal cells. The more abundant expression pattern of PRDI-BF1 and XBP-1 versus Tyr results in a more confined T cell repertoire in terms of avidity and function. Although a differential level of tolerance exists, the residual T cell repertoire with specificity for all of the corresponding Ag appears not to be adequate in

providing tumor protection. Therefore, rationale tumor immunotherapy requires interference with self tolerance while preventing or limiting the potential risk of autoimmunity. As studied for Tyr, tolerance cannot be bypassed by the employment of a peptide variant or by interfering with tolerogenic signals or mechanisms, e.g., Treg or CTLA-4 (Sutmuller et al., 2001; van Elsas et al., 1999; van Elsas et al., 2001). However, novel approaches to circumvent tolerance are promising. One strategy that takes advantage of HLA Tg mice allows the induction of tumor-reactive high-avidity T cells for all 3 target molecules. It provides therefore the appealing therapeutic possibility to transfer tumor-reactive mouse TCR into Hu T cells in order to equip the Hu T cell repertoire with high-avidity T cells that have been lost through tolerance induction (Stanislawski et al., 2001; Kessels et al., 2001b). Allo MHC-restricted TCR (Sadovnikova and Stauss, 1996; Stanislawski et al., 2001), *in vitro* mutated and selected TCR of high affinity (Holler et al., 2000; Kessels et al., 2000) and TCR-like molecules (Chames et al., 2002) offer alternative therapeutic instruments.

5. Summary

The aim of cytotoxic T lymphocyte (CTL)-based immunotherapy for malignant disease is the long term eradication of tumor cells and depends on T cells bearing high-affinity T cell receptors (TCR) with specificity for tumor-associated antigens (Ag). Because tumor-associated Ag are not specifically expressed in tumors but are also present in nontransformed cells, high-avidity CTL are often lost from the responding T cell repertoire due to self tolerance. Thus, to design effective immunotherapeutics, the level of tolerance for a given peptide Ag requires extensive immunological analysis.

The first part of this work focused on the human (Hu) tyrosinase (hTyr) (369-377) CTL epitope that is presented by malignant melanoma and various nontransformed cells in association with Hu histocompatibility leukocyte Ag (HLA)-A*0201 (A2.1) and used for vaccination-based immunotherapy of melanoma patients. Its mouse homologue, mTyr (369-377), is naturally processed and bound by A2.1 with equivalent efficacy and thus enabled the investigation of the effect of self tolerance on Tyr-specific T cells in different lines of A2.1 transgenic (Tg) mice and man. It was found that self Tyr-reactive CTL in Tg mice and, importantly, in man were affected by partial tolerance resulting in only residual T lymphocytes of higher avidity for self Tyr along with low-avidity T cells to be present in the periphery. Immunizing mice with the xenogeneic non-self Tyr peptide facilitated the generation of self Tyr-reactive CTL. As compared to Tyr-reactive CTL induced by high amounts of the self Tyr epitope, however, the non-self Ag had no effect on improving the avidity of self Tyr-specific mouse and Hu T cells. Depleting mice of $CD25^+$ T cells with and without CTL-associated Ag 4 (CTLA-4) blockade demonstrated that tolerance of Tyr-specific CTL was not regulated by $CD4^+CD25^+$ T regulatory cells (Treg) or CTLA-4.

In contrast to malignant melanoma, immunotherapy for the fatal and non-curative hematological malignancy multiple myeloma (MM), is at a very early stage of research and development because only few, if any, promising MM Ag have yet been described. To identify universal MM-associated Ag in the second part of this work, two transcription factors, the X box-binding protein 1 (XBP-1) and the mouse B lymphocyte-induced maturation protein 1 (Blimp-1) and its Hu homologue the positive regulatory domain I-binding factor 1 (PRDI-BF1), governing B cell terminal differentiation into malignant and non-malignant plasma cells were evaluated. A2.1 Tg

mice were used to identify A2.1-presented CTL epitopes derived from these proteins. CTL specific for several PRDI-BF1 and XBP-1 peptides were able to efficiently kill various MM cell lines. Based on their higher avidity, XBP-1-specific effector T cells were also capable of recognizing freshly isolated malignant myeloma cells. However, CTL recognition was not limited to myeloma targets but also occurred with various malignant cells and, albeit to a much lower extent, with a few types of normal cells. The Hu T cell repertoire was found to be profoundly affected by self tolerance. Ag-based stimulation of Hu T cells resulted in PRDI-BF1- and XBP-1-specific effector cells that were able to produce interferon (IFN)-γ in response to peptide-pulsed targets but had an impaired capacity to kill targets or to secrete IFN-γ in the presence of MM cells. These results clearly demonstrate that plasma cell-associated transcription factors can serve as MM-associated and even broader tumor Ag.

The delivery into Hu T-lymphocytes of appropriate TCR selected by circumventing self tolerance offers the appealing possibility to turn Hu T cells into efficient MM-reactive (PRDI-BF1/XBP-1) and melanoma-specific (Tyr) CTL. This work is an important and innovative contribution to the design of Tyr-based anti-melanoma and MM-reactive immunotherapeutics.

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7. Publications

Lotz, C., Abdel Mutallib, S., Liewer, U., Huber, C., and Theobald, M.. Plasma cell-associated transcription factors as targets for multiple myeloma-reactive cytotoxic T lymphocytes. Manuscript in preparation.

Antunes Ferreira, E., **Lotz, C.**, Heit, A., Huber, C., and Theobald, M.. Tumor-specific self tolerance to mdm2 is governed by the level of class I MHC expression in vivo. Manuscript in preparation.

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