The generation of cytotoxic T cell epitopes and their generation for cancer immunotherapy

Kessler, J.

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Jan Kessler

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Promotor	Prof. Dr. C.J.M. Melief
Overige leden	Prof. Dr. J. Borst (Universiteit van Amsterdam)
	Prof. Dr. J.B.A.G. Haanen
	Prof. Dr. F. Koning
	Prof. Dr. J.J. Neefjes
	Prof. Dr. E.J.H.J. Wiertz (Universiteit Utrecht)
	Prof. Dr. W. Stoorvogel (Universiteit Utrecht)

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The real voyage of discovery is not in seeking new landscapes but in having new eyes.

Marcel Proust, 1871 - 1922
French novelist

Beautiful are the things we see,
More beautiful those we understand,
Much the most beautiful are those we do not comprehend.

Nicolas Steno, 1638 - 1686
Danish anatomist and geologist, and bishop

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1. Prologue

More than three decades after the discovery of MHC restriction [1], two decades after the first elucidation of MHC structure [2] and the peptide in the MHC-groove [3], and 18 years after both the finding of MHC-specific peptide binding motifs [4] and the identification of the first tumor-specific cytotoxic T lymphocyte epitope [5,6], T cell mediated immunotherapy of cancer has now outgrown its infancy. Nevertheless, trials in cancer patients have not shown consistent and high percentages of clinical successes [7-9], and immunotherapy of cancer is, with a single exception [10], not yet a standard (adjuvant) therapy.

Although the T cell arm of the immune system is exquisitely equipped to eradicate virally infected cells, the similar use of T cells for the destruction of cancer cells that (over)express tumor specific proteins has still to be exploited to its full clinical potential. Our rapidly accumulating understanding of the mechanisms involved in the adequate induction of anti-tumor immunity in patients is currently being used for the design of more effective immunotherapeutic treatments that will likely raise clinical success rates. For the development of effective T cell mediated cancer therapies it is crucially important that both an optimal immunostimulatory context is realized and that the targets of the $CD8^+$ cytotoxic T lymphocytes (CTL) and $CD4^+$ T helper (Th) lymphocytes are properly chosen. These targets are the tumor-associated antigens (TAA) expressed in the tumor cells and more specifically the T cell epitopes contained in these proteins.

The studies in this thesis address the epitopes recognized by CTL: the events leading to their generation and presentation and, based on these mechanisms, the prediction and identification of cancer-specific epitopes to be used as targets in cancer immunotherapy. This introductory chapter will therefore especially review current knowledge regarding T cell immunity in cancer, its induction by immunotherapy, the identity of TAA, and the generation, prediction and identification of T cell epitopes.

Because two of the studies in this thesis are at the basis of some reports in the literature, the outline and scope of each chapter is integrated, as boxed intermezzo, at its appropriate place in the text.

2. Innate and adaptive immunity work together and are linked

The human immune system is equipped with innate and adaptive (or acquired) arms that are defending the body against foreign pathogens. The innate system, composed of primarily macrophages, dendritic cells (DC), granulocytes, natural killer cells and the complement system, is evolutionary much older and constitutes a first line of defence that is already present at the start of the immune response and immediately interacts with pathogens, foreign antigens, cells sensed as abnormal and conserved structures shared by large groups of micro-organisms (so-called pathogen-associated molecular patterns). On the other hand, the adaptive response, executed by B- and T lymphocytes, before it can exert its effector functions, first needs amplification and selection for which it uses clonal receptors with narrow specificity generated by gene rearrangements and somatic mutations. Thereby, the adaptive response, unlike the innate response, develops immunological memory. It is only since the last decade that the prescient prediction by Charles Janeway in 1989 that the innate immune system is driving the adaptive

immune system [11] has been firmly grounded and is dissected at the molecular level [12]. From a historical viewpoint, the model for adaptive immune response regulation has undergone strong changes in the last 50 years. In the Self-Nonself (SNS) model, proposed in 1959 by Burnet and Medawar, the induction of the response was completely defined at the level of the lymphocyte by the non-self nature of the antigen that is recognized (this so-called signal one is the self-nonself discriminator in this model). Under the pressure of accumulating incompatible observations that needed additional explanation, the SNS model has been strongly adapted and refined. In the 1970s, a helper cell (later found to be the T helper cell) providing help was proposed [13], and later the focus shifted to the stimulator cell that induces lymphocyte activation. The stimulator cell (now called antigen presenting cell; APC) was proposed to provide, next to the antigen-specific signal one, a necessary second costimulatory signal to the lymphocyte [14]. Ten years later, in 1986, this was confirmed empirically by Jenkins and Schwartz [15]. In 1989, Charles Janeway hypothesized that the costimulation provided by the APC first needs to be induced through ligation of so-called pattern recognition receptors (PRR) on the APC by conserved pathogen-associated molecular patterns (PAMPs) of bacteria [16], thereby linking innate and adaptive immunity. Thus, the PRR allow APC to discriminate between infectious-nonself and non-infectious-self. Therefore, this model has been coined either the infectious-Nonself model, PRR-model or Stranger-model [17,18]. In 1997, the first Toll like receptor (TLR) was identified [19] and was shown to act as PRR for components of bacteria. Since then numerous TLR that recognize a variety of conserved microbial-associated products, like lipopolysacharide (LPS), have been identified [20], thereby further unravelling the linkage between innate and adaptive immunity. Meanwhile, in 1994, Polly Matzinger proposed the so-called Danger theory (of immune activation) [21]. This theory, initially purely theoretical, made 'danger' caused to cells and tissues the central concept and added still an extra level of cells and signals to the activation of the APC. Its activation could also be induced by danger/alarm signals released from injured (necrotic) cells, thus including endogenous non-foreign signals in APC-activation and not putting the primacy on innate immunity. As Matzinger stated herself, "the danger theory, may seem to propose just one more step down the path of slowly increasingly complex cellular interactions, this small step drops us off a cliff, landing us in a totally different viewpoint, in which the 'foreignness' of a pathogen is not the important feature that triggers a response, and 'selfness' is no guarantee of tolerance" [22]. The Danger theory has been criticized by Janeway and his co-worker Medzhitov, because of its "inherent tautology". As they state it (and rather ridicule the theory): "the (adaptive) immune response is induced by a danger signal, but the danger signal is defined as just about anything that can induce an immune response" [23]. Others, as well, have pointed to the flaws in this theory, being above all its conceptual emptiness and vagueness when the concept 'danger' is not specified and thus its metaphorical generalizing character [24]. Currently in 2009, studies have revealed several endogenous non-foreign alarm signals, like heat shock proteins, interferon-α (IFNα), interleukin-1β (IL-1β) and CD40-ligand (CD154) [25]. Furthermore, TLRs have been found to engage not only pathogenic components but also those from endogenous origin. Thus, one may argue that the Danger model is the most comprehensive theory because it incorporates also endogenous APC-ac-

tivation signals. However, this model may underestimate the importance of the exogenous pathogenic signals and it was Janeway who was the first to propose, and his group the first to identify, the important linkage between the innate and adaptive immune responses inducing the costimulatory signals of the APC.
As the immune system is a diverse collection of mechanisms that have come together during the course of evolution, it is impossible to explain its complexity by a too much restricted paradigm (the Stranger model) and not helpful to do so by a too much generalized metaphorical paradigm, like the Danger model). Indeed, later Matzinger [26,27] and others [28] have tried to reconcile both models. In any case, together innate and adaptive immune mechanisms can counteract the attack of in principle all pathogens ranging from viruses and bacteria to multi-cellular parasitic organisms.

3. The T cell response in a nutshell

Whereas B lymphocytes upon antigen-encounter produce antibodies (soluble B cell receptors) that recognize pathogen-derived native proteins, polysaccharides and lipids, T lymphocytes by their cell surface expressed T cell receptor (TCR) specifically recognize short linear protein sequences, i.e. peptides, derived from either endogenous or exogenous proteins. Upon proper activation, B- and T cells divide, expand in numbers, exert their effector functions, and memory is installed.
Two major subsets of T cells collaborate to mediate an effective immune response: $CD8^+$ cytotoxic T lymphocytes (CTL) recognize short peptides of a defined length (8–12 aa) presented by HLA class I molecules on the cell surface and $CD4^+$ T helper lymphocytes (Th cells) recognize longer peptides of less defined length (15–20 aa) that are presented by HLA class II molecules. Th cells are involved in the activation and regulation of B cells, CTL and APC through secreted cytokines and cell surface expressed molecules like CD40-ligand (CD154). Th type 2 (Th2) cells are mainly involved in B cell activation and Th type 1 (Th1) cells accomplish CTL activation via their stimulatory effect on APC and by secretion of cytokines. Moreover, regulatory $CD4^+$ T cells (Treg) exist that down-regulate T cell responses, e.g. preventing autoimmunity but also suppressing anti-tumor responses [29]. CTL are the killer cells that lyse target cells expressing their cognate class I-presented peptide by perforin and/or Fas-mediated mechanisms. T cell activation is accomplished when the peptide (first signal) is presented in an appropriate costimulatory context by the APC, in particular the DC.
This second signal can be provided by any of the molecules within the B7-family [30] or TNF receptor (TNFR)-family [31] of proteins. Crucially important costimulatory molecules of the B7-family are CD80 (B7.1) and CD86 (B7.2) whose interaction with CD28 activates T cells [32]. Reversely, CD80/86 ligation of the T cell-expressed counter-regulatory receptor CTLA-4, whose expression is upregulated after T cell activation [33], attenuates T cell responses by feedback inhibition. For sustained T cell effector functions, survival and memory maintenance, additional signals are required. TNFR-family members that function after initial T cell activation to further costimulate and sustain T cell responses are CD27 [34], 4-1BB (CD137) [35], OX40 (CD134) and GITR, all expressed on (activated) T cells, interacting with CD70, 4-1BB-ligand, OX40-ligand and GITR-ligand, expressed by the APC [31]. Adhesion molecules like intercellular adhesion molecule 1 (ICAM-1) interacting with LFA-1 on T cells also contribute to T cell activation [36]. Apart from cell surface receptors that mediate co-

stimulation, the cytokine milieu composed of especially IL-2 (secreted by Th cells) [37], IL-7 [38], IL-12 (secreted by DC) [39-41], and IL-15 [42,43] has been shown to be decisive for T cell activation, function and memory. Next to positive signalling, negative regulation of T cell activation is accomplished by ligation of PD-1 (a member of the CD28-family expressed on activated T cells) with B7-family member PD-1 ligand (PD-L1 or B7-H1), which is sometimes overexpressed on tumor cells [30]. B7-family member ICOS-ligand (B7h) that engages with ICOS which is expressed on activated and resting memory T cells [44] may down regulate Th1-responses through the induction of IL-10 [45]. Together, the summation of positive and negative signals coming together in the immunological synapse between APC, in particular DC, and T cell [46] determines the activation, proliferation, effector functions and instalment of memory of T cells. Once activated, T cells loose receptors that are required for lymph node entry, in particular CD62L [47] and CCR7 [48], and accordingly can migrate from the lymph node via the blood into the peripheral tissues.

Thus, to accomplish Th and CTL activation, DC need to provide appropriate costimulation. This is induced after maturation (activation) via TLR signalling by PAMPS (e.g. lipopolysaccharide; LPS), and/or via ligation of cell surface expressed CD40 by CD40-ligand (expressed on activated Th cells) [49-52]. 12). CD40 can be seen as a master switch for T cell costimulation because of its ability to induce B7-family ligands as well as several TNF family ligands on DC [31,53]. Optimal DC maturation is enhanced by proinflamatory cytokines like tumor necrosis factor (TNF)α, interferon (IFN)β, INFγ and IL-1β [54]. Immature DC, residing in peripheral tissues – particularly in barrier organs such as the skin and bowel – but also in the blood [55], are dedicated to capturing antigens, mostly by endocytosis. DC maturation via TLRs, together with partially unresolved mechanisms [56], induce migration of DC to lymph nodes [57,58] where they acquire the 'mature' phenotype specialized at presenting antigens and stimulating T cells through enhanced expression of CD80/86 and secretion of IL-12, a cytokine crucial for CTL effector and memory formation [41,59]. DC are believed to be at the crossroads of immunity and tolerance dependent on their maturation status [60]. On the one hand, as outlined, when in an immunogenic context DC activate naive anti-foreign T cells and on the other hand, when DC have an immature phenotype, they are capable of tolerizing autoreactive T cells – which have escaped the process of central tolerance – in the periphery (a process called peripheral tolerization) [61]. Several subsets of DC exist in vivo with distinct roles in immunity to infection and maintenance of self tolerance dependent on differences in their location and intrinsic abilities to capture, process and present antigens [62].

4. Protein degradation pathways and the generation of T cell epitopes

HLA class I molecules can be found on the surface of virtually all nucleated cells, whereas HLA class II molecules are mainly expressed by APC, but also by inflamed cells and thymus epithelial cells. Peptides presented by HLA class I and class II molecules are produced through proteolysis in one of the three major intracellular protein degradation and antigen processing systems that exist: (1) Ubiquitin (Ub)-mediated protein degradation that proceeds via the proteasome, which is called the ubiquitin-proteasome system (UPS); (2) autophagy, which is an intracellular degradation system that delivers cytoplasmic constituents

via the autophagosome to the lysosome. There are at least three different types of autophagy [63]: chaperone-mediated autophagy, microautophagy, and macroautophagy, the latter being best characterized; (3) endocytosis-mediated lysosomal degradation of extracellular proteins and plasma membrane proteins.
The classical but outdated doctrine holds that class I presented peptides are derived from endogenous proteins by UPS-mediated degradation, whereas class II presented peptides result from degradation of exogenous proteins in the endocytic pathway. However, as shown in table 1, current knowledge reveals exceptions to these rules and interconnections between degradation pathways. The best known exception is the cross-presentation pathway that enables DC and macrophages to present exogenous antigens by class I molecules. Emphasis here will be on antigen processing for class I presentation by the proteasomal pathway because of its relevance for this thesis (Fig. 1). First, the autophagic, endocytic and cross-presentation pathways are addressed briefly (Fig. 1).

4.1. The autophagic pathway of antigen processing

Autophagy is in principle a nonselective process involved in removal of damaged or surplus organelles, turnover of long lived proteins, production of amino acids in nutrient emergency, and cell survival and death [73]. In autophagy part of the cytoplasm becomes surrounded by two concentric membranes. Fusion of the outer membrane of this so-called autophagosome with a lysosomal vesicle results in degradation of enclosed cytoplasmic structures and macromolecules. The autophagic process was already identified before the UPS, and its contribution to intracellular protein degradation is estimated to be as large as that of the UPS [74]. Lately, autophagy has attracted new research and its emerging roles in innate and adaptive immune responses are being unravelled [75,76]. In line with autophagy eliminating intracellular pathogens, this process has been shown to deliver viral genetic material to endosomal TLRs in plasmacytoid DC, thereby inducing IFNα secretion [77]. Importantly, autophagic sequestration of viral

Table 1. Common and uncommon antigen processing pathways for the generation of MHC class I and class II presented peptides.

	Class II presentation	Class I presentation
UPS pathway	exception (cytosolic antigens)[a]	common (cytosolic and nuclear antigens)
Autophagic pathway	common (endogenous, often foreign, antigens)[b]	exception (endogenous foreign antigens)[c]
Endocytic pathway	common (exogenous antigens)[b]	cross-presentation (exogenous antigens)[d]

[a] The UPS pathway has been shown to be connected to class II loading compartments in several studies [64-66], which are not discussed in the text and reviewed in ref. 67.
[b] The balance between the autophagic and endocytic pathways for class II presentation is not yet fully understood [68].
[c] Until now this pathway has been reported only twice, in conjunction with proteasomal degradation [69,70].
[d] Production of cross-presented peptides proceeds via the endocytic route mostly in conjunction with the UPS pathway, but can also proceed in a TAP- and proteasome-independent way [71] in the endocytic tract. Autophagy has been implicated in cross-presentation as well [72].

components can fuel MHC class II presentation to $CD4^+$ Th cells [78]. Peptide supply for class II presentation is even considered to depend significantly on autophagic degradation also of non-foreign (non-pathogenic) proteins [79]. Although from a protein trafficking perspective (autophagosomes being fused to late endosomes where class II loading occurs) it is easier to understand the role of autophagy in class II peptide-generation, autophagic degradation may also be involved in class I antigen processing, for instance by its clearance of ubiquitinated cytoplasmic protein aggregates [80]. Recently, the first evidence for the involvement of autophagy in class I presentation, intricately linked to the proteasomal route, has been demonstrated in macrophages for endogenous antigens from herpes simplex virus type 1 [70]. This study suggests an intersection between the vacuolar and MHC class I presentation pathways.

4.2. The endocytic pathway of antigen processing

APC can internalize pathogens or parts thereof, dying virally infected cells and dying tumor cells into the endocytic pathway to provide a representation of the protein environment that they encounter in the periphery to T cells. The antigens can be endocytosed by a variety of mechanisms. Immature DC are highly efficient in all forms of endocytosis, being phagocytosis (for bacteria and cells, taken up in the phagosome), marcro- and micro-pinocytosis and receptor-mediated uptake mechanisms [60]. Degradation of the antigens is accomplished in diverse endosomal-lysosomal compartments by a large collection of proteases with mostly broad substrate specificity and variable pH requirements [81]. Most of the endosomal proteases are known as cathepsins. In a late endosomal, early lysosomal compartment known as the MHC class II compartment (MIIC) the generated peptides encounter class II molecules and are loaded in the class II binding groove through exchange with the class I invariant chain peptide (so-called CLIP) [82]. Upon endocytosis of exogenous material, DC will be activated and migrate to T cell areas in the lymph nodes [83]. Thus, in general, pathogen-derived peptides produced in the endocytic pathway will allow the initiation of a $CD4^+$ T cell response.

4.3. Cross-presentation: cross-talk between antigen processing pathways

For proper activation, naïve $CD8^+$ T cells must be stimulated by signal one (the MHC-pep complex) together with costimulation provided by professional APC, such as DC. As DC are mostly not infected themselves by viruses (and neither often transformed), they must acquire the antigen exogenously in peripheral tissues and display it through a process termed cross-presentation to $CD8^+$ T cells in the lymph nodes [84]. Thus, cross-presentation is the pathway by which the $CD8^+$ T cell response can be initiated towards viral infections or mutations that exclusively occur in parenchymal cells [85]. DC are the principal cells endowed with the capacity to cross-present exogenous antigenic material. To this end, endocytosed antigenic material is transferred from the endosomal-lysosomal pathway into the cytosol for further processing by the UPS-pathway and MHC class I loading in the ER. Exogenous antigenic material can, therefore, end up stimulating either the $CD4^+$ T cell response and/or the $CD8^+$ T cell response, dependent on the intracellular antigen trafficking. The access to the cross-presentation pathway can occur already directly at the moment of endocytosis, as has been observed for antigen uptake via the mannose receptor [86], or antigens can divert

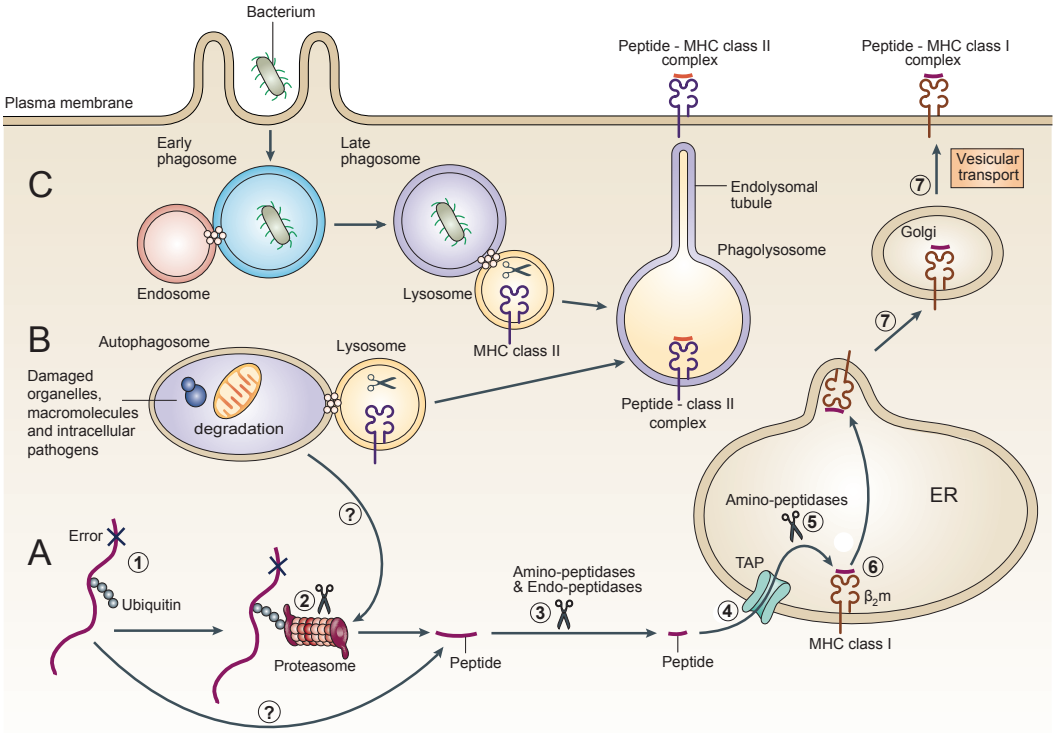

Figure 1. Overview of the common antigen processing pathways for MHC class I and II presentation. A. The UPS pathway. Posttranslational modification by ubiquitination marks defective or outlived intracellular proteins for proteolytic degradation b y the 26S proteasome(see '1'). The first degradation step is moslty accomplished by the proteasome(2) but its bypassing may happen (see '?'). Cytosolic aminopeptidases and endopeptidases act on proteasomal degradation fragments(3), rendering smaller peptides. Some of these peptides are translocated via the TAP transporter into the ER. There (further) N-terminal trimming may occur(5) and class I loading takes place(6) when peptides conform to the binding-motif of the expressed class I molecules. Finally, this trimolecular complex then moves through the Golgi apparatus(7) and is inserted in the plasma membrane. Furthermore, the autophagic pathway **(B)** and the endocytic pathway **(C)**, as explained in the text both leading to MHC class II presentation, are depicted. *Figure adapted from reference 68.*

to this pathway later. The escape of antigens from extracellular sources in the endocytic route to the cytosol may rely on diverse, as yet not completely resolved, mechanisms [68]. The delivery of proteins or peptides through a membrane pore, similar as the so-called ER-dislocon has been proposed. Likewise, fusion of the ER with phagosomes has been demonstrated explaining antigen transfer [87,88]. Alternatively, peptides and/or proteins may leak from the phagosomes into the cytosol or the phagolysosomal membrane may rupture. Moreover, evidence exist for loading of class I molecules in the endo-lysosomal compartments themselves [89] allowing cross-presentation independent of cytosolic transit and without proteasomal involvement [71]. Spatial separation of cross-presentation and endogenous class I presentation may have the advantages of speed and absence of competition [90]. Importantly, recently also the autophagic pathway has been implicated in cross-presen-

tation of tumor antigens [72]. Induction of (macro)autophagy in tumor cells was required for cross-presentation by DC in vitro and in vivo. Autophagosomes were suggested to be the antigen carriers in this study. Although the significance of cross-presentation in maintaining tolerance (cross-tolerance) and inducing immune responses (cross-priming [84]) has been disputed [91], it is now recognized as a major mechanism by which the immune system monitors the presence of foreign antigen and transformed cells in the periphery [92].

4.4. The ubiquitin-proteasome pathway for MHC class I antigen processing

The production of peptides presented by MHC class I molecules is mainly achieved during the continuous turnover of endogenous proteins in the proteasomal pathway.

Apart from non-selective lysosome-mediated degradation, it has become clear since the 1970s that selective ATP-dependent proteolysis by the ubiquitin-proteasome system (UPS [93]) is a key mechanism not only in cellular quality control, through its removal of abnormal and damaged proteins, but also in protein regulation [94]. The proteasome degrades substrates that are involved in many cellular processes such as stress response [95], cell cycle control [96,97], transcription activation, apoptosis and metabolic adaptation [98,99]. Substrates destined for degradation are first covalently modified with ubiquitin chains in an ATP-dependent cascade mediated by the E1, E2, and E3 enzymes [94]. Proteins tagged with multiubiquitin chains are then selected by the 26S proteasome holoenzyme and degraded in an ATP-dependent process [100]. Although the majority of proteasome-mediated protein degradation is considered to start with substrates that are ubiquitinated, there is accumulating recent evidence that ubiquitin-independent degradation can be accomplished by both the 20S and 26S proteasome and may have been underestimated [101,102].

4.4.1. Structure of the proteasome

Proteasomes are complex multi-subunit proteases, ubiquitously expressed and abundantly present both in cytosol and the nucleus. Several types of proteasomes exist which share a proteolytically active core, the 20S proteasome [103]. This catalytic core unit is a cylindrical structure of four stacked rings. The two inner rings each consist of seven distinct β-subunits (β1-7) and the two outer rings are assembled from seven homologous but different α-subunits (α1-7) together forming a central channel in which proteolysis takes place. Thus of each subunit two copies are present in the 20S proteasome. The channel prevents unwarranted proteolysis of cellular proteins and its access is restricted to unfolded proteins or polypeptides [104]. Although the isolated 20S proteasome can degrade peptides in an ATP-independent manner, which can be used *in vitro* for the assessment of proteasomal cleavages in model polypeptides, it can not actively unfold native proteins.

The 26S proteasome consists of the 20S core unit capped – at one or both sites – with the 19S regulatory complex [103]. The 19S cap [105] recognizes multi-ubiquitinated proteins, unfolds these substrates by an ATP-dependent mechanism, removes ubiquitin chains, and provides a passageway for threading unfolded proteins into the 20S core complex by opening the gate of the channel that is otherwise blocked by N-termini of α-subunits. The proteasome activator 28 (PA28) [105,106] is another regulatory complex which forms a cap that, like the 19S cap, can associate with one or both ends of the 20S core particle, which may lead to hybrid proteasomes [107,108]. Recently, a thymus spe-

cific proteasome type has been discovered that incorporates a different β5-subunit [109] with a modulated cleavage specificity. Consequently, the class I-presented peptide repertoire in the thymus may be significantly different which could be important for proper positive selection of $CD8^+$ T cells [110].

4.4.2. Cleavage specificity of the proteasome

The catalytic activities of the proteasome reside in three of the β-subunits, each in twofold present in the 20S unit. The β1-subunit is responsible for the so-called caspase-like activity, cleaving after acidic or small hydrophobic residues, β2 cleaves after basic or small hydrophobic amino acids (the trypsin-like activity) and the β5-subunit cuts after hydrophobic residues whether bulky or not (chemotrypsin-like activity) [111]. Consequently, and in line with its task to degrade a multitude of different substrates, the proteasome has a broad cleavage specificity with the capacity to cut in principle after or before all twenty amino acids [112-114]. However, not all amide (peptide) bonds are equally prone to be cleaved by the proteasome. For instance the proteasome does not readily cleave after a lysine [115]. Apart from the residues directly linking a scissile amide bond (indicated as P1 and P1' residues), also residues in the N-terminal and C-terminal flanking regions, up to eight residues, contribute to the propensity of the proteasome to hydrolyze a specific bond. Consequently, single residue differences in epitope-flanking regions from two related viral proteins could lead to abrogation of CTL epitope production [116,117]. Similarly, carboxy-terminal liberation of a CTL epitope from hepatitis C virus was impaired due to a mutation in the flanking region [118]. The broad cleavage specificity of the proteasome makes it extremely difficult to deduce algorithms capable to reliably predict proteasomal cleavages *in silico* (see below). Proteasomes cleave in a processive manner [119], meaning that each substrate leaves the proteasome before the next one enters. The cleavage propensity of the proteasome is stochastic in nature [112]. Thus, a certain amide bond may be either cleaved or not in different copies of the same protein, mostly leading to a cleavage pattern with partially overlapping degradation fragments having different abundances and also resulting in differences in frequencies between cleavages. The length of proteasome degradation fragments varies between 3 and 23 amino acids, and the median length products of 7 to 9 aa comprise only ~15% of the products [112,113].

Under inflammatory circumstances when INF-γ is produced, and also constitutively in cells of lymphoid organs, proteasomes exchange the normal, so-called constitutive, catalytic subunits (β1, β2 and β5) with slightly different subunits, the immuno(i)-subunits iβ1 (also called LMP2), iβ2 (MECL1) and iβ5 (LMP7), giving rise to so-called immunproteasomes [120-122]. Cleavage specificities of the immuno-subunits are quantitatively, and also slightly qualitatively, different compared to their constitutive counterparts [123]. Consequently, differences in epitope production have been found in cells expressing either of the subunit types. Some CTL epitopes were found dependent on immuno-subunits [124-126] while another epitope was only generated in cells expressing constitutive subunits [127]. Stimulation with INF-γ also has the capacity to induce PA28. The PA28 cap, together with the immuno-subunits, is up-regulated in DC upon maturation [128], although the effect on proteasome composition in DC is only very moderate due to low turnover of proteasomes [129]. The incorporation of the PA28 subunit reportedly enhanced the presentation of some

viral epitopes [126,130] but its precise influence on antigen processing is not yet completely resolved.

4.4.3. Substrates for the UPS, rapid protein turnover and the DRiP model

In 1996, it was proposed by Yewdell that a significant proportion of proteasomal substrates originate from so-called defective ribosomal products (DRiPs) [131]. This could explain the observation that after viral infection, epitopes derived from long-lived viral proteins are rapidly, within an hour, presented [132,133]. DRiPs include all proteins that fail to achieve a stable conformation due to defects in transcription, translation, post-translational modifications or protein folding [134]. Formal proof for the DRiPs hypothesis has not been provided yet [135,136], but strong support has been reported [133,137,138]. Two related studies showed that (1) blocking protein synthesis slowed the export of MHC class I molecules from the ER, indicating decreased supply of antigenic peptides [138] and (2) in acute influenza infection TAP becomes fully employed owing to the production and degradation of viral proteins [137]. Both studies indicate that an important proportion of MHC class I ligands are derived form newly synthesized proteins. The direct linkage of translation and antigen presentation would make perfect sense for immunity to acute virus infections, in which speed is of extreme importance to minimize viral replication [139]. Also because a DRiP itself has not yet been identified [140], refinements of the DRiP-model, involving the existence of an immunoribosome, have been postulated [139,141]. An alternative model proposes that a subset of nascent polypeptides is stochastically delivered to the 20S proteasome owing to neglect by the protein folding machinery, which would also explain rapid peptide presentation [136]. In mature DC, in which antigen processing and presentation is optimized, DRiPs were found to be stored rapidly in intracellular aggregates that have been termed DALIS (for dendritic cell aggresome-like induced structures) [142]. DALIS only form when protein synthesis is ongoing and this is the place where DRiPs become ubiquitinated [143]. Thus, DC regulate the degradation of DRiPs by producing DALIS. Another special source of polypeptides for cytosolic proteolysis are the products of cryptic translation [144], which include peptides encoded by introns, intron/exon junctions, 5'- and 3'- untranslated regions, and alternate translational reading frames [145]. These polypeptides with no biological function may constitute a subcategory of DRiPs [144]. Several CTL epitopes have been demonstrated to result from cryptic translation [146-149].

4.4.4. Generation of class I ligands, overview

MHC class I antigen processing involves a process that can be divided in the three most defining events: proteolysis in the cytosol and ER, transport of peptides into the endoplasmic reticulum (ER), and the assembly of the class I peptide complex.

In principle all proteins that are tagged for destruction are prone to be degraded into single residues. Next to the first degradation step by the proteasome, cytosol-resident aminopeptidases and endopeptidases accomplish further protein degradation of proteasome-degradation products (length 2–22 aa, on average 7-9 aa [113]). Cytosolic aminopeptidases that have been implicated are puromycine sensitive aminopeptidase (PSA) [150], bleomycin hydrolyse (BH) [150] and leucine aminopeptidase (LAP) [151]. Endopeptidases reportedly acting on proteasomal products and contributing to protein degradation are tripeptidyl peptidase II

(TPPII) [152] and thimet oligopeptidase (TOP) [153-155]. Various other cytosolic (endo)peptidases, like nardilysin [156], neurolysin [157], insuline degrading enzyme (IDE) [158], exist in the cytosol, but their involvement in protein degradation has not yet been demonstrated.
The generation of immunogenic peptides presented by class I molecules can be considered as a by-product of protein degradation, because upon partial degradation a small proportion of intermediate degradation products (length on average 8–16 aa) escape from very rapid destruction in the cytosol [159] by transfer into the ER where class I loading takes place. This is accomplished by the transporter associated with antigen processing (TAP), a heterodimer that translocates peptides (on average 8–16 aa in length [160]) in an ATP-dependent fashion (reviewed in ref. 161). In the ER, peptides can be N-terminally trimmed by the ER-resident aminopeptidase ERAP1/ERAAP [162,163] and, dependent on their binding affinity, assemble with MHC class I heavy chain and light chain (β2-microglobulin) in a complex folding mechanism assisted by the chaperones calnexin, calreticulin and Erp57 and the accessory protein tapasin (reviewed in ref. 164). Peptides need to fulfil the specific binding requirements of the class I molecule to which they bind. Seminal studies in the 1980s learned that residues in the peptide function as anchors, of which the side chains bind in pockets of the class I binding groove, enabling peptides to bind with high affinity to the class I molecule [4]. The combination of primary anchor residues (mostly at position two and the C-terminal position for the human class I molecules) and secondary anchors in the peptide that are required for efficient binding is defined in the peptide binding motif for each MHC/HLA class I molecule, which also allows the in silico prediction of peptide binding for any peptide with the appropriate length [165].
The extensively polymorphic HLA class I molecules – each individual expresses up to six HLA class I molecules, of which hundreds of variants are known [166]) – can be grouped in several HLA class I supertypes [167] with overlapping binding motifs, but each molecule may have its own fine specificity.

4.4.5. Generation of class I ligands, post-proteasomal processing

For class I binding, peptides need a defined length (8–12 aa, mostly 9 or 10 aa) with anchors at appropriate positions in the peptide. Seminal studies have shown the absence of C-terminal excision of model CTL epitopes in proteasome-inhibited cells [168-170]. Together with a failure to detect C-terminal trimming activities in the cytosol or ER [151,159], this has led to the current notion that the proteasome liberates the exact C-terminus of the vast majority of class I presented peptides [144,171]. Although some CTL epitopes are directly made by the proteasome, a significant fraction of class I ligands is made as N-terminally extended variant by the proteasome. The generation of the amino-terminus of these class I ligands is accomplished by aminopeptidases that reside either in the cytosol (PSA, BH, and LAP) or the ER (ERAAP/ERAP1). Redundancy in the function of these N-terminal trimming enzymes occurs [172]. For instance, the SIINFEKL epitope from ovalbumin (OVA) that was dependent on LAP in one study [151] was normally presented in LAP knock-out mice [172]. It has been shown in knockout mice that especially the trimming in the ER by ERAAP/ERAP1 is required for the presentation of many class I ligands [173-175], although other peptides can be (partially) destroyed by ERAAP activity [175,176]. Contradictory

results have been reported as to which extent ERAAP/ERAP1 influences the anti-viral $CD8^+$ T cell immunohierarchies [175,177]. From a mechanistic viewpoint, peptides in the ER may be (partially) protected from destruction by ERAAP/ERAP1 through their binding to class I molecules [178].

Cytosolic endopeptidases of which the role in class I antigen processing has been studied are TPPII and TOP. TPPII is a very large homo-oligomer of 5–6 MDa consisting of subunits of ~138 kDa that are organized as two stacks of 10 dimers each that form a twisted, spindle shape structure [103,179,180]. It was first known for its (tripeptidyl) aminopeptidase activity removing tripeptides from the substrate's N-terminus [180]. Indeed, N-terminal liberation of a CTL epitope (from RU1) was reported to depend on TPPII (in conjunction with PSA) [181]. It was found that TPPII can substitute for partially impaired proteasome function when cells are cultured under prolonged periods with proteasome inhibitors [182-185]. TPPII was identified to exert a relatively low endoproteolytic activity of the trypsin-like type next to its amino-peptidase activity [183]. Accordingly, a CTL epitope from HIV Nef was found to be produced both at its N-terminus and its C-terminus in an endoproteolytic manner by TPPII [186], and a CTL epitope from influenza virus nucleoprotein was likewise suggested to be TPPII-dependent [187,188]. The HIV Nef epitope is the first epitope of which the C-terminus is known to be produced independently of the proteasome. As TPPII was found to be responsible for the degradation of the majority of cytosolic polypeptides (> 15 aa) in vivo [152], it was suggested that this enzyme may be necessary for the post-proteasomal generation of many class I ligands. However, subsequent studies [189-191] showed that in general TPPII seems not to be required for the (C-terminal) generation of CTL epitopes (reviewed in ref. 192).

TOP is a ubiquitously expressed cytosolic metallopeptidase of which the crystal structure revealed a deep substrate-binding channel [193]. TOP has great flexibility in substrate recognition [194,195], and prefers to release 3–5 residues from the C-terminus of substrates with a preferred length of 6–17 aa [196]. In principle, this endows TOP with the capacity to either destroy or generate class I ligands dependent on the specific substrate (either a minimal epitope or a C-terminal extended epitope-precursor). A positive effect of TOP overexpression on the presentation of a specific CTL epitope (from hsp65 *M. tuberculosis*) has been reported [197]. Most studies, however, demonstrate a destructive effect of TOP on the production of class I presented peptides [154,155,198]. Overexpression of TOP (5 to 16-fold the physiological level) reduced total class I expression, and RNAi-mediated silencing of TOP (modestly) enhanced class I expression [155]. At the level of defined epitopes, destruction by TOP was only shown for one epitope (namely SIINFEKL) [155]. A recent study, again points to the possible role of TOP in both antigen destruction and antigen generation, because, using a biochemical approach, in the cytosol both the substrates and products of TOP were demonstrated to include peptides of the length of class I ligands [199].

4.4.6. Major unresolved questions in class I antigen processing

Several important issues in class I antigen processing of endogenous proteins are still considerably unknown and debated. First, as discussed before, the precise origin of class I presented peptides has not yet been unravelled. Although it is apparent that rapid presentation of epitopes derived from long lived

proteins occurs, it has not yet been established that DRiPs [140] and/or immunoribosomes [135] really exist and are the major source of class I ligands. Alternative mechanisms may account for rapid antigenic presentation by class I molecules [136].

Second, although the major role of the proteasome in antigen processing is undisputed for the majority of class I ligands, a vast number of studies have indicated that there might be a significant fraction of class I-peptides that is generated independently – or partially independently – of the proteasome by other endopeptidases. Benham et al. [200] observed that proteasome inhibitor insensitivity was allele-specific. In particular HLA-A3 and -A11 matured efficiently, whereas other alleles tested were not resistant to inhibitor treatment, suggesting that a non-proteasomal protease or peptidase may preferentially generate peptides with basic C-termini binding in the acidic F-pocket of the HLA-A3/A11-binding groove. Likewise, Luckey et al. observed a broad resistance to proteasome inhibition of cell surface expression of 13 human class I alleles [201]. They also observed relatively high levels of re-expression of class I molecules accommodating basic C-termini (HLA-A3, -A68, -B2705), but several other alleles, in particular those that bind a broad array of C-termini, displayed the same behaviour. In another study, comparison by mass spectrometry of the cell surface HLA-B2705-displayed peptide-repertoire under conditions with and without proteasome inhibition revealed that the repertoire was mainly unaffected and demonstrated the complete range of HLA-B2705 binding C-termini (including basic aa) suggesting a role for at least one non-proteasomal (endo)peptidase with a broad range of specificities [202]. It was shown that this peptidase is not TPPII [191]. Proteasome inhibition has been shown to have indifferent effects on the presentation of defined epitopes [187,188,203]. Treatment of cells with proteasome inhibitors even led to enhanced presentation of several defined epitopes [204-208]. Taken together, these results strongly suggest the existence of endoproteolytic activities that complement the proteasome in the generation of the C-terminus of class I ligands. However, by using as primary tool proteasome inhibitors that are known to be leaky, especially for the tryptic-activity of the proteasome [114], these studies do not proof the existence of proteasome-independent generation of class I ligands. Only one defined epitope from HIV Nef was found to be made in a non-proteasomal manner by TPPII [186]. However, subsequent studies rendered a broad role of this enzyme in the generation of class I ligands unlikely [192]. Thus, the extent of non-proteasomal processing and the identity of the alternative endopeptidases that are involved remain to be explored.

A third open issue is the extent to which cytosolic peptides are protected by chaperones from rapid destruction in the cytosol [144,209]. The group of Shastri has shown that post-proteasomal N-terminally extended variants of the OVA SIINFEKL epitope bind to the chaperone TRiC [210], which may increase the efficiency of presentation. In a more recent study, they showed that both N-terminal and C-terminal pre-proteasomal processing-intermediates from the same SIINFEKL-epitope are associated with the hsp90α chaperone [211]. These findings raise important questions. Because only one model CTL epitope was studied, the prevalence of the association of processing-intermediates with chaperones is not clear, and the nature of the pre-proteasomal intermediates is still unresolved as well [209].

Scope of the thesis, mechanisms of antigen processing:
In chapter 6, the non-proteasomal processing leading to class I presentation is elaborated. Alternative cytosolic endopeptidases that liberate the C-terminus of CTL epitopes were identified. Nardilysin is for the first time implicated in the C- and N-terminal generation of defined CTL epitopes. TOP is shown to function as a C-terminal trimming enzyme, generating a CTL epitope by making the final C-terminal cut. The roles of both enzymes in class I antigen processing in general are investigated.

5. Immunity to cancer and tumor immunoediting

From a historical viewpoint, the study of immunity to malignancies is deeply rooted in the treatment of tumors [212]. Based on a single observation in a patient that recovered from sarcoma after he had developed severe erysipelas, at the end of the 19th century, the New York surgeon William Coley started to treat cancer patients with bacterial vaccines resulting in sporadic regressions [213]. It was only much later that the underlying mechanisms, inducing innate immunity, were explained at the molecular level by the discovery of bacterial endotoxins [214] and tumor necrosis factor [214]. In the 1950s, studies of chemical induced tumors in syngeneic mice [215] definitively indicated the existence of tumor-specific antigens and, thus, recognisability of tumors by adaptive immunity. This raised hope for a well-grounded immunotherapy of cancer. These findings also instigated Burnet [216,217] and Thomas [218] to independently propose the theory of immunosurveillance of cancer, speculating that spontaneously arising cancer cells are often destroyed or kept in check by the immune system. A temporary setback again in the tumor immunology field was caused in the mid-1970s by a study of Hewitt and colleagues that showed absence of immunogenicity of spontaneously arising tumors [219]. However, several years later the group of Boon found that spontaneous murine leukemia cells possessed weak antigens that only led to rejection after the immune system was challenged with related more immunogenic tumor cells [220]. Based on this work and allowed by new insights in the basics of antigen presentation [1,2], in the early 1990s the same group identified by a laborious genetic approach the first mouse [221] and human [5,6] tumor antigens and their encoded CTL epitopes. Since then, the identification of tumor-specific antigens and insights in immunity to cancer in general has progressed tremendously.
Current knowledge tells that by the time cancer is clinically detectable, it likely has already been adapted to the host immune recognition and attack, so that it effectively evades any immune response. The concept of immunosurveillance – which has been doubted for a long while because no differences in tumor development were found between athymic nude mice (that later were found to not completely lack functional T cells) and syngeneic wild-type mice [222,223] – has been substantiated only recently [224,225]. For instance, a deficiency in IFNγ enhanced host susceptibility to both chemically induced and spontaneous tumors [226]. In another mouse study a genetic trait was serendipitously found that conferred resistance to a highly aggressive sarcoma cell line [227]. This was dependent on innate immunity infiltrates of natural killer cells, macrophages, and neutrophils that independently killed the tumor cells [228]. Immunosurveillance is now seen as a phase

in a broader evolutionary process of the tumor as reaction to immune pressure, called cancer immunoediting [229]. Immunoediting ranges from tumor recognition and elimination (through immunosurveillance) to tumor sculpting (by immunoselection) and escape. Both innate and adaptive cellular immunity takes part in tumor suppression and tumor shaping in a complex process influenced by multiple variables such as the tumor's type, anatomic location, stromal response, cytokine profile and inherent immunogenicity [224]. In immunoediting, before escape, an equilibrium phase is envisioned in which occult tumors are kept in check by the immune system during a period of latency, previously also called tumor dormancy [230]. A study reporting the occurrence of metastatic melanoma in two allograft recipients that had received kidneys from the same donor who had suffered from primary melanoma 16 years before her death, indeed strongly suggests an apparent equilibrium phase in the donor [231].

Often – it is not known how often – tumors escape from naturally induced immune pressure. Obviously, this also happens under circumstances of non-optimal therapeutically induced immunity. Numerous immune evasion mechanisms, all affecting the interplay of tumor and immune system, are known that contribute to escape from natural or therapeutically induced anti-cancer immunity (reviewed in ref. 232).

The tumor's inherent low capacity to appropriately stimulate the immune system, primarily caused by the absence of a proinflammatory environment and costimulatory context [233], but also often by a low density of (cancer-specific) MHC-peptide complexes, will lead to T cell ignorance, anergy or deletion, together called peripheral tolerance [234].

Cross-presentation of tumor antigens and subsequent T cell activation, needed to induce robust tumor immunity, will fail when a too low number of tumor cells are dying (either by apoptosis [235] or necrosis) or when DC are not appropriately matured [236]. Although (dying) tumor cells may sometimes inherently express danger signals, such as uric acid [237], it has now become increasingly clear that mostly the tumor microenvironment both actively (e.g. by secretion of TGF-β and IL-10) and passively suppresses the induction of tumor immunity (reviewed in ref. 238). Especially the maturation of DC is mostly lacking or incomplete in the tumor environment [239,240]. Stimulation by immature DC will lead to T cell tolerization [236,241] and may induce regulatory T cells that often play an immune suppressive role in cancer immunity [29,242]. Another mode of suppression is accomplished by tumors that express high levels of PD-L1 interacting with inhibitory B7-family member PD-1 on activated and exhausted T cells [243]. Moreover, tumor sculpting, either caused by natural immunity or by therapeutically induced immunity, may result in loss of tumor antigens [232,244] and possibly even antigenic drift [245], a mechanism common in viral immunity. Finally, lesions in molecules of antigen processing and presentation pathways, such as class I and TAP downregulation, often occur in tumors, highly likely as a result of immune pressure [105,246].

6. T cell mediated immunotherapy for cancer, modalities, and basic requirements

Immunotherapy of cancer by T cells can be divided in passive adoptive T cell transfer and active immunostimulatory vaccination strate-

gies (reviewed in refs. 247-249). Subsequently, adoptive transfer and active vaccination strategies can be categorized in antigen-non-defined or antigen/epitope-defined forms.

6.1. Adoptive transfer of undefined tumor-specific or defined epitope-specific T cells

The only routine immunotherapy for cancer in the clinic to date is the infusion of donor lymphocytes after allogeneic stem cell transplantation in leukaemia. This therapy is curative in significant percentages of patients [10]. The broad donor-derived $CD4^+$ and $CD8^+$ T cell repertoire targeting a diversity of undefined (allogeneic) leukaemia antigens is exploited in this setting. Remarkable clinical responses were observed in metastatic melanoma patients after adoptive transfer of autologous tumor-specific infiltrating lymphocytes (TIL) that were ex vivo expanded to high numbers [250]. The non-myeloablative conditioning regime in this trial may have contributed to the further expansion in vivo of the adoptively transferred T cells, by making space and also by the depletion of negative regulatory $CD4^+$ T cells or so-called myeloid derived suppressor cells [251,252]. Furthermore, the $CD4^+$ T cell component in the transferred TILs has likely helped the memory $CD8^+$ T cell population [250]. Because it is often hard to expand tumor-specific CTL at high numbers ex vivo, efforts are undertaken to introduce tumor-epitope specific T cell receptors (TCR) in peripheral blood lymphocytes (PBL) of the patient [253]. Promising clinical results were reported in patients with metastatic melanoma who were given autologous PBL retro virally transduced with the TCR specific for the well known MART-1(27-35) HLA-A2-presented epitope [254]. TCR gene transfer has the advantage that the problem of expanding enough tumor-specific T cells is bypassed. However, targeting a single epitope may lead to antigen loss variants. Therefore, adoptive transfer of PBL transduced with multiple 'off the shelf' TCRs targeting CTL epitopes in different TAA is a logical and promising next step.

6.2. Vaccination strategies with undefined antigens

Irradiated autologous tumor cells or allogeneic HLA-matched tumor cell lines that are modified to express GM-CSF, IL-2 and other cytokines or costimulatory molecules have been used as vaccines.

In various clinical trials this type of vaccine has induced immune responses [255] and clinical responses have been reported [256-259]. However, several disadvantages are connected to this strategy like the suboptimal direct antigen presenting capacity of tumor cells, absence of HLA class II presentation, uncertain cross-presentation, and often the lack of autologous tumor samples needed for preparation of the vaccine. Other forms of vaccination with the full potential of undefined antigens from the targeted tumor are tumor lysates (loaded on DC [260]), heat shock proteins (HSPs) derived from the tumor and DC transfected with amplified tumor mRNA [261,262]. The main advantage of vaccination with autologous tumor cells, or tumor derived lysates, HSPs or mRNA, is the presence of the full undefined repertoire of relevant tumor antigens, including those with mutations that are unique in the individual tumor. In this sense the strategies applying autologous tumor material are all personalized non-standardized vaccines that have to be produced for each patient separately. These therapies aim to induce T cell responses against as much as possible (undefined) tumor-specific HLA class I (and in certain settings HLA class II) presented peptides.

6.3. Vaccination strategies with defined full length tumor associated antigens

Vaccinations with recombinant viral vectors or naked DNA plasmids encoding defined full length tumor associated antigens and vaccination with recombinant tumor proteins themselves have been applied in vaccines aiming to raise humoral and T cell responses against the tumor expressing the antigen. Likewise, DC electroporated with mRNA encoding full length TAA are currently being optimized for clinical testing [263]. Vaccination strategies aiming to raise immunity to a full length antigen have the advantage that the HLA haplotype of the individual patient does not need to be considered. On the other hand, and apart from the problems related to each mode of delivery (virus, DNA, mRNA, protein; reviewed in refs. 247 and 248), vaccination with single whole antigens has the important drawback that vaccine induced immune pressure may induce escape through antigen loss variants of the tumor. In principle this could be circumvented by vaccination with multiple full length defined antigens (either in the form of DNA, mRNA [263] or protein).

6.4. Vaccination strategies with defined T cell epitope containing synthetic peptides

Since the first identification of a defined tumor-specific CTL cell epitope [6], the concept of immunizing cancer patients with synthetic peptide epitopes has been elaborated. Numerous clinical peptide vaccine trials have been conducted with sometimes promising results. The relatively poor immunogenicity of peptides per se requires them to be injected either together with adjuvants or loaded on DC (reviewed in refs. 264 and 265). Further optimization of the peptide vaccination strategy is envisaged [266]. It is now firmly established that for robust and persistent $CD8^+$ T cell responses a concomitant $CD4^+$ T helper response is needed [52,267-269]. Therefore, HLA class II presented tumor-specific epitopes are preferably incorporated in peptide vaccines to promote the CTL mediated tumor destruction. Important advantages of peptide vaccination are its defined nature and the easy manner to synthesize peptides by good manufacturing practice (GMP), enabling peptide vaccines to be used as pre-fabricated 'off the shelf' vaccines. Furthermore, modifications aiming at increasing the immunostimulatory context of the vaccine – like conjugation with synthetic Toll like receptor (TLR) ligands [270] – can easily be accomplished. Immunizations with a single (or only a few) CTL epitope(s) may induce outgrowth of antigen loss variants of the tumor. Therefore, peptide vaccines should preferably contain multiple HLA class I presented CTL epitopes derived from different target antigens together with a tumor-specific HLA class II presented $CD4^+$ T helper epitope. The use of longer (e.g. 30-mer) epitope-containing vaccine peptides that require processing which can only be accomplished efficiently by professional antigen presenting cells (DC) has been shown beneficial [271-273].

7. Tumor associated antigens and their classification

For immunotherapeutic purposes the most important criteria to classify tumor associated antigens (TAA) are: (1) broadness of expression (shared between patients and/or cancer types), (2) tumor specificity (absence of expression in healthy tissues) and (3) the function of the TAA in the oncogenic process and/or cancer survival. Additionally, (4) possible changes in turnover kinetics of the TAA are important to consider [274], as e.g. in the case of p53 [275].

With respect to broadness of expression, there is a first rough division in unique tumor antigens that are restricted to only an individual tumor in one patient – which for obvious reasons restricts their immunotherapeutic applicability – and the antigens that are shared between cancer patients. When combined with the criterion of tumor-specificity, this results in the following often used classification.
Unique tumor-specific antigens are resulting from mutations occurring in a single tumor of one patient. The first example of a unique point-mutation was found in the melanoma associated mutated antigen–1 (MUM1) gene [276] (for more examples, see listing in ref. 277).
Shared lineage-specific differentiation antigens are expressed in both the tumor and its original healthy tissue. Examples are the melanoma/melanocyte antigens (MART-1/Melan-A, gp100, tyrosinase, TRP2) and prostate antigens (PSA, kallikrein 4).
Shared tumor-specific antigens are expressed in different tumors but not in healthy tissues. The most prominent among these TAA is the group of so-called cancer-testis antigens like the MAGE, BAGE and GAGE families and NY-ESO-1, which in normal tissues are only expressed in testis and/or placental tissues. Further examples are viral oncoproteins (e.g. HPV16 E6 and E7) and the fusion-proteins encoded by translocated genes (e.g. BCR-ABL).
Shared antigens overexpressed in tumors are formally not tumor-specific but have a much higher expression level in tumors. Often these TAA are widely expressed in different cancer types, like hTERT, survivin and PRAME. Others, like carcinoembryonic antigen (CEA) and MUC1, own a more restricted expression pattern. A special case in this category of TAA is p53 because this oncoprotein is mutated in a variety of tumors and apart from being overexpressed can also show enhanced turnover, rendering it possibly applicable for immunotherapy [275]. Moreover because of its rapid degradation in normal cells, there appears to be no tolerance of p53 at the level of $CD4^+$ T cells [278].
Some antigens can be positioned in between two of the categories; e.g. PRAME is widely expressed in various cancer types and, in contrast, in healthy tissues only at very low levels in adrenals, ovaries and endometrium, next to its expression in testis and placenta [279].
Further extensive listings of TAAs can be found in the literature [280] or in databases (e.g. at www.cancerimmunity.org).

7.1. Strategies for the identification of TAA

Identification of TAA can be accomplished with different experimental strategies [281-284]. The discovery of MAGE-1 [5] in the early 1990's as the gene encoding the first tumor-specific CTL epitope [6] is one of the pillars of tumor immunology. An autologous melanoma specific CTL line was used to find the tumor specific cDNA that encodes the recognized CTL epitope from a cDNA library derived from the melanoma. Subsequently, the minimal CTL epitope was identified by cDNA truncation and peptide recognition techniques.
This classical strategy of expression profiling, which is often revered to as 'direct immunology' because it is based on natural immunity, has since then been applied for the identification of (among others) the MAGE, BAGE and GAGE families [285,286], Melan-A/MART-1 [287,288], tyrosinase [289] and gp100 [290].
In a biochemical strategy, the CTL clone can also be used to identify the HPLC-fraction of peptides isolated from the tumor cell surface that contain the epitope. Subsequently, mass spectrometry can identify the precise epitope sequence, and databank searches may lead to the identification of novel TAA [285-290].

A key characteristic of both strategies is the use of an autologous tumor specific CTL as the selection tool. The unknown tumor specific CTL epitope is used as handle to identify the source protein, and, therefore, tumor protein discovery and T cell epitope identification are intertwined in these strategies.

The serological identification of antigens by recombinant expression cloning (SEREX) strategy defines putative tumor antigens using patient-derived serum IgG antibodies to screen proteins expressed from tumor-derived cDNA libraries [291]. Tumor antigens identified by SEREX will likely contain $CD4^+$ T helper cell epitopes because isotype switching from IgM to IgG implies the presence of specific help from $CD4^+$ T cells. The cancer-testis antigen NY-ESO-1 [292] is only one example of a large array of (putative) tumor associated antigens that were identified by SEREX methodology [282].

With the rise of genomics and in silico data mining techniques, transcriptome analysis is currently used to detect tumor-specific expression profiles directly at the genetic level without the need for patient derived T cells or serum (in more detail reviewed in ref. 293). Various methods are used, like classical mRNA/cDNA subtraction techniques, representational differential analysis (RDA) [294,295], differential PCR display and comparison of cDNA profiles obtained by serial analysis of gene expression (SAGE) [296], DNAChip/microarray analysis [297-299] and expressed sequence tag (EST) databases. These studies often aim to identify expression profiles that can be used for the improved diagnosis [299], classification [297,298] or prognosis [300] of cancer. Tumor-specific expression of identified transcripts has to be confirmed at the protein level before immunogenicity studies are planned. The advent of the complete human genome sequence has enabled recent studies identifying mutational profiles in various cancers [301,302] that reveal the number and uniqueness of cancer-related mutations (within and between classes of cancer). Importantly, each mutation may give rise to (unique) cancer-specific T cell epitopes [303].

7.2. Selection of TAA for T cell immunotherapy

Which tumor associated antigens are most suitable as immunotherapeutic target? And how many TAA should be targeted simultaneously? These important questions are still being debated. First, the ideal tumor antigen target is widely expressed in different tumor types, enabling 'off the shelf' vaccines that are applicable in broad patient populations. Secondly, the function of the targeted TAA in the oncogenic process is highly relevant, although for several TAA not yet known. The phenomenon of immune escape by selection of antigen loss variants of the tumor is far beyond only theoretical consideration [304-306]. Therefore, TAA that either play a role in the oncogenic process or promote cancer cell survival are favourable targets. In this respect, the lineage specific differentiation antigens are lower ranked tumor antigens than purely oncogenic proteins like the HPV16 derived E6 and E7 proteins and the BCR-ABL fusion protein [307,308]. Overexpressed anti-apoptotic proteins like survivin are interesting because down regulation or loss of such TAA would severely impede the growth potential of the tumor cell [309,310]. Likewise, the telomerase catalytic subunit (hTERT) is involved in the pathogenic process [311] and has a reported anti-apoptotic role [312]. Another tumor antigen for which a role in tumorigenesis and metastasis has been reported is PRAME [300,313-315]. These tumor antigens (survivin, hTERT

and PRAME) are widely expressed in different tumor types and constitute also for this reason attractive tumor antigens. However, it can not be excluded that therapy-induced immune pressure may give rise to selection of antigen loss variants, even of anti-apoptotic or tumor promoting proteins, as has been observed in patients with melanomas expressing the melanoma inhibitor of apoptosis protein (ML-IAP) [255]. A third important consideration is the immunogenicity of the targeted tumor antigen. Tolerance to the non-mutated lineage specific differentiation antigens (like gp100, tyrosinase and MART-1/Melan-A), which are self proteins, may severely hamper an effective immune response against these antigens [316,317]. Such tolerance is likely affecting the immunodominant epitopes more than subdominant T cell epitopes, which is a reason why the latter category of epitopes in these differentiation antigens has attracted considerable attention (see below). To circumvent both the selection of antigen loss variants and the tolerance to differentiation antigens, targeting of multiple antigens by polyvalent vaccines (or multi-specific adoptive transfer) is essential. An additional advantage is that the full potential of the anti-tumor response in the patient is better exploited. The rule here would be: targeting more antigens is better. Lately, driven by these problems, different groups have made an argument in favour of personalized immunotherapy targeting the unique antigens caused by mutations [301] that are often only present in the tumor of one patient [303,318,319]. These tumor antigens are purely tumor specific, and therefore not tolerogenic, and are believed to be often crucial to the oncogenic process [277,303]. Furthermore, the natural immune response in some patients was found to be stronger against the unique antigens than the response against shared antigens [320]. Immunotherapies against non-defined tumor antigens such as vaccination with DC transfected with tumor-derived mRNA [262] or tumor-lysate pulsed DC are in fact personalized therapies that target both the shared and the unique antigens of each patient. However, the application of a defined patient-tailored immunotherapy will require identification of the unique antigens at the epitope level separately for each patient and meets with tremendous technical and logistic difficulties [318].

8. Identification of tumor-specific T cell epitopes

To date, a total of 180 HLA class I restricted CTL epitopes and 75 HLA class II restricted T helper epitopes in shared tumor associated antigens have been reported (according to the listing at www.cancerimmunity.org; update September 2006). Although this number of T cell epitopes seems a reasonable starting point for the design of defined immunotherapeutic vaccines, there is strong skewing to epitopes derived from antigens expressed primarily in melanoma and 75 (42%) of the HLA class I epitopes are presented in HLA-A2, leaving epitopes restricted by other HLA class I alleles severely underrepresented. This severely hampers the design and development of defined epitope-based vaccines targeting other tumors than melanoma, especially in patients lacking HLA-A2. Furthermore, the identification of HLA class II peptides recognized by T helper cells, which are indispensable as help to mount efficient $CD8^+$ effector T cell responses [52,267-269], has lagged behind (Fig. 2).

8.1. Identification of CTL epitopes starting with CTL of unknown specificity

The discovery of CTL epitopes has proceeded along two different experimental lines: either starting with a pre-existing CTL clone with unknown specificity (direct immunology), or departing from a predicted epitope (reverse immunology). In the first years all epitopes were identified by the direct immunology approach of expression cloning (Fig. 2). A patient-derived autologous tumor-specific CTL clone recognizing an unknown epitope was used to screen a tumor derived cDNA library (mostly from melanoma) which is expressed in antigen-negative (tumor) cells. Subsequently, recognition of truncated variants of the epitope-encoding cDNA and mapping of synthetic peptides revealed the minimal epitope sequence. In this procedure, next to the unknown CTL epitope, the equally unknown source tumor antigen was often discovered together with the epitope (see above) [285-290]. The major drawback of this laborious strategy is the dependence on autologous tumor-specific T cells that are either generated in mixed lymphocyte tumor cultures (MLTC) or obtained as tumor infiltrated lymphocytes (TIL). Such T cell responses are generally scarce and the induction in MLTC is dependent on the availability of autologous tumor cell lines, which have been mainly obtained from melanomas. Furthermore, CTL responses from MLTC or TIL are per definition directed to immunodominant epitopes. In recent years, the direct approach has been adapted for the identification of epitopes in known antigens without the need for autologous tumor cell lines. As elaborated by Chaux et al., TAA artificially expressed in DC were used for the generation of autologous CTL clones specifically recognizing unknown epitopes derived from the transduced antigen. Peptide-mapping experiments then again revealed the exact epitope sequence [321]. By virtue of the natural CTL response that is used this method as well will result in the identification of mostly immunodominant epitopes, and a systematic search for novel epitopes is impossible.

An alternative biochemical approach for defining the unknown specificity of tumor-reactive CTL, which are either induced against tumor cells [322,323] or e.g. against peptides eluted from tumor cells [324], starts with the immuno-affinity purification of the HLA class I – peptide complexes from the relevant tumor cell. The peptides are subsequently isolated and fractionated by (multiple rounds of) high-performance liquid chromatography (HPLC) to reduce the complexity of the peptide pool. Pinpointing the fraction that contained the epitope through recognition by the CTL together with tandem mass spectrometry (MS/MS) mediated sequencing of the peptides in that fraction then identifies the precise peptide sequence [322-327]. Subsequently, database searches may identify the unknown source antigen [324-327]. A particular advantage of this strategy is that it may identify post-translationally modified epitopes [328,329] (or special epitopes generated by protein/peptide splicing [283,284,330]). Still another way to analyse the specificity of pre-existing CTL clones is the application of synthetic peptide libraries to search for reactive mimicry epitopes. The natural epitope may subsequently be identified by screening recognition of substitution analogs, defining a recognition motif and database searching [331]. The combination of library screening-deduced T cell recognition motifs in the peptide and MS/MS sequencing of eluted peptides has also been exploited to identify a novel mouse CTL epitope [326].

8.2. Identification of CTL epitopes by reverse immunology

Since the first finding of HLA specific peptide binding motifs in the early 1990s [4], it is possible to screen known TAA for contained peptides that are predicted to be cell surface expressed. Predicted HLA class I ligands can be tested for their immunogenicity by raising $CD8^+$ T cells against the exogenously loaded peptide. Subsequently, peptide-specific CTL are tested for their recognition of tumor cells expressing the relevant TAA and restriction element to prove the natural presentation of the CTL epitope. The basis of this indirect strategy for CTL epitope identification, which was coined 'reverse immunology', is that an initial epitope-prediction-phase is followed by an epitope-validation-phase (a flow scheme is presented in Fig. 3). Nowadays, approximately 40% of the CTL epitopes in shared tumor associated antigens (Fig. 2), and also numerous CTL epitopes in viral and microbial antigens, have been identified via the reverse immunology strategy. The advantage of reverse immunology is that it is the only strategy that can be used to systematically search for novel epitopes, including subdominant ones, in known proteins and presented in any HLA molecule of interest. Both the prediction phase and the validation phase of reverse immunology have their own difficulties and weaknesses, although significant improvements have lately been implemented.

8.2.1. Prediction phase of reverse immunology

The prediction phase of the reverse immunology approach takes advantage of our growing knowledge concerning the intracellular generation of peptides presented by HLA class I molecules as outlined before in paragraph 4.4 and reviewed recently [144,171,332,333]. The determination of peptide binding motifs for the prevalent HLA class I molecules has allowed the in silico screening of TAA by computer algorithms for aa sequences with predicted

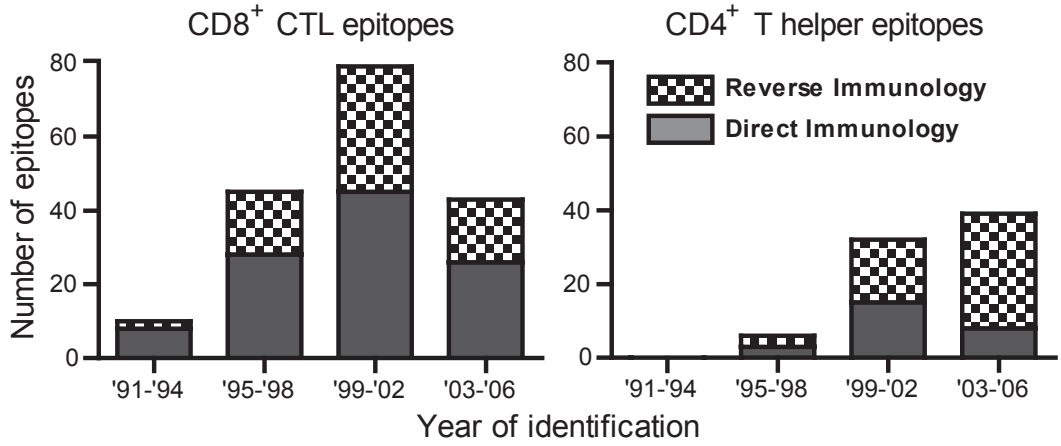

Figure 2. Numbers of CTL and T helper epitopes from shared tumor associated antigens identified by direct immunology versus reverse immunology since 1991. Source of the data are T cell epitope listings provided by the Academy of Cancer Immunology (www.cancerimmunity.org; update 2006).

binding capacity. Various HLA class I binding algorithms have been developed of which the BIMAS algorithm [165] and the SYFPEITHI algorithm [334] are freely accessible and currently the most widely used (algorithms are listed in Table 2 and see refs. 335 and 336). The algorithms employ slightly different peptide binding motifs and different arithmetic methods, based on either the contribution to binding of each aa in a peptide independently or the overall peptide structure, but are all extremely valuable to select the small percentage of peptides with potential binding capacity [337]. Guidelines for validation and comparisons between the different algorithms have been made [337,338], and significant differences in the predictions often occur (exemplified in chapter 7). Therefore, from a practical point of view, the combination of two or more methods is advisable to reduce the number of non-selected peptides with binding capacity. Experimental verification of actual binding capacity is preferred, because the ranking of the predictions does not perfectly correlate with the actual binding measurements and false positive prediction of binding occurs (exemplified in chapter 7 and chapter 4 [339]).
HLA class I binding assays exist in various forms (briefly reviewed in chapter 4 and ref. 293), and can be divided on the one hand in cell free assays (using soluble HLA) versus cellular assays using HLA class I molecules on the cell surface (chapter 4 [339]), and on the other hand in competitive assays (chapter 4 [339]) (resulting in semi-quantitative data) versus assays that do not use a (labelled) reference peptide and are therefore quantitative. Next to verification of binding capacity, the stability of peptide binding can be measured. Highly stable peptides have been shown to be more immunogenic [340], because they allow a sustained interaction with the T cell.

Scope of the thesis, development of binding assays:
In chapter 4 [339], peptide binding assays were developed for 13 prevalent HLA class I molecules. Using B-LCL expressing the class I molecule of interest and a fluorescently-labeled class I ligand with proven high affinity as reference peptide to compete with, reliable competition-based cellular assay are now available for easy measurement of peptide binding.

8.2.2. Improved CTL epitope prediction by verification of proteasomal processing and TAP translocation

The tumor-specific CTL epitopes that were identified by reverse immunology in the first years (until 2001) were predicted by taking into account only the HLA class I peptide binding capacity [341-344]. However, it was observed that numerous CTL that were raised against high affinity binding peptides did not recognize tumor cells expressing the relevant TAA and restriction element [345-349]. A major reason for this was the lack of intracellular generation of predicted peptides by the processing machinery. Thus, a refining of the epitope prediction procedure was needed.

Scope of the thesis, improved prediction of CTL epitopes:
In chapter 2 [350], the in vitro proteasome-mediated excision of class I binding peptides from their flanking regions was incorporated in the epitope prediction procedure. This allowed the selection of four C-terminally liberated peptides from 19 high affinity HLA-A2 binding peptides in TAA PRAME. These four peptides were proven to be naturally presented epitopes. The other peptides were considered to be likely not produced intracellularly, avoiding laborious T cell induc-

tions against these peptides. The same strategy was followed in chapter 5 [307] to identify CTL epitopes in the fusion regions of the BCR-ABL fusion proteins expressed in CML and ALL. Putative CTL epitopes were found, of which one was proven to be expressed. Some published epitopes were made likely to be not expressed.

This optimization of the prediction procedure (chapter 2) has greatly enhanced the accuracy of epitope predictions and has since then been applied successfully in studies identifying CTL epitopes in various tumor associated antigens (see e.g. chapter 5 [307] and refs. 351-354) and autoimmune antigens [355-357].

Four computer algorithms, which are based on different computational methods, are currently freely available via the internet for the prediction of proteasomal cleavages: MAPPP/FragPredict [358], PAProC [359], NetChop [360], and Pcleavage [361] (Table 2). Proteasomes cleave abundantly at certain sites and cleave much less abundant or do not cleave at other sites. However, due to the broad specificity of the proteasome, the stochastic nature of proteasomal digestion [112,360] (overlapping fragments are often found in the experimental systems [112,307,350]), and (partly) undefined influences of distant residues on cleavage efficiency, a qualitatively and quantitatively accurate prediction of proteasomal digestion sites is very complicated. In general, predictions may still result in a high number of improperly predicted cleavage sites [350]. Therefore, experimental determination of proteasome-mediated digestion is needed to reliably select peptides that are C-terminally liberated (see also below). An extra level of complexity here is the different forms in which proteasomes occur. Some CTL epitopes are preferentially made by immuno-proteasomes [362], which are expressed in professional antigen presenting cells [128] and

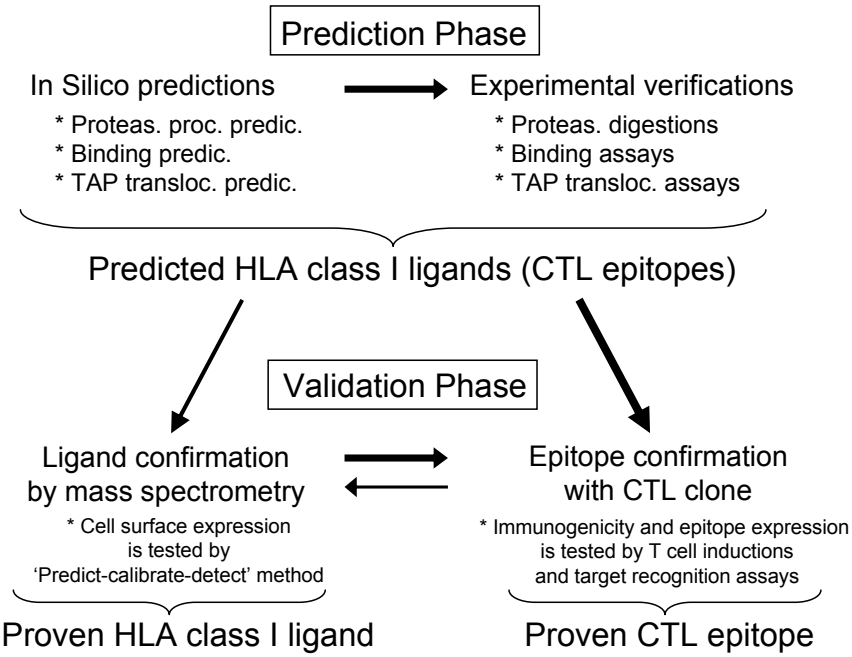

Figure 3. Flow chart of the reverse immunology approach for CTL epitope identification.

contain variant catalytic subunits with slightly different catalytic activity [123], and other epitopes are preferentially made by constitutive proteasomes [127,363], although most epitopes are liberated by both types of proteasomes. To cover both categories of epitopes, predictions and experimental verifications should use (in silico and in vitro, respectively) both types of proteasomes. Instead of first performing HLA class I binding assays [350], the proteasomal digestion pattern can also be determined first [351]. This reflects the physiological mechanistic order and has the advantage that binding of only those peptides that are C-terminally liberated by a major cleavage site needs to be verified experimentally.

Translocation of peptides into the ER via TAP is also an important event in the class I antigen presentation pathway. However, the specificity of the TAP heterodimer for peptides is much less selective because peptides meant to bind in all possible HLA class I molecules should be translocated into the ER. Specificity of TAP even seems to have evolved to fit the specificity of the proteasome [364]. Despite that, differences in translocation efficiencies between peptides exist [160] and TAP affinity has impact on HLA class I presentation [365]. Thus reasoning, in silico TAP translocation prediction algorithms have been developed [366-368] (Table 2) to incorporate TAP translocation efficiency in the overall HLA class I ligand prediction. However, a problem related to determination of TAP translocation efficiencies (in silico or in vitro) is the amino-terminal trimming that can occur both in the cytosol and in the ER. Therefore it is not a priori known which peptides should be tested. In general, TAP translocation efficiency, either predicted or experimentally verified, has until now been incorporated in only few reverse immunology studies identifying novel CTL epitopes [356].

Recently, five integrated in silico CTL epitope prediction tools have been developed that combine predictions of proteasomal cleavages, TAP translocation and HLA class I binding [115,369-373] (see Table 2 and further discussed in chapter 7). In addition, an algorithm was developed that directly predicts CTL epitopes using large datasets of T cell epitopes and non-epitopes as training data for the algorithm (CTLPred) [374].

8.2.3. Validation phase of reverse immunology

In the validation phase of the reverse immunology approach, the natural presentation and immunogenicity of the putative epitope should be demonstrated. In principle, two roads are open (Fig. 3). First, the biochemical purification of HLA-peptide complexes – from cells expressing the relevant TAA and HLA class I molecule – followed by the mass spectrometric search for the predicted peptides in the eluted HLA class I bound ligands (the 'predict-calibrate-detect' method; see below) [375,376]. However, this method validates cell surface expression of predicted HLA class I ligands but not their immunogenicity. Therefore, in the vast majority of studies the prediction phase is directly followed by the induction of (naïve) T cells against the exogenously loaded predicted epitope. Peptide-specific T cells are then used as tool to test the natural presentation of the epitope. T cells have mostly been induced in vitro using human peripheral blood lymphocytes (PBL) from healthy donors [307,350]. Furthermore, PBL [353,377] or TIL [351] from patients with the relevant tumor antigen and restriction element have been used. An alternative approach is the induction of T cell responses in HLA class I transgenic mice [378]. Peptide specific T cells should be used at the clonal level to enhance specificity of the response and to reduce aspecific back-

ground recognition of target cells that lack tumor antigen or restriction element. Next to tumor cells expressing the relevant TAA and HLA class I molecule, target cells should preferably include transfected target pairs with or without the tumor antigen and lacking or expressing the relevant HLA molecule. This enables exclusion of aspecific recognition effects by T cell clones that may be cross-reactive to irrelevant antigens. The sensitivity of the CTL clone for the peptide should be high, which is to be determined with peptide titration, before a definitive judgement of the natural presentation of the epitope can be made. While CTL recognition of properly chosen target cells will prove the natural presentation of the predicted epitope, it should be noted that, in principle, sometimes a length variant of the predicted epitope (with comparable binding capacity) may be the actually recognized peptide. Is such a case suspected, then tandem mass spectrometry is required to determine the exact aa sequence of the epitope-variant after its isolation from the cell surface (Fig. 3).

8.3. Identification of HLA class II presented T helper epitopes

Like CTL epitope identification, the identification of HLA class II presented T helper cell epitopes can either start with a $CD4^+$ T cell recognizing an unknown epitope or may depart from T helper cells which are induced against either predicted epitopes or a complete set of overlapping peptides in a reverse immunology setting (Fig. 2).

In vivo sensitized $CD4^+$ T cells that recognize a tumor-antigen have been employed to identify epitopes by expression cloning [379,380]. Contrary to HLA class I ligands, peptides binding in HLA class II are relatively long and different length variants of a T helper epitope are often recognizable by a single T helper clone. This characteristic has often advantageously been exploited to screen the T helper cell reactivity against a complete set of overlapping peptides of a TAA using responder $CD4^+$ T cells that were derived from either patients or healthy donors. Subsequently, peptide-specific T helper clones were tested for their recognition of endogenously processed TAA to validate the epitope [381-383]. The application of in silico algorithms for the prediction of HLA class II presented epitopes has lagged behind the use of predictions for CTL epitope identification. Binding requirements for peptides in HLA class II molecules are much less restricted than for class I molecules. Thus, the HLA class II binding motifs share a certain degree of degeneracy, and prediction of binding is less straightforward. Furthermore, the peptides bound in HLA class II have a broad length spectrum (9–25 aa) and class II antigen processing pathways are only incompletely defined making any assessment of processing uncertain. In addition to the classical endosomal-lysosomal pathway of exogenous and transmembrane proteins, alternate and partially overlapping routes for class II ligand generation exist in the cytosol [67]. Both proteasome-dependent and proteasome-independent cytosolic generation of class II ligands derived from either exogenous or endogenous sources have been reported [64-66,384,385]. Furthermore, HLA class II antigen processing can differ depending on the route of delivery of exogenous antigens [64] and the precise antigen presenting cells in which it takes place [66]. Despite this complexity in the processing, several HLA class II binding algorithms have been developed [334,386-388] (listed in Table 2). The degeneracy of HLA class II binding motifs allows the search and prediction of promiscuous pan-class II binding peptides, which are obviously more widely applicable

Table 2. Web-based algorithms for prediction of HLA class I and class II ligands and ligand/epitope databases.

Name[a]	URL	Possibilities / additional information[b]
HLA Peptide Binding Prediction		
BIMAS	http://bimas.dcrt.nih.gov/molbio/hla_bind	MHC class I
SYFPEITHI	http://www.uni-tuebingen.de/uni/kxi/	MHC class I and II
NetMHC	http://www.cbs.dtu.dk/services/NetMHC	MHC class I
PREDEP	http://margalit.huji.ac.il/	MHC class I
ProPred-I	http://www.imtech.res.in/raghava/propred1/index.html	MHC class I (with proteasome cleavage filter)
nHLAPred	http://www.imtech.res.in/raghava/nhlapred/	MHC class I (two methods)
IEDB (HLA class I)	http://tools.immuneepitope.org/analyze/html/mhc_binding.html	HLA class I (different methods)
HLA-A2 (No name)	http://zlab.bu.edu/SMM/	HLA-A2, 9- and 10-mers only
Multipred	http://research.i2r.a-star.edu.sg/multipred/	HLA-A2, -A3 and -DR supertypes
MHCPred	http://www.jenner.ac.uk/MHCPred	HLA class I and II (and TAP)
IEDB (MHC class II)	http://tools.immuneepitope.org/tools/matrix/iedb_input?matrixClass=II	MHC class II
MHC-BPS	http://bidd.cz3.nus.edu.sg/mhc/	HLA class I and class II (flexible length)
SVMHC	http://www-bs.informatik.uni-tuebingen.de/SVMHC/	MHC class I and HLA class II
SVRMHC	http://svrmhc.umn.edu/SVRMHCdb/	HLA class I and class II
ProPred (class II)	http://www.imtech.res.in/raghava/propred	HLA class II
MHC2Pred	http://www.imtech.res.in/raghava/mhc2pred/	MHC class II (promiscous binding)
Proteasomal Cleavage sites Prediction		
NetChop	http://www.cbs.dtu.dk/services/NetChop	Based on artificial neural network
PAProC	http://paproc.de	Based on evolutionary algorithm
MAPPP (FragPredict)	http://www.mpiib-berlin.mpg.de/MAPPP/cleavage.html	Based on peptide cleavage data
MAPPP (combined)	http://www.mpiib-berlin.mpg.de/MAPPP/expertquery.html	Combined with MHC class I binding
Pcleavage	http://www.imtech.res.in/raghava/pcleavage/	Based on cleavage data or ligands
TAP translocation Prediction		
TAPPred	http://www.imtech.res.in/raghava/tappred/	TAP transporter affinity
MHCPred	http://www.jenner.ac.uk/MHCPred	TAP transporter affinity
$PRED^{TAP}$	http://antigen.i2r.a-star.edu.sg/predTAP/	TAP transporter affinity
Integrated Proteasomal cleavage, TAP translocation and Binding prediction		
EpiJen	http://www.jenner.ac.uk/EpiJen/	Uses MHCPred
WAPP	http://www-bs.informatik.uni-tuebingen.de/WAPP	Uses SVMHC
NetCTL	http://www.cbs.dtu.dk/services/NetCTL/	Uses NetMHC and NetChop
MHC-Pathway	http://70.167.3.42/	Older version of IEDB (combined)
IEDB (combined)	http://tools.immuneepitope.org/analyze/html/mhc_processing.html	Newer version of MHC-Pathway
CTLPred	http://www.imtech.res.in/raghava/ctlpred/	Directly based on epitope databases
Databases of HLA class I and II ligands and tumor T cell epitopes		
SYFPEITHI	http://www.uni-tuebingen.de/uni/kxi/	HLA ligands and binding motifs, T cell epitopes
T-cell tumor epitopes	http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm	T cell epitopes derived from TAA
IEDB	http://beta.immuneepitope.org/home.do	T cell epitopes (among others)
MHCBN	http://bioinformatics.uams.edu/mirror/mhcbn/index.html	MHC binding and non-binding peptides

[a] Full name, institute and references can be found on webpage. Several references are also provided in the text of the current review.
[b] Method and additional information to be found on webpage

for vaccine development. HLA class II peptide binding predictions have successfully led to the identification of promiscuous T helper epitopes in among others NY-ESO-1 [389], TRP-2 [390], and hTERT [391], and Melan-A/MART-1 [392].

8.4. Identification of HLA class I and HLA class II ligands by tandem mass spectrometry

As discussed above, starting from pre-existing CTL with unknown or known specificity, the combination of microscale liquid chromatography coupled to tandem mass spectrometry (MS/MS) with functional immunoassays has been used for the identification or confirma-

tion of tumor-specific CTL epitopes, respectively [322-328].
Without the availability of an epitope-specific T cell, tandem mass spectrometry can also be applied for the validation of predicted HLA class I ligands (Fig 3). This alternative reverse immunology strategy was coined the 'predict-calibrate-detect' (PCD) method [376,378]. Predicted ligands are synthesized and used for the calibration of an HPLC – mass spectrometry system to identify co-eluting natural ligands of identical mass of which the precise identity is then verified by MS/MS sequencing. In this manner, Stevanovic and colleagues identified class I ligands from p53, CEA and MAGE-A1 in peptides extracted from tumor tissue or tumor cell lines [375,378]. Although technically demanding, the PCD method advantageously does not depend on the often cumbersome generation of peptide-specific CTL clones and allows the identification of low abundant peptides. It is, therefore, a secure intermediate station in the identification of CTL epitopes. The immunogenicity of the ligands still needs to be determined by T cell inductions [378,393].
With the aim to identify as many novel tumor-specific HLA ligands as possible in a single tumor sample, mass spectrometric ligand identification has been coupled to gene expression profiling to reveal antigens (over)expressed in the tumor but not in healthy tissue from the same patient [319]. This method has recently been used to identify HLA class I ligands derived from both universal and novel renal cell carcinoma (RCC) associated antigens [394]. These HLA class I ligands were in part unique for the patient and can therefore in principle be used in a personalized therapy.
Subtractive mass spectrometric approaches have been used for the direct identification of differentially expressed HLA class I and class II ligands with the aim to identify disease-related (e.g. TAA derived) ligands. Peptides in HLA-DR4 from an diabetes auto-antigen were identified by mass spectrometric comparison of HPLC-fractionated peptides purified from either untreated cells or cells that endogenously processed the antigen after it was delivered via a lectin-based method [395]. Lemmel et al. were the first to use differential stable isotope labelling (e.g. differential acetylation) of HLA class I bound peptides extracted from colon carcinoma versus regular colon tissue to quantify their ratio by mass spectrometry [396]. A variant of this subtractive analysis applied differential stable isotope labelling to two isoforms of a meningococcal outer membrane protein before their uptake by DC, again to create a so-called mass-tag (resulting in spectral doublets) that simplified the comparisons between HLA class II ligands extracted from the two sources [397]. The same group applied metabolic labelling by culturing virus-infected cells with stable isotope-labelled amino acids. Comparisons between the labelled peptides extracted from infected cells and the unlabeled peptides from non-infected cells have led to the identification of viral and infection-induced HLA class I ligands [398]. These pairwise comparative methods of mass spectrometric analysis could also be applied to tumor antigens.
As most tumor cells do not express HLA class II, the direct isolation of class II bound peptides from tumor cells was not considered feasible. Instead, several strategies have been used to target antigens into antigen presenting cells [395,399,400]. A recent study, however, reports the successful identification of (tumor-associated) HLA class II ligands from dissected primary tumor samples that were found to express HLA class II [401].
The advent of proteomics in the identification of HLA bound ligands is typically technology-

driven [402,403]. With the development of improved mass spectrometry technologies that are more sensitive and more accurate the identification by MS/MS of many more peptides in one sample is now possible. High throughput MS/MS analyses in an automated data-dependent mode followed by database searches allows the identification of a significant percentage of the full HLA bound 'ligandome' of a sample without prior focussing on predicted epitopes. It is already now possible to identify up to 3000 peptides per allele from one cell line (personal communication P.A. van Veelen, unpublished data). Further improvements in sample preparation and separation techniques, and data analysis will still boost the results [404]. This will lead to the identification of novel tumor-specific HLA class I ligands (likely immunogenic T cell epitopes) derived from both known and as yet unknown universal tumor associated antigens and also from unique mutated antigens. Basic insights and the development of anti-cancer immunotherapies, which may include individualized vaccinations with defined tumor-specific epitopes that are partly unique for the patient [318], will greatly benefit from these developments.

9. The need to define more T cell epitopes

Several reasons exist to further broaden the repertoire of defined tumor specific CTL and T helper cell epitopes. First of all, the immunomonitoring of cancer patients treated with any T cell mediated therapy is required to assess the effectiveness of the treatment. Monitoring can also be performed to determine the existence of precursors in healthy donors against a specific candidate target CTL epitope. Monitoring of T cell responses is mostly accomplished ex vivo by measuring T cell populations with tetramers of the tumor-epitope or by measuring specific cytokine (e.g. IFNγ) production upon stimulation with defined T cell epitopes [405]. In the case of vaccination with undefined antigens (tumor cells, tumor lysates or e.g. tumor cell derived mRNA) or full length TAA (irrespective of the vehicle), the monitoring of T cell responses against multiple T cell epitopes will better reveal the effects induced by the therapy. Even in the case of vaccination with a single minimal epitope, immunomonitoring of a T cell response induced by so-called 'antigenic spread' against a non-vaccine CTL epitope that is expressed on the tumor may be necessary to correctly assess the efficacy of the therapy [406].

Scope of the thesis, immunomonitoring: In chapter 3 [407], we screened HLA-A*0201-subtyped healthy individuals and advanced melanoma patients for the existence of $CD8^+$ T cells directed against the four HLA-A0201-restricted CTL epitopes from PRAME that were identified in chapter 2. IFNγ enzyme-linked immunosorbent spot assays and tetramer staining were used to detect CTL reactivity. T cell reactivity was found to be directed especially towards the $PRA^{100-108}$ epitope.

Recent studies, as well, have assessed above mentioned PRAME epitopes as candidate targets for immunotherapy. It was shown that $CD8^+$ T cells against all four HLA-A2 restricted epitopes were detectable in healthy donors [408,409], and in patients suffering from CML [408-410].

A strong argument can be made for a defined multi-epitope and multi-TAA directed T cell immunotherapeutic approach, either by using adoptive transfer of PBL transduced with multiple TCR or by applying vaccination strat-

egies. Targeting multiple TAA will enhance the barrier against escape of antigen loss variants of the tumor and will exploit more fully the anti-tumor T cell potential of the patient (in the case of vaccination). Loss of HLA class I molecules on tumor cells, which can be another reason for immune escape, is often restricted to only one or a few alleles [411]. Targeting multiple epitopes restricted by different class I molecules of the patient will circumvent such an escape mechanism. Where the latter goals may also be reached by vaccination with multiple full length TAAs (expressed in the tumor), it has been shown that the use of optimal epitopes can induce immune responses with increased potency compared with the response induced by the same epitopes in the context of the full length protein [412].
Given the pivotal role of $CD4^+$ T cells in promoting the primary and secondary $CD8^+$ T cell responses through the induction of DC maturation and the production of cytokines [52,267-269], the inclusion of T helper epitopes in a multi-epitope based vaccine will have strong beneficial effects. Furthermore, vaccination with minimal CTL peptide epitopes, unless administered on DC, may cause T cell tolerance through their systemic spread and presentation on non-professional antigen presenting cells [413,414]. To circumvent this, vaccines should contain longer epitope-containing peptides that require processing which can only be accomplished efficiently by professional antigen presenting cells (DC).
When enough CTL and T helper epitopes – derived from different TAA and presented in various prevalent HLA molecules – are identified, it would be feasible to combine these epitopes in a defined epitope-based vaccine (as peptide vaccine or e.g. recombinant 'string-of-bead' viral delivery system [415]) that is tailored to the TAA expression pattern and HLA haplotype of each patient. Importantly, vaccination with longer (35-mer) peptides, containing both CTL and T helper epitopes in their natural protein context, leads to a far more robust $CD8^+$ T cell response and therapeutic immunity in a mouse model [271]. Both the induction of a concurrent $CD4^+$ T cell response and the restricted processing and presentation of the long peptides only by professional APC contributed to this enhanced efficacy [416].
Vaccines based on defined epitopes have the additional advantage that the binding and TCR recognition characteristics of the epitopes can be optimized by aa replacements. In the case of differentiation TAA, tolerance against the immunodominant epitopes is expected, and these are therefore not first choice. The subdominant epitopes, however, mostly have a lower binding capacity rendering them less immunogenic. Designing modified analogs of the epitope, also called altered peptide ligands, with improved binding characteristics can be used to efficiently recruit a non-tolerized T cell repertoire [417,418]. However, care should be taken that vaccination with epitope analogs does not induce CTL that are incapable of recognizing tumor cells as has been observed in patients vaccinated with optimized variants of MART and gp100 CTL epitopes [419]. Obviously, any epitope contained in epitope-based vaccines should be thoroughly checked for its natural processing and cell surface presentation to avoid responses against so-called cryptic epitopes that are not presented on the tumor cells [420,421].

10. Purpose and chronology of the thesis

The research presented in this thesis has been initiated to identify CTL epitopes in the leuke-

mia specific BCR-ABL fusion regions (chapter 5). As these fusion regions contain only a low number of epitopes, the priority shifted to epitope identification in tumor associated antigen PRAME using an optimized epitope-prediction procedure (chapter 2). To facilitate further epitope discovery, HLA class I binding assays were developed (chapter 4), and the potential usefulness of the epitopes that were identified was examined in healthy donors and patients (chapter 3). Meanwhile a study was started to further unravel the proteolytic mechanisms involved in CTL epitope generation (chapter 6).

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CHAPTER 2

CHAPTER 5

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Efficient Identification of Novel HLA-A*0201–presented Cytotoxic T Lymphocyte Epitopes in the Widely Expressed Tumor Antigen PRAME by Proteasome-mediated Digestion Analysis

By Jan H. Kessler,* Nico J. Beekman,* Sandra A. Bres-Vloemans,* Pauline Verdijk,* Peter A. van Veelen,* Antoinette M. Kloosterman-Joosten,* Debby C.J. Vissers,* George J.A. ten Bosch,* Michel G.D. Kester,* Alice Sijts,‡ Jan Wouter Drijfhout,* Ferry Ossendorp,* Rienk Offringa,* and Cornelis J.M. Melief*

*From the *Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, 2300 RC Leiden, The Netherlands; and the ‡Institute of Biochemistry, Charité, Humboldt University, D-10117 Berlin, Germany*

Abstract

We report the efficient identification of four human histocompatibility leukocyte antigen (HLA)-A*0201–presented cytotoxic T lymphocyte (CTL) epitopes in the tumor-associated antigen PRAME using an improved "reverse immunology" strategy. Next to motif-based HLA-A*0201 binding prediction and actual binding and stability assays, analysis of in vitro proteasome-mediated digestions of polypeptides encompassing candidate epitopes was incorporated in the epitope prediction procedure. Proteasome cleavage pattern analysis, in particular determination of correct COOH-terminal cleavage of the putative epitope, allows a far more accurate and selective prediction of CTL epitopes. Only 4 of 19 high affinity HLA-A*0201 binding peptides (21%) were found to be efficiently generated by the proteasome in vitro. This approach avoids laborious CTL response inductions against high affinity binding peptides that are not processed and limits the number of peptides to be assayed for binding. CTL clones induced against the four identified epitopes (VLDGLDVLL, $PRA^{100–108}$; SLYSFPEPEA, $PRA^{142–151}$; ALYVDSLFFL, $PRA^{300–309}$; and SLLQHLIGL, $PRA^{425–433}$) lysed melanoma, renal cell carcinoma, lung carcinoma, and mammary carcinoma cell lines expressing PRAME and HLA-A*0201. This indicates that these epitopes are expressed on cancer cells of diverse histologic origin, making them attractive targets for immunotherapy of cancer.

Key words: antigen presentation • antigen processing • cytotoxic T lymphocyte induction • human histocompatibility leukocyte antigen class I binding • tumor immunotherapy

Introduction

T cell–based immunotherapy of cancer has been successful in numerous mouse tumor model systems (1) and so far efficacious in a limited number of clinical conditions (2–6). Specific T cell–mediated immunotherapy requires the identification of tumor-specific antigens carrying T cell epitopes presented in the context of HLA class I and/or HLA class II molecules (for reviews, see references 1 and 7–9). The strategy, pioneered by Boon and coworkers, of screening melanoma cDNA libraries with CTLs derived from tumor infiltrating lymphocytes, has been successful in the identification of CTL epitopes in unknown tumor-associated proteins. The MAGE, BAGE, and GAGE families of tumor-associated testis-specific antigens (10–13), as well as the melanocyte differentiation antigens overexpressed in tumors like tyrosinase, Melan-A/MART-1, and gp100 (14–17) have been identified in this manner. This strategy requires the availability of CTL clones from mixed leukocyte tumor cultures of cancer patients. Consequently, any CTL epitope that is not successful in activating CTLs

Address correspondence to J. Kessler, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Building 1: E3-Q, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Phone: 31-71-526-1671; Fax: 31-71-521-6751; E-mail: kesslerj@worldonline.nl

that can be expanded in vitro (e.g., subdominant epitopes) will be missed. For the systematic detection of CTL epitopes presented in a broad range of HLA class I molecules, there is a great need for an efficient strategy. The strategy of predicting potential CTL epitopes in already identified tumor-associated proteins followed by in vitro sensitization of CTLs against these putative epitopes (also designated "reverse immunology") has the advantage that it does not utilize patient-derived T cells as a primary screen and therefore allows a more systematic search for new CTL epitopes. Reverse immunology has been used to identify new CTL epitopes in MAGE-1 (18), MAGE-2 (19), MAGE-3 (20–23), TRP2 (24), gp100 (25), and HER-2/neu (19). However, so far this strategy has been rather inefficient, mainly because CTLs raised against putative epitopes were often unable to recognize tumor cells expressing the source protein (26–30). Explanations for these failures are both an insufficient affinity of the induced CTLs for their MHC–peptide complex but more often the lack of processing of the presumed epitope (9). Recently, Chaux et al. successfully applied an alternative strategy of in vitro CTL inductions against dendritic cells (DCs)[1] transduced with MAGE-1, abandoning the strategy of epitope prediction and allowing processing of the relevant epitopes to take place by the APC (31).

In this study, we chose to improve the epitope prediction strategy by verifying the proteasome-mediated generation of peptides in order to identify HLA-A*0201–presented CTL epitopes in the so-called PRAME protein. The main intracellular mechanisms that define the exact amino acid (aa) sequence of a CTL epitope include enzymatic breakdown of the protein by the proteasome, transporter-associated with antigen processing (TAP)-mediated translocation into the endoplasmic reticulum (ER) and binding of the peptide with sufficient affinity in the groove of an MHC class I molecule (for reviews, see references 32 and 33). The COOH terminus of CTL epitopes requires exact cleavage by the proteasome (34–37), whereas NH_2-terminal extensions of the epitope can apparently be trimmed by putative aminopeptidase activity mainly in the ER (36, 38–41) or in the cytosol (34, 42). In vitro proteasome-mediated digestions are known to reliably yield MHC class I ligands from viral and model protein-derived polypeptides (43–47). Therefore, after identification of HLA-A*0201 binding peptides, we now incorporated in vitro proteasome-mediated digestions of 27-mer polypeptides encompassing high affinity binding peptides in the epitope prediction procedure. Digestion pattern analysis permitted assessment of efficient COOH-terminal generation of putative epitopes and, in addition, enabled evaluation of possible premature destruction by major cleavage sites within the epitope, as observed by us in a variant viral sequence (47).

[1]*Abbreviations used in this paper:* aa, amino acid; B-LCL, B lymphoblastoid cell line; DC, dendritic cell; ER, endoplasmic reticulum; FI, fluorescence index; FL, fluorescein; HBV, hepatitis B virus; MS, mass spectrometry; RT, reverse transcription; TAP, transporter-associated with antigen processing.

The tumor-associated PRAME protein (48) is a particularly attractive target of T cell–based immunotherapy of cancer because of its expression in a wide variety of tumors, including melanoma (95% of patients), renal cell cancer (41%), lung cancer (50%), mammary cancer (27%), acute leukemias (30%), and multiple myeloma (52%; references 49–51), and because of its absence from normal tissues, except testis, and its very low levels in endometrium, ovaries and adrenals. We focussed on HLA-A*0201 as a restriction element because of its high prevalence (45.8%) among the Caucasian population (52). Via the improved multistep epitope prediction procedure, we report here the identification of four naturally processed HLA-A*0201–presented CTL epitopes in PRAME that are recognized by CTLs on cell lines derived from tumors of various histologic origins. This study underscores the importance of incorporating processing criteria for accurate identification of CTL epitopes.

Materials and Methods

Cell Lines and Culture Conditions. The EBV-transformed B lymphoblastoid cell line (B-LCL) JY was cultured in complete culture medium consisting of IMDM (BioWhittaker) supplemented with 8% FCS (Greiner), 100 IU/ml penicillin, and 2 mM L-glutamine. The processing-defective T2 cell line was a gift from Dr. P. Cresswell (Yale University, New Haven, CT). Melanoma cell lines (Mel603, M453, and FM3), renal cell carcinoma cell lines (MZ1851, MZ1774, and MZ1257), and mammary carcinoma cell line MCF7 were provided by Dr. P. Schrier (Leiden University Medical Center). Mammary carcinoma cell line ZR-75-1 was obtained from the American Type Culture Collection. Lung carcinoma cell lines GLC02 and GLC36 were provided by Dr. L. de Leij (University of Groningen, Groningen, The Netherlands). The CD40 ligand–transfected mouse L cell line (53) that was used for generation of activated B cells was donated by Dr. C. van Kooten (Leiden University Medical Center). PRAME cDNA was provided by Dr. P. Coulie (Ludwig Institute for Cancer Research, Brussels, Belgium). The PRAME-encoding insert was cloned into vector pDR2 (Invitrogen), conferring hygromycine resistance. The renal cell carcinoma cell line MZ1851 was transfected with pDR2-PRAME using Fugene (Boehringer) as transfection reagent. After 48 h, hygromycine (100 μg/ml) was added to select transfected cells. Hygromycine-resistant cells were tested by reverse transcription (RT)-PCR for PRAME expression.

Peptides. Peptides were synthesized by solid phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. Short peptides for CTL inductions were dissolved in 20 μl DMSO, diluted in 0.9% NaCl to a peptide concentration of 1 mg/ml, and stored at −20°C before usage. The fluorescein (FL)-labeled reference peptide as used in the competition-based HLA-A*0201 binding assay was synthesized, labeled, and characterized as described earlier (54). The sequence of the FL-labeled peptide was FLPSDYFPSV (hepatitis B virus [HBV] nucleocapsid 18–27; reference 55) wherein we substituted the tyrosine with a cysteine to tag an FL group to the peptide: FLPSDC(FL)FPSV (54). The 27-mer polypeptides used for in vitro proteasome digestion were synthesized as described above, purified by reversed phase-HPLC in an acetonitrile–water gradient and lyophilized from acetonitrile–water overnight. Purity was confirmed by mass spectrometry.

*Cellular Competition–based HLA-A*0201 Peptide Binding Assay.* The affinity of peptides for HLA-A*0201 was analyzed using the homozygous HLA-A*0201$^+$ B-LCL JY as described previously (54), with minor adaptations. In brief, naturally bound peptides were stripped from the HLA-A*0201 molecules by exposing the JY cells for 90 s to ice-cold citric acid buffer with pH 3.1 (1:1 mixture of 0.263 M citric acid and 0.123 M Na_2HPO_4). Cells were immediately buffered with ice-cold IMDM containing 2% FCS, washed twice in the same medium, and resuspended in 2% FCS/IMDM containing 2 μg/ml human β_2-microglublin (Sigma-Aldrich). Subsequently, the stripped JY cells were plated at 4×10^4/well in a 96-well V-bottomed plate together with 150 nM of a known high affinity HLA-A2*0201 binding FL-labeled reference peptide (55) and titrated concentrations of competitor test peptide. After incubation for 24 h at 4°C, cells were washed three times in PBS containing 1% BSA, fixed with 0.5% paraformaldehyde, and analyzed on a FACScan™ flow cytometer (Becton Dickinson). The percentage inhibition of FL-labeled reference peptide binding was calculated using the following formula: $[1 - (MF_{\text{reference and competitor peptide}} - MF_{\text{no reference peptide}})/(MF_{\text{reference peptide}} - MF_{\text{no reference peptide}})] \times 100\%$. The binding affinity of competitor peptide is expressed as the concentration needed to inhibit 50% binding of the FL-labeled reference peptide (IC_{50}). An $IC_{50} \leq 5$ μM was considered high affinity binding, 5 μM $< IC_{50} \leq 15$ μM was considered intermediate affinity binding, 15 μM $< IC_{50} \leq 100$ μM was judged low affinity binding, and $IC_{50} > 100$ μM was considered not binding.

Peptide–MHC Complex Dissociation Assay. Binding stability at 37°C of peptides complexed with HLA-A*0201 was measured as described previously (56). In short, JY cells were treated with 10^{-4} M emetine (Sigma-Aldrich) for 1 h at 37°C to stop de novo synthesis of MHC class I molecules. Subsequently, endogenous bound peptides in HLA-A*0201 were removed by mild acid treatment (see above) and reconstituted with the peptide of interest at 200 μM in 2% FCS/IMDM containing 2 μg/ml human β_2-microglublin (Sigma-Aldrich) for 1.5 h at room temperature. Hereafter, cells were washed twice to remove free peptide and incubated at 37°C for 0, 2, 4, and 6 h. Subsequently, expressed HLA-A*0201–peptide complexes on JY cells were stained using the conformation-specific Moab BB7.2 and goat anti–mouse IgG–FITC and analyzed on a FACScan™ flow cytometer. The fluorescence index (FI) was calculated for each sample as: $(MF_{\text{sample}} - MF_{\text{background}})/MF_{\text{background}}$, where $MF_{\text{background}}$ is the value without peptide. The percentage of residual HLA-A*0201 molecules was calculated by equating for each peptide the FI of $t = 2$ h to 100% and then using the formula: % remaining $= (FI_{t=n}/FI_{t=2}) \times 100\%$. Because the dissociation of peptides from MHC is a linear process, the stability of the peptide–MHC complexes was measured as the time required for 50% of the molecules to decay (DT_{50}), starting from $t = 2$. By linear regression analysis of the sequential measurements plotted against the percentage of remaining HLA-A*0201 molecules, the DT_{50} was calculated. As a positive control, the known highly stable HBV nucleocapsid 18–27 epitope was used.

In Vitro Proteasome-mediated Digestions. 20S proteasomes were purified from a B-LCL cell line as described (57). This cell type is known to contain immunoproteasomes (58). High LMP2 and LMP7 content was confirmed by two-dimensional immunoblotting (data not shown). To assess kinetics, digestions were performed with different incubation periods. Peptides (27 mer, 20 μg) were incubated with 1 μg of purified proteasome at 37°C for 1, 4, and 24 h in 300 μl proteasome digestion buffer as described (44). TFA (30 μl) was added to stop the digestion and samples were stored at −20°C before mass spectrometric analysis.

Mass Spectrometry. Electrospray ionization mass spectrometry was performed on a hybrid quadrupole time of flight mass spectrometer, a Q-TOF (Micromass), equipped with an on-line nanoelectrospray interface (capillary tip, 20-μm internal diameter × 90-μm outer diameter) with an approximate flow rate of 250 nl/min. This flow was obtained by splitting of the 0.4 ml/min flow of a conventional high pressure gradient system, using an Acurate flow splitter (LC Packings). Injections were done with a dedicated micro/nano HPLC autosampler, the FAMOS (LC Packings), equipped with two additional valves for phase system switching experiments. Digestion solutions were diluted five times in water/methanol/acetic acid (95:5:1, vol/vol/vol), and 1 μl was trapped on the precolumn (MCA-300-05-C8; LC Packings) in water/methanol/acetic acid (95:5:1, vol/vol/vol). Washing of the precolumn was done for 3 min to remove the buffers present in the digests. Subsequently, the trapped analytes were eluted with a steep gradient going from 70% B to 90% B in 10 min, with a flow of 250 nl/min (A, water/methanol/acetic acid [95:5:1, vol/vol/vol]; B, water/methanol/acetic acid [10:90:1, vol/vol/vol]). This low elution rate allows for a few additional mass spectrometry (MS)/MS experiments if necessary during the same elution. Mass spectra were recorded from mass 50–2,000 daltons every second with a resolution of 5,000 full width/half maximum (FWHM). The resolution allows direct determination of the monoisotopic mass, also from multiple charged ions. In MS/MS mode, ions were selected with a window of 2 daltons with the first quadrupole and fragments were collected with high efficiency with the orthogonal time of flight mass spectrometer. The collision gas applied was argon (4×10^{-5} mbar), and the collision voltage ~30 V. The peaks in the mass spectrum were searched in the digested precursor peptide using the Biolynx/proteins software (Micromass) supplied with the mass spectrometer. The intensity of the peaks in the mass spectra was used to establish the relative amounts of peptides generated after proteasome digestion. The relative amounts of the peptides are given as a percentage of the total amount of peptide digested by the proteasome at the indicated incubation time.

RT-PCR Assay for PRAME Expression. Analysis of PRAME mRNA expression was determined by RT-PCR. Total cellular RNA was isolated with Trizol (GIBCO BRL) according to the manufacturer's procedure. RT reaction was performed on 5 μg of total RNA in a reaction volume of 25 μl with 5 μl of 5× reverse transcriptase buffer (Promega), 2.5 μl each of 10 mM deoxynucleotides (Amersham Pharmacia Biotech), 0.5 μg oligo dT_{15} primer, 25 U of RNAsin (Promega), and 15 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega). The RT reaction was incubated at 42°C for 60 min, heat inactivated for 10 min at 70°C, and diluted two times with water. For PCR amplification, 1 μl of reverse transcribed cDNA reaction mixture was used as a template. PCR primers used for the analysis of PRAME expression were OPC 189 (sense primer, 5′-CTGTACTCATTTCCAGAGCCAGA-3′) and OPC 190 (antisense primer, 5′-TATTGAGAGGGTTTCCAAGGGGTT-3′; reference 48). PCR conditions were 5 min at 94°C followed by 34 cycles consisting of 30 s at 94°C, 2 min at 64°C, and 3 min at 72°C.

In Vitro CTL Response Induction and Generation of CTL Clones. PBMCs of two HLA-A*0201$^+$ healthy donors (one donor for induction against $PRA^{300–309}$ and the other donor for inductions against $PRA^{100–108}$, $PRA^{142–151}$, $PRA^{425–433}$, and $PRA^{47–56}$) were obtained by the Ficoll-Paque method and used for CTL in-

ductions. To optimally use all APCs present in PBMCs, we developed a culture system that yields a mix of activated B cells and mature DCs to be used as APCs during the primary induction step. PBMCs were separated in a T cell fraction and a fraction containing B cells and monocytes by SRBC rosetting. The T cell fraction was cryopreserved. The mixture of monocytes and B cells was cultured in 24-well plates at a concentration of 10^6 cells/well in complete culture medium containing 800 U/ml GM-CSF (provided by S. Osanto, Leiden University Medical Center), 500 U/ml IL-4 (PeproTech), and 500 ng/ml CD40 mAb (clone B-B20; Serotec) for 6 d. This culture system achieved a threefold effect: (a) GM-CSF and IL-4 induced differentiation of monocytes into immature DCs (59), (b) IL-4 and CD40 mAbs caused activation and proliferation of B cells (60), and (c) CD40 mAb mediated maturation of immature DCs (61). At day 3, cytokines and CD40 mAb were replenished. To further promote CTL-inducing capacity, the APC mix was cultured for an additional 2 d with 0.4 ng/ml LPS (Difco Labs), 500 U/ml IFN-γ (Boehringer Mannheim), and 500 ng/ml CD40 mAb. At day 8 the APC mix was pulsed with 50 μg/ml peptide (each peptide separately) for 4 h at room temperature, irradiated (30 Gy), and washed to remove free peptide. The cryopreserved autologous T cell fraction was thawed and depleted from $CD4^+$ T cells using magnetic beads (Dynal). The primary induction was performed in 96-well U-bottomed plates. APCs at a concentration of 10,000/well were cocultured with 50,000 $CD8^+$ T cells/well in culture medium containing 10% human pooled serum (HPS), 5 ng/ml IL-7 (PeproTech), and 0.1 ng/ml IL-12 (Sigma-Aldrich). At day 7 after initiation of induction, the CTL microcultures were harvested (pooled), washed, and restimulated at a concentration of 40,000 responder cells/well of 96-well U-bottomed plates in culture medium containing 10% HPS, 5 ng/ml IL-7, and 0.1 ng/ml IL-12. Autologous-activated B cells, generated via the protocol described by Schultze et al. (60), irradiated (75 Gy), and peptide pulsed (50 μg/ml) for 4 h at room temperature in culture medium containing 2% FCS and 3 μg/ml β_2-microglublin (Sigma-Aldrich) after mild acid elution to remove naturally presented peptides from the MHC class I molecules (see Materials and Methods, MHC binding assay), were used at a concentration of 10,000 cells/well as restimulator APCs. Restimulations were repeated at day 14 and 21 in a similar way, with the exception of IL-7 being replaced by 20 IU/ml IL-2 (Chiron Corp.). At day 29, the CTL bulk culture was cloned by standard limiting dilution procedures. CTL clones were maintained by aspecific stimulation every 7 to 12 d using a feeder mixture consisting of allogeneic PBMCs and B-LCL in culture medium containing 10% FCS, 1.5% leucoagglutinin (Sigma-Aldrich), and 240 IU/ml IL-2.

^{51}Cr Cytotoxicity Assay, HLA Class I Blocking, and Proteasome Inhibition. CTL activity was measured in standard chromium release assays. In brief, after ^{51}Cr labeling (1 h), target cells (2,000/well) were added to various numbers of effector cells in a final volume of 100 μl complete culture medium in 96-well U-bottomed plates. After 4 h incubation at 37°C, supernatants were harvested. The mean percentage of specific lysis of triplicate wells was calculated according to: (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100%. For peptide titration experiments, ^{51}Cr-labeled target cells (2,000/well) were pulsed with titrated amounts of peptide for 1 h at 37°C in 96-well plates. Subsequently, CTLs were added at an E/T ratio of 10. HLA class I blocking was accomplished by treatment of 2×10^5 ^{51}Cr-labeled M453 melanoma cells for 1 h with mAb W6.32 or control IgG2a at room temperature before addition to effector cells. Inhibition of proteasome function in melanoma FM3 was performed by treatment with 10 μM of lactacystin (Calbiochem) for 17 h during culture at 37°C. Thereafter, FM3 cells were harvested and ^{51}Cr labeled for use in the cytotoxicity assay. Reconstitution of lysis by peptide was performed by pulsing lactacystin-treated and ^{51}Cr-labeled FM3 cells for 30 min with 5 μM peptide.

Results

*Identification of HLA-A*0201 Binding Peptides from PRAME.* To select candidate HLA-A*0201 binding peptides from PRAME, its aa sequence was screened for HLA-A*0201 binding motif containing peptides with a combination of two known binding prediction algorithms (62, 63). Only peptides of 9 or 10 aa length were included, taking into account the low prevalence of HLA-A*0201–restricted CTL epitopes of 8 or 11 aa length (64). In total, 128 peptides (65 nonamers and 63 decamers) were synthesized in order to determine their actual binding affinity for HLA-A*0201 using a competition-based cellular binding assay (54). 19 high affinity binding peptides were identified ($IC_{50} \leq 6$ μM), and 27 peptides bound with intermediate affinity (6 μM $< IC_{50} \leq 15$ μM), whereas the other peptides displayed a low (15 μM $< IC_{50} \leq 100$ μM) or undetectable binding capacity ($IC_{50} > 100$ μM; Table I). To more precisely define binding characteristics, peptide–MHC stability was assessed by measuring the dissociation rate of high affinity binding peptides complexed with HLA-A*0201 at 37°C (56). Two of the tested high affinity binding peptides ($PRA^{292–301}$ and $PRA^{190–199}$) showed a high off rate from HLA-A*0201, because <10% of HLA-A*0201–peptide complexes were detectable after 2 h incubation at 37°C. In previous work we have detected a strong correlation between MHC–peptide binding stability and immunogenicity in vivo (56). Therefore, $PRA^{292–301}$ and $PRA^{190–199}$ are, with respect to their binding characteristics, not likely to be efficiently presented in HLA-A*0201. For all other peptides, the 50% decay time (DT_{50}) was 2.5 h or longer (Table II), indicating a stable association with HLA-A*0201.

*In Vitro Proteasome-mediated Digestions of 27-mer Polypeptides Encompassing HLA-A*0201 Binding Peptides.* The two most important requirements for a peptide to be naturally presented as CTL epitope are: (a) proper excision from the protein by the proteolytic machinery and (b) sufficient binding affinity for HLA class I molecules. Therefore, we analyzed in vitro proteasome-mediated digestions of 27-mer polypeptides encompassing the 19 identified high affinity HLA-A*0201 binding peptides. Potential epitopes were primarily assessed for efficient liberation (i.e., by a major cleavage site at 1 h incubation) of their precise COOH terminus, which is a first requirement for the generation of most CTL epitopes (34–37). Intactness of the candidate epitope was evaluated as a secondary factor favoring efficient processing and presentation. 20S proteasomes isolated from a human EBV–transformed B cell line were used for digestions with 1-, 4-, and 24-h incubation periods, and mass spectrometry profiles of the digestion products were analyzed. Digestion patterns of four 27-mer

Table I. *Binding Affinity for HLA-A*0201 of 128 Nonamers and Decamers Derived from PRAME*

Start*	Sequence	Length	IC_{50}‡	Start	Sequence	Length	IC_{50}	Start	Sequence	Length	IC_{50}
300	ALYVDSLFFL	10	1.7	466	RLRELLCEL	9	14.0	86	LMKGQHLHL	9	62.3
142	SLYSFPEPEA	10	1.9	33	SLLKDEALAI	10	14.0	240	VTCTWKLPTL	10	67.2
47	LLPRELFPPL	10	2.1	422	ALQSLLQHL	9	14.2	44	ALELLPREL	9	71.3
435	NLTHVLYPV	9	2.5	103	GLDVLLAQEV	10	15.2	379	TLQDLVFDEC	10	71.9
292	FLSLQCLQAL	10	2.5	231	QLDSIEDLEV	10	15.5	371	ALLERASAT	9	72.0
394	QLLALLPSL	9	2.9	312	RLDQLLRHV	9	15.7	353	VLSLSGVMLT	10	74.6
182	FLKEGACDEL	10	3.0	493	RTFYDPEPI	9	15.8	305	SLFFLRGRL	9	79.4
294	SLQCLQALYV	10	3.2	308	FLRGRLDQL	9	16.1	409	TTLSFYGNSI	10	80.8
422	ALQSLLQHLI	10	3.2	429	HLIGLSNLT	9	16.3	93	HLETFKAVL	9	89.0
425	SLLQHLIGL	9	3.7	85	VLMKGQHLHL	10	17.0	319	HVMNPLETL	9	90.3
258	QMINLRRLLL	10	4.0	316	LLRHVMNPL	9	17.4	18	SVWTSPRRLV	10	>100
190	ELFSYLIEKV	10	4.5	353	VLSLSGVML	9	17.4	20	WTSPRRLVEL	10	>100
248	TLAKFSPYL	9	4.6	172	FIPVEVLVDL	10	17.5	26	LVELAGQSL	9	>100
39	ALAIAALEL	9	5.1	134	TVWSGNRASL	10	18.4	51	ELFPPLFMA	9	>100
100	VLDGLDVLL	9	5.2	339	VMHLSQSPSV	10	18.5	67	QTLKAMVQA	9	>100
333	RLSEGDVMHL	10	5.4	72	MVQAWPFTC	9	18.5	70	KAMVQAWPFT	10	>100
462	YLHARLREL	9	5.4	390	ITDDQLLALL	10	18.9	78	FTCLPLGVL	9	>100
360	MLTDVSPEPL	10	5.6	18	SVWTSPRRL	9	19.1	84	GVLMKGQHL	9	>100
419	SISALQSLL	9	5.7	315	QLLRHVMNPL	10	19.7	95	ETFKAVLDGL	10	>100
432	GLSNLTHVL	9	6.8	71	AMVQAWPFT	9	20.0	133	WTVWSGNRA	9	>100
214	KIFAMPMQDI	10	7.2	207	RLCCKKLKI	9	20.8	155	MTKKRKVDGL	10	>100
320	VMNPLETLSI	10	8.6	247	PTLAKFSPYL	10	21.1	165	STEAEQPFI	9	>100
39	ALAIAALELL	10	9.0	219	PMQDIKMIL	9	23.9	180	DLFLKEGAC	9	>100
390	ITDDQLLAL	9	9.2	459	RLAYLHARL	9	24.3	198	KVKRKKNVL	9	>100
242	CTWKLPTLA	9	9.3	264	RLLLSHIHA	9	24.6	205	VLRLCCKKL	9	>100
99	AVLDGLDVLL	10	9.4	217	AMPMQDIKMI	10	24.6	222	DIKMILKMV	9	>100
308	FLRGRLDQLL	10	9.6	361	LTDVSPEPL	9	26.8	224	KMILKMVQL	9	>100
355	SLSGVMLTDV	10	9.9	430	LIGLSNLTHV	10	27.2	234	SIEDLEVTC	9	>100
34	LLKDEALAI	9	10.2	33	SLLKDEALA	9	29.2	234	SIEDLEVTCT	10	>100
284	YIAQFTSQFL	10	10.4	258	QMINLRRLL	9	31.2	237	DLEVTCTWKL	10	>100
71	AMVQAWPFTC	10	10.4	91	HLHLETFKAV	10	31.8	240	VTCTWKLPT	9	>100
470	LLCELGRPSM	10	10.5	297	CLQALYVDSL	10	33.8	261	NLRRLLLSHI	10	>100
186	GACDELFSYL	10	10.6	372	LLERASATL	9	35.0	325	ETLSITNCRL	10	>100
410	TLSFYGNSI	9	11.0	401	SLSHCSQLT	9	36.9	368	PLQALLERA	9	>100
25	RLVELAGQSL	10	11.1	397	ALLPSLSHC	9	42.6	382	DLVFDECGI	9	>100
91	HLHLETFKA	9	11.1	389	GITDDQLLAL	10	47.3	382	DLVFDECGIT	10	>100
100	VLDGLDVLLA	10	11.9	417	SISISALQSL	10	48.2	383	LVFDECGIT	9	>100
454	TLHLERLAYL	10	12.2	259	MINLRRLLL	9	48.2	389	GITDDQLLA	9	>100
371	ALLERASATL	10	12.9	479	MVWLSANPC	9	49.0	401	SLSHCSQLTT	10	>100
326	TLSITNCRL	9	13.2	160	KVDGLSTEA	9	51.2	473	ELGRPSMVWL	10	>100
462	YLHARLRELL	10	13.3	436	LTHVLYPVPL	10	53.0	481	WLSANPCPHC	10	>100
350	QLSVLSLSGV	10	13.3	226	ILKMVQLDSI	10	56.1	493	RTFYDPEPIL	10	>100
99	AVLDGLDVL	9	13.4	292	FLSLQCLQA	9	58.7				

*Position in PRAME of the NH_2-terminal aa of the peptide. Peptides are listed in order of their IC_{50}.
‡IC_{50} is peptide concentration needed to inhibit binding of FL-labeled reference peptide for 50% (IC_{50} in μM).

polypeptides, all containing potential high affinity HLA-A*0201 binding epitopes, are shown in Fig. 1.

Fig. 1 A shows the digestions of PRAME 90–116, which harbors five HLA-A*0201 binding peptides (Table I) with their natural flanking residues. Of the COOH-terminal residues of the five HLA-A*0201 binding peptides, only Leu-108 was generated (fragments containing this COOH terminus added up to 8% at 1 h digestion). Therefore, both the 9-mer VLDGLDVLL ($PRA^{100–108}$) and the 10-mer AVLDGLDVLL ($PRA^{99–108}$) represent potential CTL epitopes. The NH_2 terminus of the epitope precursor is likely to be Lys-98, because the fragments most frequently generated were aa 90–97 and its complement aa 98–116, indicating an abundantly cleaved site after Phe-97.

Fig. 1 B shows the digestions of 27-mer PRAME 133–159, which contains 10-mer SLYSFPEPEA ($PRA^{142–151}$), the second best HLA-A*0201 binding peptide (Table I). Fragments sharing Ala-151 as COOH terminus added up to 29% at 1 h digestion, indicating an abundantly cleaved site after this residue. Furthermore, fragment aa 142–159 and the complementary fragment aa 133–141 were abundantly present, pointing to a major cleavage site after Ala-141. Thus, major cleavage sites were present just after and before SLYSFPEPEA, rendering this peptide a potential efficiently generated CTL epitope.

Table II. *Stability of High Affinity Binding Peptides in HLA-A*0201*

Start*	Sequence‡	Affinity IC_{50}§	Stability DT_{50}‖
		μM	*h*
300	ALYVDSLFFL	1.7	>4
142	SLYSFPEPEA	1.9	3
47	LLPRELFPPL	2.1	2.5
435	NLTHVLYPV	2.5	3
292	FLSLQCLQAL	2.5	N.S.¶
394	QLLALLPSL	2.9	>4
182	FLKEGACDEL	3.0	3
294	SLQCLQALYV	3.2	>4
422	ALQSLLQHLI	3.2	2.5
425	SLLQHLIGL	3.7	>4
258	QMINLRRLLL	4.0	>4
190	ELFSYLIEKV	4.5	N.S.
248	TLAKFSPYL	4.6	>4
100	VLDGLDVLL	5.2	2.5
	HBV control**		>4

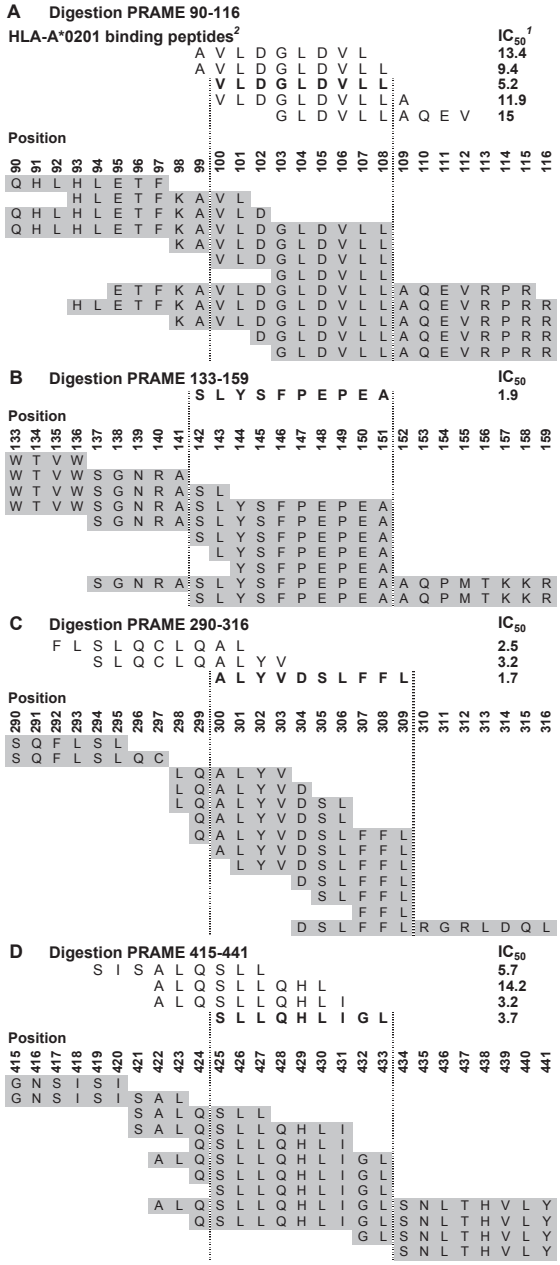
*Start aa position of peptide in PRAME.
‡In addition to all high affinity binding peptides, PRAME 100–108 and 371–380 are tested.
§Binding affinity expressed as IC_{50} (see Table I).
‖DT_{50} is given starting from $t = 2$ h at 37°C.
¶N.S., not stable; <10% of HLA molecules detectable after 2 h incubation at 37°C.
**HBV nucleocapsid 18–27 is used as control peptide.

Fig. 1 C depicts digestions of PRAME 290–316 encompassing 10-mer ALYVDSLFFL ($PRA^{300–309}$), which bound best in HLA-A*0201 (Table I) and has its COOH terminus (Leu-309) in common with the already described HLA-A24–presented 9-mer LYVDSLFFL ($PRA^{301–309}$; reference 48). As might be expected on that basis, a cleavage site after Leu-309 was observed, because digestion fragments sharing this COOH terminus were abundantly generated after 1 h incubation. However, $PRA^{300–309}$ itself was found intact only after 24 h incubation at low quantities. This is probably due to cleavage sites within this potential epitope (after Val-303, Asp-304, and Leu-306). HLA-A*0201 binding peptides $PRA^{292–301}$ and $PRA^{294–303}$ (also in PRAME 290–316) were, respectively, not COOH-terminally generated and not found as intact fragment, indicating that these peptides are not likely to be naturally generated in the processing pathway.

Fig. 1 D shows the digestion pattern of PRAME 415–441, which harbors four peptides binding in HLA-A*0201 (Table I). The NH_2-terminally elongated decameric precursor $_{424}$QSLLQHLIGL$_{433}$ of high affinity binding 9-mer SLLQHLIGL ($PRA^{425–433}$) was efficiently generated. The abundant generation of the COOH-terminal and NH_2-terminal counterparts of this 10-mer precursor (fragments aa 434–441 and aa 415–423, respectively) were also pointing to major cleavage sites just after and before $_{424}$QSLLQHLIGL$_{433}$, indicating $PRA^{425–433}$, a potential CTL epitope. The three other HLA-A*0201 binding peptides were either not COOH-terminally excised ($PRA^{419–427}$ and $PRA^{422–430}$) or the correct COOH terminus was found only after 4 h incubation ($PRA^{422–431}$).

A concise representation of digestion analysis of 27-mers harboring all 19 high affinity binding peptides, including those discussed above, is shown in Fig. 2. Summarizing, 11 HLA-A*0201 binding peptides were either not COOH-terminally excised ($PRA^{47–56}$, $PRA^{435–443}$, $PRA^{292–301}$, $PRA^{182–191}$, $PRA^{248–256}$, $PRA^{100–108}$, and $PRA^{360–369}$) or the correct COOH terminus was generated only after 4 h incubation by a minor cleavage site ($PRA^{394–402}$, $PRA^{422–431}$, $PRA^{190–199}$, and $PRA^{419–427}$). The absence or late appearance of fragments containing the correct COOH terminus render these 11 peptides very unlikely to constitute naturally processed epitopes. Furthermore, three peptides were COOH-terminally liberated at 1 h digestion but only in very low quantities (<1%, data not shown; $PRA^{258–267}$, $PRA^{333–342}$, and $PRA^{462–470}$), whereas $PRA^{294–303}$ was not found as intact fragment. Consequently, it is doubtful that the latter peptides are efficiently generated in vivo. Only the high affinity binding peptides $PRA^{100–108}$, $PRA^{142–151}$, $PRA^{300–309}$, and $PRA^{425–433}$ were COOH-terminally excised by a major cleavage site at 1 h incubation and found intact in digestion fragments, indicating possible CTL epitopes (Figs. 1 and 2). Therefore, these four peptides were chosen for CTL inductions.

*In Vitro Human CTL Inductions against Four Putative HLA-A*0201–restricted Epitopes.* Separate CTL inductions, using PBMCs of healthy donors, were performed against VLDGLDVLL ($PRA^{100–108}$), SLYSFPEPEA



Fragment[3,4]		Intensity (% digested)[5]		
Start	End	1 h	4 h	24 h
90	97	26	19	29
93	101	8	2	3
90	102	5	2	2
90	108	2	1	0
98	108	2	1	3
100	108	0	0.1	0.2
103	108	4	1	2
95	115	3	4	3
93	116	7	31	18
98	116	29	28	27
102	116	8	5	6
103	116	7	5	6

Fragment		Intensity (% digested)		
Start	End	1 h	4 h	24 h
133	136	22	35	26
133	141	21	12	24
133	143	5	4	3
133	151	18	11	10
137	151	4	3	5
142	151	4	2	6
143	151	2	2	3
144	151	1	1	1
137	159	10	23	11
142	159	12	7	12

Fragment		Intensity (% digested)		
Start	End	1 h	4 h	24 h
290	295	40	45	32
290	297	4	4	1
298	303	5	4	6
298	304	5	4	5
298	306	2	2	2
299	306	4	3	4
299	309	0	0	0.4
300	309	0	0	0.3
301	309	0	0	0.7
304	309	4	5	9
305	309	8	9	24
307	309	21	19	13
304	316	7	5	3

Fragment		Intensity (% digested)		
Start	End	1 h	4 h	24 h
415	420	13	12	6
415	423	17	15	14
421	427	0	2	1
421	431	0	4	2
424	431	0	8	3
422	433	0	1	7
424	433	17	10	8
425	433	0	1	2
422	441	0	0	8
424	441	0	3	3
432	441	0	5	3
434	441	54	39	42

Figure 1. In vitro proteasome-mediated digestions of four 27-mer PRAME polypeptides containing potential HLA-A*0201–restricted epitopes. 20S proteasomes isolated from an EBV-transformed B cell line were coincubated with 27-mer PRAME peptides at 37°C for the indicated time points. Digestion mixtures were analyzed by mass spectrometry as described in Materials and Methods. Generated digestions fragments are depicted as shaded sequences. The digestion of 27-mer PRAME 90–116 is represented in A, digestion of PRAME 133–159 is depicted in B, in C the digestion of PRAME 290–316 is showed, and D represents the digestion of PRAME 415–441. Notes: (1) IC_{50} as determined in competition binding assay (see Table I); (2) peptides binding with high or intermediate affinity to HLA-A*0201 are shown. Predicted epitopes used for CTL induction are printed in bold; (3) digestion fragments are shaded and ordered according to their COOH terminus; (4) digestion fragments generated for <1% at 1 h digestion or <3% at 4 and 24 h incubation and not relevant for epitope prediction are not shown; (5) intensity is expressed as percentage of total summed mass-peak intensities of digested 27-mer at the indicated incubation time.

(PRA$^{142–151}$), ALYVDSLFFL (PRA$^{300–309}$), and SLLQHLIGL (PRA$^{425–433}$) according to the protocol described in Materials and Methods. At day 28, the CTL bulk cultures were tested in a ^{51}Cr release assay to asses peptide specificity. The CTL cultures raised against SLYSFPEPEA and SLLQHLIGL showed high specificity for targets loaded with the inducing peptide (at an E/T ratio of 5, both cultures reached 80% lysis compared with 20% lysis of targets loaded with a control peptide), whereas the other two CTL cultures displayed only slightly increased lysis of targets loaded with the relevant peptide (data not shown). The four CTL bulk cultures were cloned by limiting dilution at day 29. The peptide specificity of generated CTL clones was initially assessed in a split-well cytotoxicity assay. Despite low peptide specificity of CTL bulk cultures against VLDGLDVLL and ALYVDSLFFL, CTL clones specific for

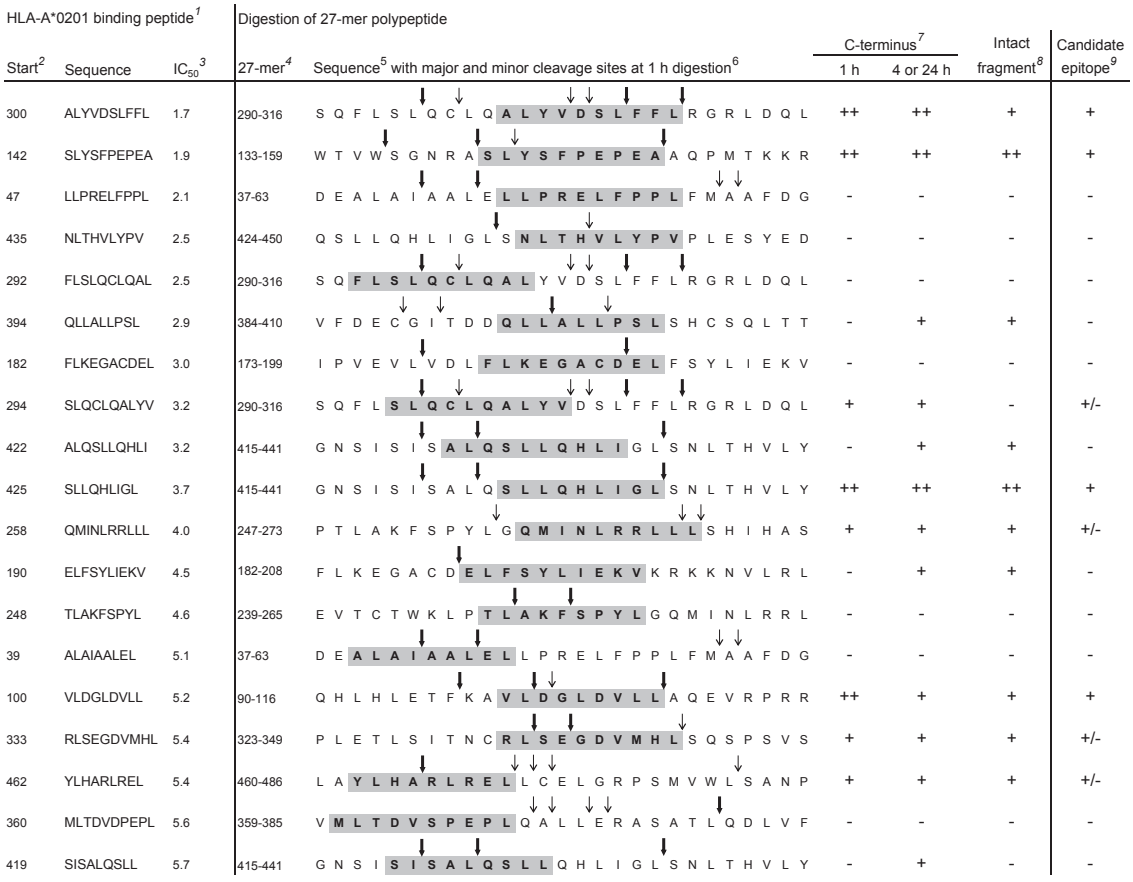


Figure 2. Proteasome-mediated cleavage patterns of 27-mer peptides encompassing 19 high affinity HLA-A*0201 binding PRAME peptides. 20S proteasomes isolated from an EBV-transformed B cell line were coincubated with 27-mer PRAME peptides at 37°C for the indicated time points. Digestion mixtures were analyzed by mass spectrometry as described in Materials and Methods. Major and minor cleavages sites at 1 h digestion are depicted. Notes: (1) all 19 high affinity binding peptides ($IC_{50} < 6$ μM) are listed and ranked according to their binding affinity for HLA-A*0201; (2) start aa position in PRAME of the HLA-A*0201 binding peptide. (3) IC_{50} (in μM) as determined in competition binding assay (see Table I); (4) start and end aa position in PRAME of 27-mer polypeptide encompassing the high affinity binding peptide; (5) aa sequence of 27-mer polypeptide (aa sequence of HLA-A*0201 binding peptide is printed in bold and shaded); (6) major (bold arrows) and minor (thin arrows) cleavage sites at 1 h incubation are depicted, classified according to the following definitions. Major site: fragments containing as COOH terminus the residue NH_2-terminal from the cleavage are present for ≥5% at 1 h incubation. Minor site: fragments containing as COOH terminus the residue NH_2-terminal from the cleavage are present for <5% at 1 h incubation. (7) Generation by digestion of fragments containing the correct COOH terminus of the HLA-A*0201 binding peptide. Generation at 1 h digestion or after a longer incubation period is separately indicated. Classification: (++) present for ≥5%, (+) present for <5%, (−) no fragments containing the correct COOH terminus were found. (8) Generation by digestion of fragments containing the intact HLA-A*0201 binding peptide and/or NH_2-terminal elongated precursors of the peptide. Classification: (++) present for ≥5% at 1 h incubation, (+) present for <5% at 1 h or only detectable after 4 or 24 h, (−) no fragments containing the HLA-A*0201 binding peptide were found. (9) Epitope prediction based on digestion results. Classification: (+) most likely an epitope, (+/−) doubtful epitope, (−) not an epitope.

all four peptides were found. In summary, 51 of 576 (9%) CTL clones induced against VLDGLDVLL ($PRA^{100–108}$) showed specific lysis of peptide pulsed targets and 19 of 202 (9%) CTL clones raised against ALYVDSLFFL ($PRA^{300–309}$) displayed peptide specificity. As may be expected, higher percentages, namely 92% (279 of 304), of clones against SLYSFPEPEA ($PRA^{142–151}$) and 29% (97 of 336) of clones against SLLQHLIGL ($PRA^{425–433}$) showed peptide specificity. Based on peptide reactivity and growth characteristics, several CTL clones were functionally characterized in detail. For each specificity, one CTL clone is presented in this study. As shown in Fig. 3 A, representative CTL clones raised against the four different peptides all showed highly specific and efficient lysis of T2 cells pulsed with 5 μM of their inducing peptides at low E/T ratios. Peptide sensitivity of the CTL clones was determined in peptide titration experiments (Fig. 3 B). CTL no. 551 (anti-$PRA^{100–108}$) was able to half-maximally lyse targets loaded with VLDGLD-

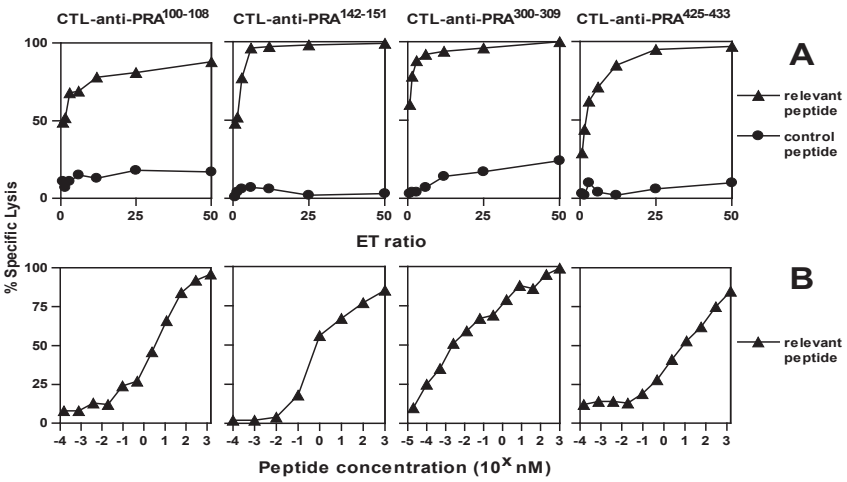


Figure 3. HLA-A*0201–restricted peptide specificity and sensitivity of CTL clones raised against four PRAME peptides. (A) Lysis by CTL clones no. 551 anti-PRA$^{100–108}$, no. 314 anti-PRA$^{142–151}$, no. 460 anti-PRA$^{300–309}$, and no. 1257 anti-PRA$^{425–433}$ of ^{51}Cr-labeled T2 cells loaded with 5 μM of the relevant peptide (▲) vs. an irrelevant HLA-A*0201 binding peptide (●) at different E/T ratios ranging from 50 to 0.75. (B) Lysis by the same set of CTL clones of ^{51}Cr-labeled T2 cells pulsed for 1 h with titrated concentrations of relevant peptide (▲). The CTL clones were used at an E/T ratio of 10. Results of one representative experiment out of three performed are shown.

VLL at ~5 nM peptide. CTL no. 314, raised against SLYSFPEPEA (PRA$^{142–151}$), lysed T2 cells at half-maximal level when pulsed with <1 nM of the inducing peptide. CTL no. 460 (anti-PRA$^{300–309}$) was extremely sensitive in lysing T2 cells pulsed with ALYVDSLFFL: half-maximal lysis was reached at ~3 pM peptide concentration. Finally, CTL no. 1257 (anti-PRA$^{425–433}$) was able to half-maximally lyse targets loaded with SLLQHLIGL at <12 nM. To analyze clonality of the CTL clones under investigation, we performed RT-PCR analysis with a panel of 24 primers of junctional regions of TCRB transcripts from 22 well-established TCRBV families to determine Vβ usage of the TCR (65). All CTL clones were shown to use a single Vβ, confirming clonality of the clones (data not shown).

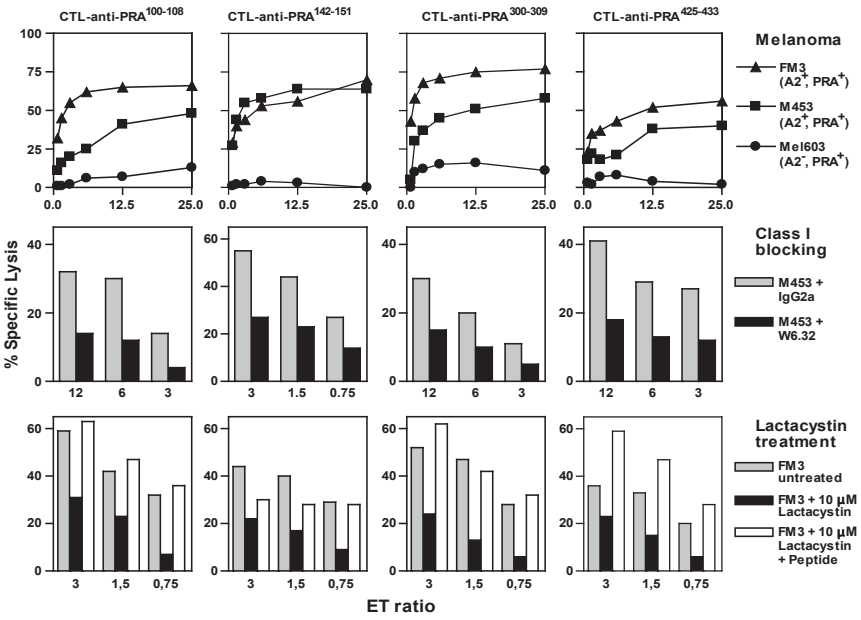


Figure 4. Recognition by CTL clones of four endogenously processed PRAME epitopes presented on melanoma cell lines in an HLA class I–restricted and proteasome-dependent fashion. (Top) Lysis of ^{51}Cr-labeled melanoma cell line Mel603, expressing PRAME but lacking HLA-A*0201 expression (●), was tested vs. lysis of M453 (■) and FM3 (▲), both expressing PRAME and HLA-A*0201 together. CTL clones no. 551 anti-PRA$^{100–108}$, no. 314 anti-PRA$^{142–151}$, no. 460 anti-PRA$^{300–309}$, and no. 1257 anti-PRA$^{425–433}$ were used at E/T ratios ranging from 25 to 0.75. (Middle) Lysis of ^{51}Cr-labeled M453 was tested after 1 h preincubation with HLA class I blocking mAb W6.32 (black bars) or an IgG2a control Ab (gray bars). (Bottom) Lysis of ^{51}Cr-labeled FM3 was tested after 17 h treatment with 10 μM of the proteasome inhibitor lactacystin (black bars) or without treatment (gray bars). As control, the lactacystin-treated cells were loaded with the relevant peptide (white bars). Results of one representative experiment of at least three performed are shown.

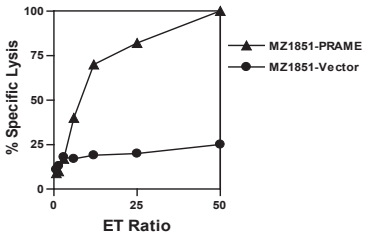


Figure 5. Lysis of PRAME transfected renal cell carcinoma cell line MZ1851 by CTL anti-PRA$^{300–309}$. CTL no. 460 directed against PRA$^{300–309}$ was tested on ^{51}Cr-labeled MZ1851 (HLA-A*0201^{+} but lacking PRAME expression) transfected with PRAME cDNA (▲) vs. MZ1851 transfected with the empty vector (●). CTL no. 460 was used at E/T ratios ranging from 50 to 0.75. Results of one representative experiment of three performed are shown.

*CTLs Raised against Four PRAME Peptides Recognize Melanoma Cell Lines Coexpressing HLA-A*0201 and PRAME in an HLA I–restricted and Proteasome-dependent Fashion.* Endogenous presentation of the candidate epitopes PRA$^{100–108}$, PRA$^{142–151}$, PRA$^{300–309}$, and PRA$^{425–433}$ in HLA-A*0201 was explored by assessing the ability of CTL clones that were raised against these peptides to specifically lyse melanoma cell lines M453 and FM3 expressing HLA-A*0201 (confirmed by flow cytometry, data not shown) and PRAME (determined by Northern blotting, data not shown). Both melanoma cell lines were efficiently lysed by all four CTL clones as measured in a ^{51}Cr release assay, whereas the melanoma Mel603 expressing PRAME (assayed with RT-PCR, data not shown) but lacking HLA-A*0201 was not killed above background level (Fig. 4, top panel). Lysis of M453 was significantly inhibited after treatment of this target with HLA class I blocking mAb W6.32 (Fig. 4, middle panel), indicating that killing of M453 by these CTL clones involved HLA class I–restricted recognition. Furthermore, proteasome inhibition experiments with lactacystin were performed. Lysis of FM3 pretreated for 17 h with lactacystin (10 μM) was significantly diminished (Fig. 4, bottom panel). This indicated, in concordance with our in vitro proteasome digestion data, that generation of the four epitopes is proteasome dependent. To confirm PRAME as source of antigen naturally presented in HLA-A*0201, renal cell carcinoma cell line MZ1851, which is HLA-*0201^{+} but lacks PRAME expression, was transfected with full-length PRAME cDNA (MZ1851-PRAME). As shown in Fig. 5, PRAME expression (confirmed by RT-PCR) sensitized MZ1851-PRAME for lysis by a CTL clone directed against PRA$^{300–309}$.

*CTLs Reactive with Four PRAME Epitopes Lyse a Broad Array of Tumor Cell Lines Expressing HLA-A*0201 and PRAME.* To investigate HLA-A*0201–restricted presentation of PRA$^{100–108}$, PRA$^{142–151}$, PRA$^{300–309}$, and PRA$^{425–433}$ on tumor cells from histologic origins other than melanoma, we used panels of cell lines derived from various tu-

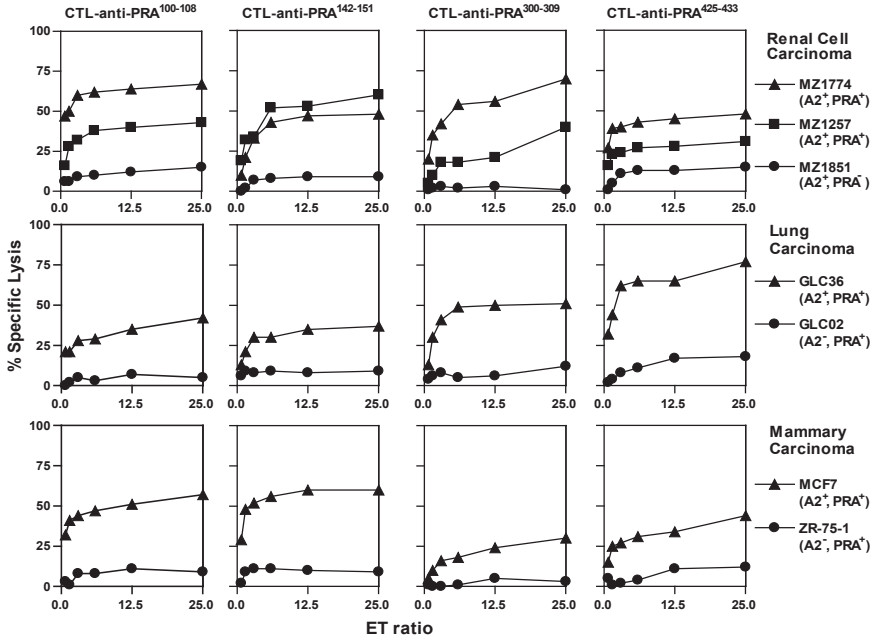


Figure 6. Lysis of tumor cell lines from multiple histologic origins by CTL anti-PRAME in a PRAME-specific and HLA-A*0201–restricted fashion. (Top) Lysis of ^{51}Cr-labeled renal cell carcinoma cell lines MZ1851, expressing HLA-A*0201 but PRAME negative (●), MZ1257 (PRAME^{+} and HLA-A*0201^{+}) (■), and MZ1774 (PRAME^{+} and HLA-A*0201^{+}) (▲) was compared. (Middle) Lysis of ^{51}Cr-labeled lung carcinoma cell lines. GLC02, expressing PRAME but HLA-A*0201 negative (●) and GLC36 (▲) expressing both PRAME and HLA-A*0201 was compared. (Bottom) ^{51}Cr-labeled mammary carcinoma cell lines MCF7 (HLA-*0201^{+} and PRAME^{+}) (●) and ZR-75-1, expressing PRAME but lacking HLA-A*0201 (▲), were tested. The CTL clones no. 551 anti-PRA$^{100–108}$, no. 314 anti-PRA$^{142–151}$, no. 460 anti-PRA$^{300–309}$, and no. 1257 anti-PRA$^{425–433}$ were used at E/T ratios ranging from 25 to 0.75. Results of one representative experiment of at least three performed are shown.

mor types which have been reported to express PRAME (48, 50).

Lysis by the selected CTL clones of tumor cell lines with or without HLA-A*0201 expression and naturally expressing PRAME or lacking PRAME expression was compared. HLA-A*0201 expression was confirmed by flow cytometry (data not shown) and PRAME expression by RT-PCR or Northern blotting (data not shown). Lysis of the HLA-A*0201$^+$ renal cell carcinoma (RCC) cell line MZ1851, which lacks PRAME expression, was compared with lysis of RCC cell lines MZ1257 and MZ1774, both expressing HLA-A*0201 and PRAME. The CTL clones reactive against the four different PRAME peptides showed significant lysis of the two PRAME$^+$ cell lines but not of MZ1851, again confirming PRAME as the source of target antigens (Fig. 6, top panel). Likewise, lysis of lung carcinoma cell lines was HLA-A*0201 restricted and PRAME specific, because only GLC36 expressing HLA-A*0201$^+$ and PRAME$^+$, and not GLC02, which is PRAME$^+$ but lacks HLA-A*0201 expression, was killed (Fig. 6, middle panel). Mammary carcinoma cell line MCF7 (HLA-A*0201$^+$ and PRAME$^+$) was killed efficiently as well, whereas cell line ZR-75-1, which lacks HLA-A*0201 but expresses PRAME, was not lysed above background level (Fig. 6, bottom panel). Finally, HPV16$^+$ cervix carcinoma cell line C33 and osteosarcoma cell line SAOS, both HLA-A*0201$^+$ and PRAME$^+$, were efficiently killed by the CTL clones (data not shown), underscoring the broad expression pattern of the PRAME epitopes by tumors. Taken together, we observed a consistent lysis of tumor cell lines when both the relevant MHC molecule and the tumor Ag were expressed. These results indicate that PRA$^{100–108}$, PRA$^{142–151}$, PRA$^{300–309}$, and PRA$^{425–433}$ are presented by HLA-A*0201 on a broad array of tumor cell lines.

*CTL Clones Recognizing a Nonprocessed High Affinity Binding Peptide Do Not Lyse PRAME and HLA-A*0201–expressing Tumor Cell Lines.* To further validate our epitope prediction procedure and investigate possible false negative epitope prediction, CTLs were induced against the highest affinity HLA-A*0201 binding peptide that was not generated in vitro by proteasome-mediated breakdown: LLPRELFPPL (PRA$^{47–56}$; Table I and Fig. 2). Digestion of the 27-mer PRAME 37–63 showed that the COOH terminus of LLPRELFPPL was not generated. Instead, cleavage sites were observed after Leu-45, Met-58, and Ala-59 (Fig. 2). Dual cleavage fragments $_{46}$ELLPRELFPPLFM$_{58}$ and $_{46}$ELLPRELFPPLFMA$_{59}$ were abundantly found at 1 h digestion (data not shown). Using the same induction protocol as used for the four identified epitopes, CTLs were generated against LLPRELFPPL. Three CTL clones recognizing this peptide with high affinity were tested for lysis of PRAME and HLA-A*0201–expressing tumor cell lines at different E/T ratios (Table III). For comparison, three CTL clones recognizing the SLYSFPEPEA epitope (PRA$^{142–151}$) with equal affinity as the affinity of CTL anti-PRA$^{47–56}$ for LLPRELFPPL were included in the same experiment. The results show that CTLs raised against LLPRELFPPL do not lyse any of the tumor cell lines, whereas the same targets were efficiently killed by the control CTLs directed against SLYSFPEPEA (Table III). These data strongly suggest that LLPRELFPPL is not endogenously generated, supporting the accuracy of our epitope prediction procedure and the relevance of the proteasome digestion analysis. In addition to these data, high affinity CTL clones generated against three different BCR-ABL fusion protein–derived peptides failed to recognize BCR-ABL–expressing target cells (data not shown). Subsequent analysis of in vitro processing of these peptides showed that the proteasome did not generate the COOH terminus of any of these high affinity HLA class I binding peptides. These results confirm the importance of proper proteasomal cleavage for the generation of HLA class I–presented epitopes.

Table III. *Percent Specific Lysis of PRAME- and HLA-A*0201–Expressing Tumor Cell Lines by CTL Clones Specific for LLPRELFPPL (Not Processed) and SLYSFPEPEA (Processed) as Determined in a ^{51}Cr Cytotoxicity Assay*

		CTL clones anti-LLPRELFPPL			CTL clones anti-SLYSFPEPEA		
Tumor cell line*	E/T ratio	No. 3	No. 61	No. 120	No. 314	No. 343	No. 509
M453	6	2	2	10	33	39	26
	3	3	3	11	26	32	16
	1, 5	1	2	9	27	17	15
MZ1257	6	1	9	0	52	28	27
	3	1	8	4	34	16	26
	1, 5	2	8	2	32	12	12
MZ1774	6	7	8	10	43	42	31
	3	8	9	10	33	25	16
	1, 5	4	4	8	21	13	10
GLC36	6	2	10	7	26	28	18
	3	7	11	9	30	22	17
	1, 5	5	9	7	21	20	16
MCF7	6	5	8	7	56	26	32
	3	6	9	7	52	24	19
	1, 5	4	9	5	48	21	13
CTL sensitivity‡		0.1–1	0.1–1	0.1–1	0.1–1	0.1–1	0.1–1

*Cell lines used as targets are derived from melanoma (M453), renal cell cancer (MZ1257 and MZ1774), lung cancer (GLC36), and mammary cancer (MCF7).

‡CTL sensitivity for peptide expressed as [peptide] in nM at which the indicated CTL clone half-maximally lysed T2 cells pulsed with titrated amounts of peptide.

Discussion

In a systematic search for new CTL epitopes in known protein sequences with tumor restricted expression, the strategy of in vitro stimulation of CTLs with predicted epitopes (also coined "reverse immunology") has successfully led to the identification of several epitopes (18–25), but has met with many failures and is generally inefficient (our unpublished results and references 26–30). The current study reports the identification of four novel HLA-A*0201–restricted CTL epitopes in PRAME ($PRA^{100–108}$, $PRA^{142–151}$, $PRA^{300–309}$, and $PRA^{425–433}$) by an improved multistep epitope prediction procedure. Using in vitro proteasome-mediated digestion pattern analysis, the four epitopes were chosen for CTL inductions and shown to be naturally presented. In addition, we show that CTL clones with high sensitivity for high affinity binding peptide $PRA^{47–56}$, which was not produced in vitro by proteasome-mediated digestion (Fig. 2), were unable to lyse PRAME and HLA-A*0201–expressing tumor cell lines (Table III), indicating a lack of endogenous processing of this peptide. Taken together, both findings imply the accuracy and relevance of the proteasome-mediated digestion pattern analysis.

Importantly, only 4 out of the 19 peptides ($PRA^{100–108}$, $PRA^{142–151}$, $PRA^{300–309}$, and $PRA^{425–433}$) were COOH-terminally excised by a major cleavage site at 1 h incubation and were contained intact in digestion fragments as well, indicating possible abundantly expressed CTL epitopes. This reduction to 21% of high affinity HLA-A*0201 binding peptides being efficiently processed, which is in concordance with an estimation by Yewdell et al. (66), permitted us to avoid laborious and time consuming CTL inductions against unlikely epitopes. Indeed, the four predicted epitopes were all shown to be endogenously processed and presented (Figs. 4–6). For future epitope predictions in other proteins, it will be worthwhile to first systematically characterize proteasome digestion patterns of a complete set of overlapping long (e.g., 30-mer) polypeptides and subsequently determine binding affinities for MHC class I molecules of interest of only those peptides that are shown to be COOH-terminally excised by a major cleavage site. This experimental order reflects the physiological sequence of events, with the primacy of CTL epitope generation at antigen processing and not at MHC binding as indeed has been observed for MHC II epitope presentation (67).

Possibly, in the future, reliable proteasome cleavage prediction algorithms will allow by-passing of experimental digestions. Efforts to develop such algorithms have been reported (68, 69). By prediction algorithms of Kuttler et al. (69), three of the four epitopes identified in our study ($PRA^{142–151}$, $PRA^{300–309}$, and $PRA^{425–433}$) were predicted to be correctly COOH-terminally liberated. In contrast, the COOH terminus of $PRA^{100–108}$, which is generated by a major cleavage site in our study, was not predicted. Of the 25 major cleavage sites observed (Fig. 2), 17 sites (68%) were correctly predicted by the most optimal algorithm variant (type II) as well as 10 of the 23 (43%) minor cleavage sites observed at 1 h digestion (by algorithm variant type III). Furthermore, many cleavage sites were falsely predicted by the algorithms, including erroneous internal epitope destruction sites, thus reducing the value of the current algorithms. Although these differences may be partly attributable to the different types of proteasomes used (immunoproteasomes in this study versus constitutive proteasomes in reference 69), we conclude that this first accessible proteasome cleavage prediction algorithm is not yet accurate enough to be used without experimental verification. As suggested by Kuttler et al. (69), a further enlargement of the training data used to educate the algorithm is likely to improve the accuracy of prediction.

With respect to the utilization and interpretation of proteasome-mediated digestion patterns for epitope prediction as reported here, several points must be raised. For proteasome-mediated digestions, we used 20S proteasomes isolated from an EBV-transformed B cell line known to contain mainly so-called immunoproteasomes with LMP2, LMP7, and MECL1 subunits (58). This implies that the four identified epitopes are likely to be presented on full-length PRAME expressing mature DCs containing immunoproteasomes next to their expression on tumor cells (containing constitutive proteasomes). For whole antigen vaccine development, such epitopes are favorable in contrast to a type of CTL epitope of which the presentation has been reported to be abrogated in mature DCs (70). The reported reverse effect, an inefficient processing in cells containing constitutive proteasomes (71–74), is excluded for our CTL epitopes by the functional data (Figs. 4–6).

The selection of candidate epitopes was mainly based on generation of the correct COOH terminus by an early major cleavage site, which is considered a sine qua none for efficient epitope generation (34–37). Late emergence (at 4 or 24 h) of the correct COOH terminus or generation by a minor cleavage site is not expected to yield epitopes or only at very low density, respectively. In contrast, cleavage sites within the epitope were less heavily weighed in the epitope prediction. In particular, ALYVDSLFFL ($PRA^{300–309}$) was found to be cleaved at several sites within the epitope (Fig. 1 C). Although this phenomenon does not exclude epitope formation, as reported for epitopes of murine leukemia virus and CMV (37, 75), partial destruction of an epitope can severely hamper its efficient presentation (for a review by Niedermann et al., see reference 76). Despite that, the $PRA^{300–309}$ peptide was included in our CTL inductions for several reasons: (a) generation of its COOH terminus by a major cleavage site (Fig. 1 C), (b) reported presentation of the 9-mer length variant $PRA^{301–309}$ (LYVDSLFFL) in HLA-A24 (48), and (c) favorable binding capacity (Table I). Furthermore, (d) it can not be excluded that in vitro digestions are prone to a "recharging-effect": longer epitope precursor fragments (containing the correct COOH terminus) can be further degraded by reentry in the proteasome, a phenomenon which will presumably not occur in vivo, because translocation of polypeptides by

TAP to the ER has been shown to occur as soon as 15 min after protein synthesis (and consequently proteasome digestion [77]). However, our control experiments did not reveal a significant effect of peptide substrate concentration on the relative digestion fragment kinetics (data not shown), making pronounced effects of recharging unlikely and indicating that the observed kinetics at least partially reflect the primary cleavage pattern and fragment abundancy. Finally, (e) cleavage within this epitope may be diminished when digested with constitutive proteasomes instead of immunoproteasomes, as enhanced cleavages after leucine and valine (hydrophobic residues) by the latter type of proteasomes have been described (76).

Our data support the current notion that a significant proportion of CTL epitopes is produced by the proteasome as NH_2-terminally extended precursor (36, 38–41). Digestion analysis of PRAME 415–441 revealed that 9-mer $PRA^{425–433}$ is abundantly generated as NH_2-terminally elongated 10-mer $_{424}$QSLLQHLIGL$_{433}$ (Fig. 1 D), rendering it likely that this fragment is translocated by TAP to the ER and is trimmed there to its final length. Likewise, 9-mer $PRA^{100–108}$ is presumably formed as 11-mer $_{98}$KAVLDGLDVLL$_{108}$, which also indicates that the intermediate binding 10-mer $PRA^{99–108}$ may be presented as well (Fig. 1 A). In contrast, SLYSFPEPEA ($PRA^{142–151}$) is likely available for TAP translocation in its precise length, because this peptide was found in significant quantities as digestion fragment at 1 h digestion (Fig. 1 B). This is in concordance with a recent report showing that the proteasome can generate both COOH and NH_2 termini of some epitopes (78).

PRAME is a particularly attractive tumor-associated antigen because it is widely expressed in many different tumor types (48–51), but not in normal tissues, except testis, and at very low levels in endometrium, ovaries, and adrenals (48). Indeed, CTL clones recognizing the four novel HLA-A*0201–restricted PRAME epitopes specifically lysed melanoma, renal cell, lung, mammary, and cervical carcinoma cell lines (Figs. 4 and 6). Therefore, and given the high prevalence of HLA-A*0201 among the Caucasian population, these epitopes are expected to be applicable for immunotherapeutic purposes (adoptive CTL therapy, vaccine design, and/or immunomonitoring) in a high percentage of cancer patients. Undesirable autoimmune CTL reactivity against the few tissues expressing PRAME at low levels is not to be expected, because expression levels are most likely too low to ensure CTL recognition as shown in vitro with human MAGE-specific CTLs by Lethe et al. (79) and in vivo in a murine p53 model by our group (80). Nevertheless, control recognition studies with normal endometrium, ovary, or adrenal tissues should ascertain absence of harmful responses towards healthy tissues expressing PRAME at low levels (expression level <3–5% of that found in melanoma, with the exception of endometrium, which expresses up to 30% of the melanoma level [48]). So far we have been unable to establish sufficient primary cell cultures of those sources for functional analysis.

Although not the principle objective of this study, we found a remarkable immunogenicity in healthy donors of the four epitopes, because CTL inductions against the four peptides (performed with blood of two separate donors, see Materials and Methods) were all successful. Particularly, $PRA^{142–151}$ and $PRA^{425–433}$ vigorously induced CTL bulk cultures recognizing these peptides as endogenously expressed PRAME epitopes. Apparently, the low level expression of PRAME in some healthy tissues did not induce irreversible tolerance against the four identified epitopes. Future comparison of CTL frequencies in healthy donors versus cancer patients, as determined by, e.g., tetramer studies or enzyme-linked immunospot analysis, will reveal whether cancer patients are naturally primed against the four epitopes. Furthermore, such experiments may allow an immunodominance ranking of the epitopes. Our digestion data suggest a ranking in efficiencies of proteasome-mediated generation of the four epitopes, which is a major factor determining immunodominance (66, 76). Because of the higher abundance of epitope precursor fragments and absence of major cleavage sites within the epitopes, $PRA^{142–151}$ and $PRA^{425–433}$ are probably more efficiently generated than $PRA^{100–108}$ and $PRA^{300–309}$ (Figs. 1 and 2).

Finally, we expect that our novel epitope prediction methodology will help to rapidly identify PRAME-derived CTL epitopes presented in HLA class I molecules other than HLA-A*0201 and will boost the reverse immunology approach for other tumor specific proteins as well. Such a systematic identification of new CTL epitopes in different tumor antigens will allow the development of multiantigen (epitope-based) tumor vaccines, covering all HLA class I haplotypes, which is probably needed to circumvent tumor escape by antigen loss variants.

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CHAPTER 3

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Detection and Functional Analysis of CD8$^+$ T Cells Specific for PRAME: a Target for T-Cell Therapy

Marieke Griffioen,[1] Jan H. Kessler,[2] Martina Borghi,[1] Ronald A. van Soest,[3] Caroline E. van der Minne,[1] Jan Nouta,[2] Sjoerd H. van der Burg,[2] Jan Paul Medema,[1] Peter I. Schrier,[1] J.H. Frederik Falkenburg,[3] Susanne Osanto,[1] and Cornelis J.M. Melief[2]

Abstract

Purpose: Preferentially expressed antigen on melanomas (PRAME) is an interesting antigen for T-cell therapy because it is frequently expressed in melanomas (95%) and other tumor types. Moreover, due to its role in oncogenic transformation, PRAME-negative tumor cells are not expected to easily arise and escape from T-cell immunity. The purpose of this study is to investigate the usefulness of PRAME as target for anticancer T-cell therapies.

Experimental Design: HLA-A*0201-subtyped healthy individuals and advanced melanoma patients were screened for CD8$^+$ T cells directed against previously identified HLA-A*0201-binding PRAME peptides by IFN-γ enzyme-linked immunosorbent spot assays and tetramer staining. PRAME-specific T-cell clones were isolated and tested for recognition of melanoma and acute lymphoid leukemia (ALL) cell lines. PRAME mRNA expression was determined by quantitative real-time reverse transcription-PCR.

Results: In 30% to 40% of healthy individuals and patients, PRA$^{100-108}$-specific CD8$^+$ T cells were detected both after *in vitro* stimulation and directly *ex vivo* after isolation by magnetic microbeads. Although CD45RA$^-$ memory PRA$^{100-108}$-specific T cells were found in some individuals, the majority of PRA$^{100-108}$-tetramer$^+$ T cells expressed CD45RA, suggesting a naive phenotype. PRA$^{100-108}$-tetramer$^+$ T-cell clones were shown to recognize and lyse HLA-A*0201$^+$ and PRAME$^+$ melanoma but not ALL cell lines. Quantitative real-time reverse transcription-PCR showed significantly lower PRAME mRNA levels in ALL than in melanoma cell lines, suggesting that PRAME expression in ALL is below the recognition threshold of our PRA$^{100-108}$-tetramer$^+$ T cells.

Conclusion: These data support the usefulness of PRAME and in particular the PRA$^{100-108}$ epitope as target for T-cell therapy of PRAME-overexpressing cancers.

Tumor antigens that are used as targets in clinical studies belong to the melanocyte differentiation antigens, cancer-testis antigens, or antigens overexpressed in tumors (1–5). Cancer-testis antigens are expressed in different tumors but not in normal tissues, except for testis, and are therefore useful targets for T-cell therapy. Most cancer-testis antigens, however, are expressed at low frequencies (30-50%).

Preferentially expressed antigen on melanomas (PRAME) has been identified as an antigen recognized by an HLA-A24-restricted CTL isolated from a melanoma patient (6). Semi-quantitative reverse transcription-PCR (RT-PCR) analysis showed frequent PRAME expression in melanomas (95%) and other tumor types, including lung and breast tumors and leukemias but not in healthy tissues, except for testis and low expression in endometrium, ovaries, and adrenals. DNA microarray analysis revealed the gene encoding PRAME as one of the genes of an expression profile for poor prognosis in breast carcinoma (7). Recently, the function of PRAME has been elucidated by Epping et al. (8). PRAME binds to retinoic acid receptor α, thereby inhibiting retinoic acid–induced differentiation, growth arrest, and apoptosis. Suppression of high levels of endogenous PRAME in retinoic acid–resistant melanoma cells by RNA interference restores sensitivity to the antiproliferative effects of retinoic acid, suggesting that PRAME overexpression contributes to oncogenesis by inhibiting retinoic acid signaling.

In this study, HLA-A*0201-subtyped healthy individuals and advanced melanoma patients were screened for T cells directed against four previously identified HLA-A*0201-binding PRAME epitopes (9) by IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays and tetramer staining. T cells specific for PRA$^{100-108}$ were most frequently found both after *in vitro* stimulation and directly *ex vivo* after isolation by magnetic microbeads. Furthermore, PRA$^{100-108}$-tetramer$^+$ T-cell clones

Authors' Affiliations: Departments of [1]Clinical Oncology, [2]Immunohematology and Blood Transfusion, and [3]Hematology, Leiden University Medical Center, Leiden, the Netherlands

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Note: S. Osanto and C. Melief contributed equally to the work.

Requests for reprints: Marieke Griffioen, Department of Hematology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, the Netherlands. Phone: 31-71-5262640; Fax: 31-71-5266755; E-mail: M.Griffioen@lumc.nl.

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were isolated and shown to recognize and lyse tumor cells expressing high levels of PRAME, supporting the usefulness of PRAME as target for immunotherapy.

Materials and Methods

Cell lines and culture conditions. Melanoma cell lines 453A0, 513D, 518A2, and IGR39D were established in our laboratory. Cell lines FM6 and FM3 were kindly provided by J. Zeuthen (Copenhagen, Denmark). Breast carcinoma cell line MDA-231 was obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured in DMEM (Invitrogen, Breda, the Netherlands) supplemented with 8% FCS, 4 mmol/L L-glutamine, 50 μg/mL penicillin, and 50 μg/mL streptomycin. The Leiden acute lymphoid leukemia (L-ALL) cell lines L-ALL-BV, L-ALL-RL, L-ALL-VG, L-ALL-CR, L-ALL-HP, L-ALL-KW, L-ALL-PH, L-ALL-SK, L-ALL-VB, and L-CML-B were cultured as described (10). L-CML-B is derived from a patient with lymphoblastic crisis of chronic myeloid leukemia (CML) but has phenotypic characteristics of ALL. K562-A2 and K562-A3 are generated by transduction of cell line K562 with retroviral vectors encoding HLA-A*0201 and A*0301. K562-A2, K562-A3 and EBV-B cell lines were cultured in RPMI with penicillin/streptomycin and 8% FCS. The T2 cell line was cultured in Iscove's modified Dulbecco's medium (IMDM) with penicillin/streptomycin and 5% FCS.

In vitro ***stimulation of peripheral blood mononuclear cells.*** Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood from healthy individuals and melanoma patients (American Joint Cancer Committee stages III and IV) by Ficoll gradient centrifugation. The study was approved by the local Medical Ethical Committee, and informed written consent was given by all individuals. Individuals were subtyped as HLA-A*0201 by PCR. PBMCs were seeded at 3×10^6 per well in 24-well plates in T-cell medium [IMDM with penicillin/streptomycin; 116 μg/mL L-arginine, 36 μg/mL L-asparagine, and 215 μg/mL L-glutamine; 10% human serum; and 150 IU/mL human recombinant interleukin-2 (IL-2)], containing 2 μg/mL of PRAME ($PRA^{100-108}$, $PRA^{142-151}$, $PRA^{300-309}$, and $PRA^{425-433}$) or influenza (FLU^{58-66}) peptides (9, 11). At day 11, PBMCs were collected, washed, and seeded at 3×10^6 per well in 24-well plates in T-cell medium without peptides. At day 13, PBMCs were tested for specific $CD8^+$ T cells by IFN-γ ELISPOT assays and tetramer staining.

IFN-γ ELISPOT assay. The IFN-γ ELISPOT assay was done as described (12) using 96-well nylon Silent Screen plates (Nunc Gmbh and Co. KG, Weisbaden, Germany) and AP-conjugate substrate kit (Bio-Rad, Hercules, CA). Peptide-stimulated PBMCs were seeded at 5×10^4, 2.5×10^4, and 1×10^4 per well together with 5×10^4 (peptide pulsed) T2 cells in triplicate in IMDM with 5% human serum overnight at 37°C. T2 cells were pulsed with peptides (5 μg/mL) in IMDM for 2 hours at 37°C.

Tetramer staining. Peptide-stimulated PBMCs ($1\text{-}5 \times 10^5$) and $CD8^+$ T-cell clones ($1\text{-}5 \times 10^4$) were washed with PBS supplemented with 0.5% bovine serum albumin (PBA) and incubated with phycoerythrin-labeled $PRA^{100-108}$, $PRA^{142-151}$, $PRA^{300-309}$, and FLU^{58-66} or APC-labeled $PRA^{425-433}$ tetrameric complexes (10 pmol/mL) in PBA for 1 hour at room temperature. After subsequent incubation with anti-CD8-FITC for 20 minutes at 4°C, cells were washed and resuspended in PBA for fluorescence-activated cell sorting analysis. Propidium iodide (PI) was added (5 μg/mL) to exclude dead cells.

Isolation of tetramer$^+$ T cells by magnetic microbeads. Total PBMCs (30×10^6) were stained with phycoerythrin-labeled $PRA^{100-108}$-tetramer (10 pmol/mL) in PBA for 1 hour at room temperature. After washing, cells were incubated with magnetic anti-phycoerythrin microbeads and applied on MS^+ columns, according to manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Total PBMCs before isolation (1×10^6) and all PBMCs after isolation were stained with anti-CD8-FITC and anti-CD45RA-APC in PBA for 30 minutes at 4°C. After washing, cells were analyzed by fluorescence-activated cell sorting. PI was added (5 μg/mL) to exclude dead cells.

Generation and isolation of $PRA^{100-108}$-specific $CD8^+$ T cells. Adherent monocytes from HD13 were cultured in AIM-V (Invitrogen, Breda, the Netherlands) with L-arginine/L-asparagine/L-glutamine and penicillin/streptomycin supplemented with 500 units/mL IL-4 (Peprotech, Inc., Rocky Hill, NJ) and 800 units/mL granulocyte macrophage colony-stimulating factor (Behringwerke, Marburg, Germany) for 8 days. Dendritic cells were pulsed with 10 μg/mL $PRA^{100-108}$ peptide in IMDM with penicillin/streptomycin for 4 hours, washed, and subsequently incubated with autologous nonadherent PBMCs in IMDM with penicillin/streptomycin, L-arginine/L-asparagine/L-glutamine, 10% human serum, 10 ng/mL IL-7 (Peprotech), and 100 pg/mL IL-12 (Sigma-Aldrich, Zwijndrecht, the Netherlands). T-cell cultures were weekly restimulated with autologous dendritic cells pulsed with $PRA^{100-108}$ as described above in T-cell medium. At day 21, $PRA^{100-108}$-tetramer$^+$ $CD8^+$ T cells were sorted by fluorescence-activated cell sorting and seeded at 1 per well in 96-well plates, each well containing 1×10^5 irradiated (50 Gy) allogeneic PBMCs, 5×10^3 irradiated (100 Gy) EBV-B cells, and 5×10^3 irradiated (100 Gy) $PRA^{100-108}$-pulsed EBV-B cells. Growing T-cell clones were isolated and weekly restimulated as described above.

Cocultures of tumor cells and $PRA^{100-108}$-specific $CD8^+$ T cells. Tumor cells (5×10^4) were seeded together with $PRA^{100-108}$-specific T cells (5×10^4) in triplicate in 96-well flat-bottomed plates in T-cell medium. As controls, triplicate wells were incubated with tumor cells (5×10^4) in the absence of T cells. After overnight incubation at 37°C, pools of supernatants of triplicate wells were analyzed for release of cytokines by human Th1/Th2 Cytokine Cytometric Bead Array (BD Biosciences, Franklin Lakes, NJ). After 48 hours of incubation, all individual cocultures were collected, washed with PBA, and stained with anti-CD3-FITC. Total numbers of viable tumor cells were analyzed by fluorescence-activated cell sorting after addition of PI (5 μg/mL). The percentage of viable tumor cells (% cell viability) was calculated as follows: [mean number of viable tumor cells (PI^- and $CD3\text{-}FITC^-$) after coincubation with T cells / mean number of viable tumor cells in the absence of T cells] × 100.

Quantitative real-time RT-PCR. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Random-primed cDNA was synthesized according to manufacturer's instructions (Roche, Indianapolis, IN). The PRAME 5′-TCACTTGTTGCCACGCACGTCTGA-3′ and housekeeping porphobilogen deaminase (PBGD) 5′-CTCATCTTTGGGCTGTT-TTCTTCCGCC-3′ probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) and labeled with reporter dye TET at the 5′ end and quencher dye TAMRA at 3′ end (Eurogentec, Seraing, Belgium). The forward PRAME primer 5′-GTCAACAGCAACTTCGCGGT-3′ was selected on the junction of exons 1 and 2 at position 94 bp (GeneID 23532), and the reverse PRAME primer 5′-AATGGAACCCCA-CAAACGC-3′ was selected on the junction of exons 3 and 4 at position 265 bp. The forward and reverse primers for PBGD (GeneID 3145) are 5′-GGCAATGCGGCTGCAA-3′ and 5′-GGGTACCCACGCGAATCAC-3′, respectively. PCR was done using the qPCR core kit (Eurogentec). Amplification was started with 10 minutes at 95°C followed by 55 cycles of 15 seconds at 95°C, 30 seconds at 65°C, and 30 seconds at 65°C. The 171-bp PRAME product was confirmed by DNA sequencing. PRAME expression was normalized to expression of the *PBGD* gene.

Results

In vitro ***expansion of PRAME-specific $CD8^+$ T cells.*** To investigate whether human individuals have circulating T cells specific for PRAME, total PBMCs from HLA-A*0201-subtyped healthy individuals and melanoma patients were stimulated *in vitro* with four previously identified HLA-A*0201-binding PRAME peptides (9) and tested for PRAME-specific T cells by IFN-γ ELISPOT assays and tetramer staining.

Of the four PRAME peptides, T cells specific for PRA$^{100\text{-}108}$ were most frequently found by ELISPOT and tetramer staining (Fig. 1; i.e., in 4 of 14 healthy individuals and 4 of 11 patients). None of the donors and patients had measurable T cells specific for PRA$^{142\text{-}151}$ (data not shown). T cells specific for PRA$^{300\text{-}309}$ were detected in one healthy individual and one patient by ELISPOT but not by tetramer staining (data not shown). T cells specific for PRA$^{425\text{-}433}$ were detected in two healthy individuals by tetramer staining but not in ELISPOT assays (data not shown). FLU$^{58\text{-}66}$-specific T cells were detected in 13 of 14 healthy individuals and 8 of 11 patients by ELISPOT and in two additional patients by tetramer staining (data not shown).

Ex vivo ***detection of PRA$^{100\text{-}108}$-specific CD8$^+$ T cells.*** To investigate the frequency and phenotype of PRA$^{100\text{-}108}$-specific T cells, total PBMCs (30×10^6) from the same healthy individuals and melanoma patients were stained with phycoerythrin-labeled PRA$^{100\text{-}108}$-tetramer and subsequently isolated by magnetic anti-phycoerythrin microbeads. By this method, FLU$^{58\text{-}66}$-tetramer$^+$ CD8$^+$ T cells were enriched ~200-fold (Fig. 2A). Of the FLU$^{58\text{-}66}$-specific T cells, 70% to 80% were CD45RA$^-$, indicating a memory phenotype. Figure 2B shows the results of all individuals with percentages of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells higher than the mean percentage + 3× SD as obtained in eight HLA-A*0201$^-$ individuals. PRA$^{100\text{-}108}$-specific CD8$^+$ T cells were detected directly *ex vivo* after isolation by magnetic microbeads in 4 of 14 healthy individuals and 4 of 11 patients. The percentages of CD8$^+$ cells that are PRA$^{100\text{-}108}$-tetramer$^+$ after isolation are 0.0 (HD1, 2, 4-6, 8-10 and P2, 8, 10), 0.1 (HD3, 12 and P4-6, 9) 0.5 (P7), 0.9 (HD14), 1.2 (P3), 1.3 (HD11), 1.5 (HD7), 1.8 (HD13), 4.3 (P11), and 6.5 (P1). The individuals with detectable PRA$^{100\text{-}108}$-tetramer$^+$ T cells *ex vivo* (Fig. 2B) were the same as those showing expansion of PRA$^{100\text{-}108}$-specific T cells after *in vitro* stimulation (Fig. 1). Variable numbers of memory (CD45RA$^-$) PRA$^{100\text{-}108}$-tetramer$^+$ T cells were found in two healthy individuals (37% in HD7 and 89% in HD14) and one patient (57% in P1). The majority of PRA$^{100\text{-}108}$-tetramer$^+$ T cells, however, expressed CD45RA, suggesting a naive phenotype. Frequencies ranged between 13 and 111 PRA$^{100\text{-}108}$-specific T cells isolated from 30×10^6 total PBMCs, indicating a low frequency of ~4 to 40 antigen-specific T cells in 1×10^6 CD8$^+$ cells.

Generation and isolation of PRA$^{100\text{-}108}$-specific CD8$^+$ T cell clones. Because PRAME and in particular the PRA$^{100\text{-}108}$ epitope are interesting targets for T-cell therapy, PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells were further investigated for their ability to recognize and lyse tumor cells. PRA$^{100\text{-}108}$-tetramer$^+$ T cells were induced *in vitro* by stimulating monocyte-depleted PBMCs from HD13 with autologous dendritic cells pulsed with PRA$^{100\text{-}108}$ peptide (Fig. 3). After two *in vitro* stimulations, PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells were sorted at 1 per well in the presence of irradiated feeder cells. Fourteen growing PRA$^{100\text{-}108}$-specific T-cell clones were isolated, and four clones (clones 3, 7, 33, and 34) were tested for specific cytokine release by Cytometric Bead Array. All clones expressed CD8, CD45RO, and TCRVβ7.1 but not CD27, CD28, and CCR7 (data not shown).

Figure 4A shows that PRA$^{100\text{-}108}$-tetramer$^+$ T-cell clones released significant levels of IL-4, IL-5, and IFN-γ and low

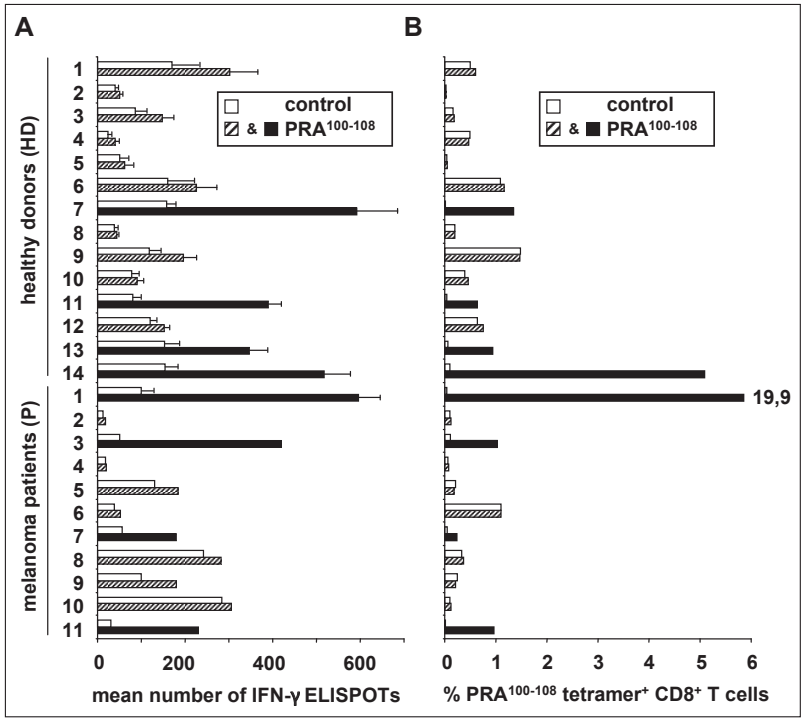

Fig. 1. Detection of PRA$^{100\text{-}108}$-specific CD8$^+$ T cells after *in vitro* stimulation. Total PBMCs from HLA-A*0201-subtyped healthy individuals and melanoma patients were stimulated *in vitro* with PRA$^{100\text{-}108}$ peptide. At day 13, PBMCs were tested for specific T cells by IFN-γ ELISPOT assays and tetramer staining. *A,* PRA$^{100\text{-}108}$-stimulated PBMCs were seeded at 5, 2.5, and 1×10^4 per well together with 5×10^4 nonpulsed (control) or PRA$^{100\text{-}108}$-pulsed T2 cells in triplicate wells in IFN-γ ELISPOT assays. Columns, mean numbers of spots produced by 10^5 peptide-stimulated PBMCs of triplicate wells; bars, SD. Solid columns are shown for mean numbers of specific spots higher than the mean number of background spots + 3× SD and $P < 0.05$ using the Student's *t* test for unpaired samples. *B,* PRA$^{100\text{-}108}$-stimulated PBMCs and PBMCs stimulated with other peptides (control = PRA$^{142\text{-}151}$, PRA$^{300\text{-}309}$, or PRA$^{425\text{-}433}$) were stained with anti-CD8 and PRA$^{100\text{-}108}$-tetramer. Percentages of CD8$^+$ cells that are PRA$^{100\text{-}108}$-tetramer$^+$. Solid columns are shown for PBMC cultures containing percentages of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells higher than 4× the percentages in PBMC cultures stimulated with control peptides.

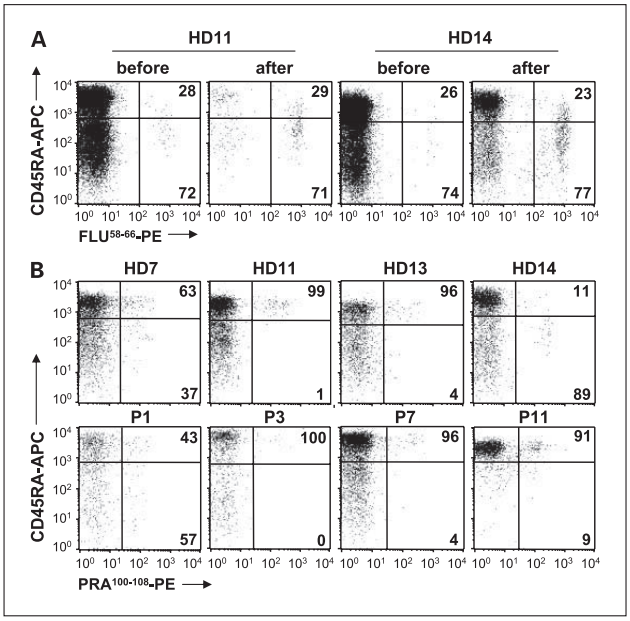

Fig. 2. *Ex vivo* detection of tetramer$^+$ CD8$^+$ T cells. *A,* total PBMCs (30×10^6) from two healthy individuals were stained with phycoerythrin-labeled FLU$^{58\text{-}66}$-tetramer and subsequently isolated by magnetic anti-phycoerythrin microbeads. Total PBMCs (2.5×10^5) before isolation and all PBMCs after isolation were stained with anti-CD8-FITC and anti-CD45RA-APC and analyzed by fluorescence-activated cell sorting. FLU$^{58\text{-}66}$-PE tetramer and anti-CD45RA-APC staining for gated viable (PI$^-$) CD8$^+$ cells. Percentages of FLU$^{58\text{-}66}$-tetramer$^+$ T cells that are CD45RA$^+$ and CD45RA$^-$. *B,* total PBMCs (30×10^6) from HLA-A*0201-subtyped healthy individuals and melanoma patients were stained with phycoerythrin-labeled PRA$^{100\text{-}108}$ tetramer and isolated by magnetic anti-phycoerythrin microbeads. All isolated cells were stained with anti-CD8-FITC and anti-CD45RA-APC and analyzed by fluorescence-activated cell sorting. PRA$^{100\text{-}108}$-PE tetramer and anti-CD45RA-APC staining for gated viable (PI$^-$) CD8$^+$ cells. Percentages of PRA$^{100\text{-}108}$-tetramer$^+$ T cells that are CD45RA$^+$ and CD45RA$^-$. For HD7, 11, 13, 14 and P1, 3, 7, 11 with percentages of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells higher than the mean percentage + 3× SD as obtained in eight HLA-A*0201$^-$ healthy donors ($0.06 + 3 \times 0.04 = 0.18$).

levels of IL-2, IL-10, and tumor necrosis factor-α upon incubation with breast carcinoma cell line MDA231 (HLA-A*0201$^+$, PRAME$^-$) pulsed with PRA$^{100\text{-}108}$ peptide or infected with an adenoviral vector encoding PRAME (data not shown). Nonpulsed MDA231 cells and MDA231 cells pulsed with control peptide (PRA$^{300\text{-}309}$) or empty adenoviral vector were not recognized. PRA$^{100\text{-}108}$-tetramer$^+$ T-cell clones also released cytokines upon incubation with K562 (MHC class I$^-$, PRAME$^+$) transduced with HLA-A*0201 (K562-A2), whereas K562 transduced with HLA-A*0301 (K562-A3) was not recognized. These results show that PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells recognize endogenously processed PRA$^{100\text{-}108}$ in the context of HLA-A*0201.

Specific cytokine release upon stimulation with PRA$^{100\text{-}108}$ peptide and K562-A2 cells was also shown for PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cell clones isolated from peptide-stimulated PBMCs from HD7 (five clones) and P3 (two clones) as well as PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells from HD14 tested immediately after isolation from peptide-stimulated PBMCs (data not shown). However, all T cells isolated from peptide-stimulated PBMCs, as shown in Fig. 1, failed to expand to sufficient numbers for detailed analysis.

The avidities of the PRA$^{100\text{-}108}$-tetramer$^+$ T cell clones were determined by titrating the PRA$^{100\text{-}108}$ peptide on HLA-A*0201$^+$ T2 cells (Fig. 4B). Specific cytokine release could be measured at 1 ng/mL of PRA$^{100\text{-}108}$ peptide. Moreover, at suboptimal peptide concentrations, PRA$^{100\text{-}108}$-specific T cell clones released more IL-4 and IL-5 than IFN-γ.

Recognition of melanoma and ALL cell lines by PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T-cell clones. Next, the PRA$^{100\text{-}108}$-tetramer$^+$ T cell clones were tested for recognition of a panel of melanoma and ALL cell lines. The T-cell clones released IL-4, IL-5, IFN-γ (Fig. 4C), and granzyme B (data not shown) upon incubation with HLA-A*0201$^+$ and PRAME$^+$ melanoma (453A0, FM6, 513D, 518A2, and FM3), but not ALL (L-ALL-BV and L-CML-B) cell lines. Negative control cell lines lacking expression of HLA-A*0201 (IGR39D and L-ALL-VG) or PRAME (MDA231 and L-ALL-RL) were not recognized. Finally, PRA$^{100\text{-}108}$-specific T cells not only recognized but also lysed HLA-A*0201$^+$ and PRAME$^+$ melanoma cell lines, as shown by reduced numbers of viable tumor cells after 48 hours of coincubation

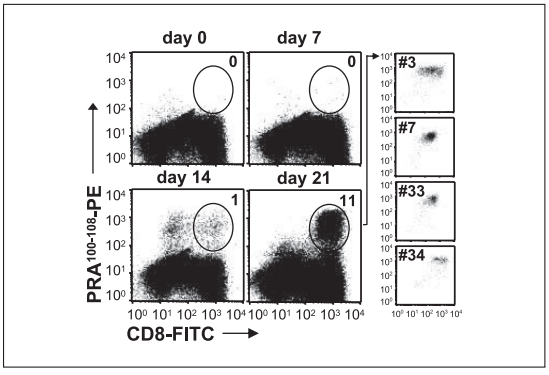

Fig. 3. Generation and isolation of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells. Dendritic cells from HD13 were pulsed with PRA$^{100\text{-}108}$ peptide and incubated with autologous nonadherent PBMCs at a ratio of 1:10. At days 7 and 14, T-cell cultures were restimulated with PRA$^{100\text{-}108}$-pulsed dendritic cells and analyzed for numbers of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells by fluorescence-activated cell sorting. Numbers indicate percentages of CD8$^+$ cells that are PRA$^{100\text{-}108}$-tetramer$^+$. At day 21, PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells were sorted at 1 per well. Fourteen growing T-cell clones, among which clones 3, 7, 33, and 34, were isolated.

with PRA$^{100\text{-}108}$-tetramer$^+$ T cells (Fig. 4D) and increased uptake of PI (data not shown).

PRAME mRNA expression by quantitative real-time RT-PCR. Total mRNA was isolated from melanoma and ALL cell lines to investigate PRAME expression levels by quantitative real-time RT-PCR (data not shown). All melanoma cell lines expressed high levels of PRAME mRNA (~10-fold lower than in K562). PRAME mRNA levels in ALL cell lines, however, were significantly lower (~100- to 1,000-fold) than in melanoma cell lines.

Discussion

The human PRAME protein is frequently expressed in tumors of various histologic origins. In this report, we investigated the usefulness of PRAME as target for anticancer T-cell therapy. HLA-A*0201-subtyped healthy individuals and advanced melanoma patients were screened for CD8$^+$ T cells directed against four previously identified PRAME epitopes (9). The results show that T cells specific for PRA$^{100\text{-}108}$ are most frequently found in 30% to 40% of healthy individuals and melanoma patients after *in vitro* stimulation. PRA$^{100\text{-}108}$-tetramer$^+$ T cells could not be detected directly *ex vivo* before isolation by magnetic microbeads due to the low frequency of these T cells in peripheral blood (~4 to 40 antigen-specific T cells in 1×10^6 CD8$^+$ cells). After isolation by magnetic microbeads, however, PRA$^{100\text{-}108}$-tetramer$^+$ T cells were found in the same individuals as after *in vitro* stimulation, showing that magnetic microbeads allow rapid *ex vivo* isolation and phenotyping of low-frequency tetramer$^+$ T cells.

Variable numbers of memory (CD45RA$^-$) PRA$^{100\text{-}108}$-tetramer$^+$ T cells were found in two healthy individuals and one melanoma patient. The majority of PRA$^{100\text{-}108}$-tetramer$^+$ T cells, however, expressed CD45RA, suggesting a naive phenotype. The presence of naive PRA$^{100\text{-}108}$-tetramer$^+$ T cells in melanoma patients indicates that these T cells have not been activated *in vivo* by PRAME-expressing tumor cells possibly due to lack of danger signals, defective dendritic cell function, or production of immunosuppressive factors (13–15). The presence of memory PRA$^{100\text{-}108}$-tetramer$^+$ T cells in a minority of individuals remains unclear. These T cells might have been activated *in vivo* by PRAME-expressing benign or malignant cells or by foreign antigen mimicry, as shown for the MART-1/Melan-A$^{27\text{-}35}$ peptide (16).

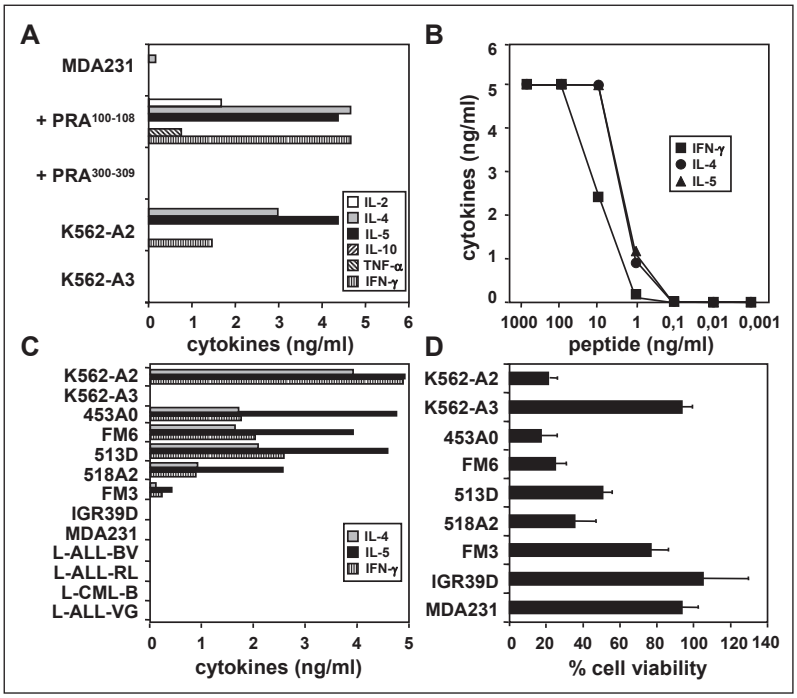

Fig. 4. Functional analysis of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T-cell clones. *A,* PRA$^{100\text{-}108}$-tetramer$^+$ T-cell clone 34 was incubated with breast carcinoma cell line MDA231 (PRAME$^-$, HLA-A*0201$^+$) pulsed with 10 μg/mL PRA$^{100\text{-}108}$ or control (PRA$^{300\text{-}309}$) peptide and with K562 (PRAME$^+$) cell lines expressing HLA-A*0201 (K562-A2) and HLA-A*0301 (K562-A3). After overnight incubation, cytokine release in supernatants was measured by human Th1/Th2 cytokine CBA. Similar results have been obtained with T-cell clones 3, 7, and 33 (data not shown). *B,* peptide sensitivity of T-cell clone 34 was tested by titrating PRA$^{100\text{-}108}$ peptide on T2 cells. After overnight incubation, cytokine release in supernatants was measured by Cytometric Bead Array. Similar results have been obtained for T-cell clone 3 (data not shown). *C,* T-cell clone 7 was incubated together with human tumor cell lines in triplicate wells. K562-A2; melanoma cell lines 453A0, FM6, 513D, 518A2, and FM3; and ALL cell lines L-ALL-BV and L-CML-B are PRAME$^+$ and HLA-A*0201$^+$. K562-A3, melanoma cell line IGR39D, and cell line L-ALL-VG are PRAME$^+$ and HLA-A*0201$^-$. Cell lines MDA231 and L-ALL-RL are PRAME$^-$ and HLA-A*0201$^+$. After overnight incubation, supernatants were analyzed for release of cytokines by Cytometric Bead Array. Similar results have been obtained for T-cell clone 3 (data not shown). *D,* after 48 hours of incubation, all cocultures, as described in (*C*), were collected, stained with anti-CD3-FITC, and analyzed for numbers of viable (PI$^-$) tumor cells by fluorescence-activated cell sorting. Columns, mean % cell viability = [number of viable tumor cells (PI$^-$ and CD3-FITC$^-$) after coincubation with T cells / mean number of viable tumor cells in the absence of T cells] × 100 (triplicate wells); bars, SD. Similar results have been obtained for T-cell clone 3 (data not shown).

Natural T-cell immunity against tumor cells seems to occur frequently (17), but the tumor antigens recognized are often unknown. MART-1/Melan-A-tetramer$^+$ T cells are readily detectable directly *ex vivo* in melanoma patients and healthy individuals due to the high frequency of these T cells in peripheral blood ($\sim$400 to 4,000 antigen-specific T cells in 1×10^6 CD8$^+$ cells). These MART-1/Melan-A-specific T cells have been shown to display a memory or naive phenotype (18, 19). It has been suggested that MART-1/Melan-A-specific T cells with a memory phenotype are induced *in vivo* upon tumor progression due to the presence of high antigen load (20). At that stage, the antigen-specific T cells apparently fail to counter tumor progression; therefore, therapies boosting naturally acquired antitumor responses are not expected to be very effective. Because circulating PRA$^{100\text{-}108}$-specific T cells display a naive phenotype in most patients, *in vivo* immunization with peptides covering the PRA$^{100\text{-}108}$ epitope may be an effective strategy to stimulate antitumor T-cell reactivity.

To investigate whether PRA$^{100\text{-}108}$-tetramer$^+$ T cells are able to recognize and lyse tumor cells, PRA$^{100\text{-}108}$-specific T cells were induced *in vitro* by stimulating PBMCs from HD13 with peptide-pulsed dendritic cells. All T-cell clones released type 1 (IFN-γ) and type 2 (IL-4 and IL-5) cytokines. This mixed Tc1/Tc2 cytokine pattern may be associated with the naive phenotype of these T cells in nonstimulated PBMCs from HD13, because PRA$^{100\text{-}108}$-specific T cells from HD14, displaying a memory phenotype in nonstimulated PBMCs, were shown to have a more Tc1 cytokine profile, predominantly releasing IFN-γ and tumor necrosis factor-α (data not shown).

All PRA$^{100\text{-}108}$-specific T-cell clones recognized HLA-A*0201$^+$ and PRAME$^+$ melanoma but not ALL cell lines. Quantitative real-time RT-PCR showed significantly lower ($\sim$100- to 1,000-fold) PRAME mRNA levels in ALL than in melanoma cell lines. These results do not support previous studies showing high levels of PRAME expression in ALL (21, 22). A possible explanation for this discrepancy may be the use of different primer pairs in PCR reactions. A 171-bp PRAME fragment from positions 94 to 265 bp was amplified by our real-time RT-PCR, whereas others amplified a 560-bp fragment from positions 662 to 1,222 bp (21, 22). Five PRAME mRNA variants have been described, all encoding the same PRAME protein. Two of these variants have an alternative exon 1 and may have been missed in our RT-PCR reactions due to partial homology to the forward primer. It can be speculated that high PRAME mRNA expression in ALL is caused by selective overexpression of these two PRAME variants. The differential recognition of melanoma and ALL cell lines by our PRA$^{100\text{-}108}$-specific CD8$^+$ T cell clones, however, makes this possibility unlikely and supports the finding that PRAME mRNA expression is significantly lower in ALL than in melanoma cell lines.

In standard 4-hour ^{51}Cr-release assays, we consistently observed low specific lysis of K562-A2 (10-20%) versus K562-A3 (0%) by PRA$^{100\text{-}108}$-tetramer$^+$ T cells (data not shown), whereas these cells were lysed up to 80% after 2 days of coincubation. Similarly, with the exception of cell line 453A0, no specific lysis of melanoma cell lines could be measured in ^{51}Cr-release assays, whereas all HLA-A*0201$^+$ and PRAME$^+$ melanoma cell lines were recognized in Cytometric Bead Array assays and killed after 2 days of coincubation with PRA$^{100\text{-}108}$-tetramer$^+$ T cells. The slow killing mechanism might be attributed to release of low levels of granzyme B and perforin. Because the T-cell clones clearly recognise HLA-A*0201$^+$ and PRAME$^+$ tumor cell lines in cytokine release assays, indicating that the affinity of the TCR is sufficiently high to recognize endogenously processed PRAME, transfer of the PRA$^{100\text{-}108}$-specific TCR to CD8$^+$ T cells with more powerful cytolytic machineries might be an effective strategy to generate potent tumor-specific T cells.

Acknowledgments

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CHAPTER 4

CHAPTER 4

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Competition-Based Cellular Peptide Binding Assays for 13 Prevalent HLA Class I Alleles Using Fluorescein-Labeled Synthetic Peptides

Jan H. Kessler, Bregje Mommaas, Tuna Mutis, Ivo Huijbers, Debby Vissers, Willemien E. Benckhuijsen, Geziena M. Th. Schreuder, Rienk Offringa, Els Goulmy, Cornelis J. M. Melief, Sjoerd H. van der Burg, and Jan W. Drijfhout

ABSTRACT: We report the development, validation, and application of competition-based peptide binding assays for 13 prevalent human leukocyte antigen (HLA) class I alleles. The assays are based on peptide binding to HLA molecules on living cells carrying the particular allele. Competition for binding between the test peptide of interest and a fluorescein-labeled HLA class I binding peptide is used as read out. The use of cell membrane–bound HLA class I molecules circumvents the need for laborious biochemical purification of these molecules in soluble form. Previously, we have applied this principle for HLA-A2 and HLA-A3. We now describe the assays for HLA-A1, HLA-A11, HLA-A24, HLA-A68, HLA-B7, HLA-B8, HLA-B14, HLA-B35, HLA-B60, HLA-B61, and HLA-B62. Together with HLA-A2 and HLA-A3, these alleles cover more than 95% of the Caucasian population. Several allele-specific parameters were determined for each assay. Using these assays, we identified novel HLA class I high-affinity binding peptides from HIVpol, p53, PRAME, and minor histocompatibility antigen HA-1. Thus these convenient and accurate peptide-binding assays will be useful for the identification of putative cytotoxic T lymphocyte epitopes presented on a diverse array of HLA class I molecules. *Human Immunology 64, 245–255 (2003).* © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Science Inc.

KEYWORDS: peptide binding; HLA class I; MHC class I; fluorescent peptide; cellular peptide binding assay; competition

ABBREVIATIONS

HLA	human leukocyte antigen
aa	amino acid
β_2M	β_2-microglobulin
Fl-labeled	fluorescein-labeled
B-LCL	B-lymphoblastoid cell line
FCS	fetal calf serum
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
MF	mean fluorescence

INTRODUCTION

The identification of human leukocyte antigen (HLA)-restricted cytotoxic T lymphocyte (CTL) epitopes is crucial for our understanding of immunity in bacterial or viral infections, autoimmune diseases, and cancer as well as for the development of defined vaccines that induce CTL and the monitoring of such immunotherapies. The peptide-binding based prediction of CTL epitopes in protein sequences has led to the identification of CTL epitopes in viral proteins [1,2], bacterial proteins [3], and tumor antigens [4]. Often, these CTL epitopes are presented in HLA-A2, being the class I allele that predominates in the

From the Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands.

Jan H. Kessler and Bregje Mommaas contributed equally to this study.

Address reprint requests to: Dr. Jan W. Drijfhout, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Building 1: E3-Q, P.O. Box 9600, 2300 RC Leiden, The Netherlands; Tel: (31) 71-5261737; Fax: (31) 71-5216751; E-mail: J.W.Drijfhout@lumc.nl.

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Caucasian population. However, an ongoing search for new CTL epitopes restricted by other prevalent HLA class I molecules is necessary for the development of immunotherapies covering all class I haplotypes and multi-epitope vaccines (Table 1).

The peptide-binding groove of HLA molecules contains highly polymorphic allele-specific pockets that accommodate side chains of the so-called anchor residues of the bound peptide [5, 6]. The peptide-binding groove of HLA class I molecules is closed at both sides [6] and thus HLA class I accommodates peptides with a length of 8–11 amino acids. Allele-specific peptide-binding motifs were defined by the analysis of naturally presented peptide pools eluted from class I molecules [7, 8]. Each HLA class I molecule displays a preference for certain aa at the major (primary) peptide anchor positions (relative position 2 and the C-terminus for most HLA class I molecules) that bind in the binding pockets. Amino acids at other positions in the peptide can significantly contribute to binding by their engagement in secondary pockets [9–16]. The knowledge of allele-specific peptide-binding motifs has led to the development of peptide-binding prediction algorithms by several groups [17–19]. Although these algorithms are extremely helpful to select potential HLA class I–binding peptides, experimental determination of the HLA class I–binding capacity is still considered necessary because of the partly undefined contributions to binding of each possible aa in every position of the peptide.

Peptide-HLA class I–binding assays employ either cell-bound class I molecules [20–28] or solubilized class I molecules [29–34]. Assays using cell-bound HLA class I molecules are either based on upregulation of class I molecules in processing defective cell lines [22, 23, 26] or on reconstitution of HLA class I molecules [24, 25, 27, 28]. Cell-free assays are quantitative and are based on competition for binding between a labeled reference peptide and a test peptide [32]. We previously applied the competition principle in easy-to-perform cell-bound HLA class I–binding assays for HLA-A2 and HLA-A3 [27]. In these assays, Epstein-Barr virus (EBV)-transformed B cell lines (B-LCLs) expressing the class I allele of interest are used, from which naturally bound class I peptides are eluted to obtain free class I molecules. Subsequently, B-LCLs are incubated with a mixture of a fluorescein (Fl)-labeled reference peptide, known to bind efficiently to the allele of interest, and titrated amounts of a competing test peptide. Cell-bound fluorescence is determined by flow cytometry and the inhibition of binding of the Fl-reference peptide is calculated as readout. We now report the adoption of this principle for an additional set of highly prevalent HLA class I alleles (HLA-A1, -A11, -A24, -A68, -B7, -B8, -B14, -B35, -B60, -B61, and -B62). Together with HLA-A2 and HLA-A3, these alleles cover more than 95% of the Caucasian populations. For each assay, the following allele-specific parameters were established: (1) a suitable reference peptide with known binding capacity for the allele; (2) the optimal position of the Fl-label in the reference peptide; (3) the required concentration of the labeled peptide; (4) the pH of the elution buffer used for acid stripping of class I molecules; (5) a B-LCL expressing the HLA class I molecule of interest; and (6) exclusion of binding of Fl-reference peptide to coexpressed class I molecules on the used B-LCL. The assays were used to identify several HLA class I–binding peptides derived from HIV-1pol, p53, PRAME, and minor histocompatibility antigen (mHag) HA-1. Finally, we analyzed the predictive power of a commonly used peptide-binding prediction algorithm for a set of HLA-A2–binding peptides to assess the need to actually assay the peptide-binding affinity after prediction of binding.

TABLE 1 Phenotype frequency distribution of HLA-I antigens for which assays were developed expressed as percentages among major populations[a]

HLA class I	Population			
	Black	Caucasoid	Asian	Amerindian
A1	9	26	7	11
A2	29	44	47	43
A3	13	22	6	8
A11	3	13	30	4
A24	6	20	42	52
A68	18	8	3	12
B7	15	17	7	5
B8	9	14	3	2
B14	7	6	1	3
B35	11	20	10	32
B60	1	6	17	5
B61	0	6	9	23
B62	2	8	16	21

[a] Phenotype frequencies for the HLA antigens have been deduced using the gene frequencies as given by Marsh *et al.* [38]).

MATERIALS AND METHODS

Cell Lines

The EBV-transformed B-LCL used for the binding assays were either obtained from the international histocompatibility workshop cell line repository or newly generated from peripheral blood mononuclear cells (PBMC) of healthy blood donors. All B-LCLs were cultured in complete culture medium consisting of IMDM (Biowhittaker, Verviers, Belgium) supplemented with 8% fetal calf serum (FCS) (Gibco BRL, Breda, The Netherlands), 100 IU/ml penicillin, and 2 mM L-glutamine.

TABLE 2 Allele-specific characteristics of HLA class I binding assays

HLA class allele[a]	Reference peptides used in the assays				Assay cell line		pH[e]
	Fl-labeled seq.[b]	[Fl-pep.]	Original seq.	Ref.[c]	Name	HLA class I type	
A1 (A*0l01)	YLEPAC (F1)AKY	150nM	YLEPAIAKY	32	CAA	A*0101, B*0801, CW*0701	3.1
A2 (A*0201)[d]	FLPSDC(F1)FPSV	150nM	FLPSDFFPSV	39	JY	A*0201, B*0702, CW*0702	3.2
A3 (A*0301)[d]	KVFPC(F1)ALINK	150nM	KVFPYALINK	32	EKR	A*0301, B*0702, Cw*0702	2.9
A11 (A*1101)	KVFPC(F1)ALINK	150nM	KVFPYALINK	32	BVR	A*1101, B*3501, Cw*0401	3.1
A24 (A*2402)	RYLKC(F1)QQLL	150nM	RYLKDQQLL	40	Vijf	A*2401, B*0702, Cw*0702	3.1
A68 (A*6801)	KTGGPIC(F1)KR	150nM	KTGGPIYKR	41	A68HI	A*6801, B*4402, Cw*0704	3.1
B7 (B*0702)	APAPAPC(F1)WPL	150nM	APAPAPSWPL	NP	JY	A*0201, B*0702, Cw*0702	3.1
B8 (B*0801)	FLRGRAC(F1)GL	50nM	FLRGRAYGL	42	Vavy	A*0101, B*0801, Cw*0701	3.1
B14 (B*1402)	DRYIHAC(F1)LL	150nM	DRYIHAVLL	43	CHE	A*2402, A*3301, B*1402, Cw*0201	4.0
B35 (B*3501)	NPDIVC(F1)YQY	150nM	NPDIVIYQY	44	BVR	A*1101, B*3501, Cw*0401	2.9
B60 (B*4001)	KESTC(F1)HLVL	125nM	KESTLHLVL	36	DKB	A*2402, B*4001, Cw*0304	3.1
B61 (B*4002)	GEFGGC(F1)GSV	50nM	GEFGGFGSV	36	Swei007	A*2902, B*4002, Cw*0202	3.1
B62 (B*1501)	YLGEFSC(F1)TY	150nM	YLGEFSITY	36	BSM	A*0201, B*1501, Cw*0304	2.9

[a] HLA class I allele of binding assay. Mostly, B-LCLs were used expressing the most common subtype of the allele (see HLA class I type).

[b] A nonanchor residue was substituted with a cysteine derivatized by a fluorescein group, denoted as C(F1).

[c] Most reference peptides were derived from the SYFPHEITI database (see ref. 19); here the original reference is cited. For HLA-B7, APAPAPSWPL (human p53 84-93) was found as high affinity binder in a molecular binding assay. NP = no published.

[d] Characteristics of HLA-A2 and HLA-A3 binding assays have been published before (ref. 27).

[e] Optimal pH of the elution buffer used for stripping naturally bound peptides.

Peptides

Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Syro II, Multi-Syntech, Witten, Germany) using Fmoc-chemistry. Peptides were analyzed by reversed-phase high performance liquid chromatography (HPLC) and mass spectrometry, dissolved in 50 μl dimethyl sulfoxide, diluted in 0.9% NaCl to a peptide concentration of 1 mM and stored at −20 °C until use. Fl-labeled reference peptides were synthesized as Cys-derivative. Labeling was performed with 5-(iodoacetamido)fluorescein (Fluka Chemie AG, Buchs, Switzerland) at pH 7.5 (Na-phosphate in water/acetonitrile 1:1 vol/vol). The labeled peptides were desalted over Sephadex G-10 and further purified by C18 RP-HPLC. Labeled peptides were analyzed by mass spectrometry.

Selection of Test Peptides for Binding Assays

Peptides derived from HIV-1pol, p53, PRAME, and mHag HA-1 that contain HLA class I peptide binding motifs were selected using either the BIMAS peptide-binding algorithm available via the Internet (http://bimas.cit.nih.gov/molbio/hla_bind/) [18] or an algorithm that was developed in our department [17].

Mild Acid Elution of HLA Class I–bound Peptides on B-LCL

Mild acid treatment of B-LCL to remove naturally HLA class I bound peptides was performed with minor modifications according to the principle first described by Sugawara *et al*. [35] and elaborated by our group [27]. B-LCLs were harvested and washed in phosphate buffered saline (PBS) and the pellet (2–15 × 10^6 cells) was put on ice for 5 minutes. The elution was performed by incubating the cells for exactly 90 seconds in ice-cold citric acid buffer (1:1 mixture of 0.263 M citric acid and 0.123 M Na_2HPO_4, adjusted to the pH listed in Table 2). Immediately thereafter, cells were buffered with ice-cold IMDM containing 2% FCS, washed once more in the same medium, and resuspended at a concentration of 4 × 10^5 cells/ml in IMDM medium containing 2% FCS and 2 μg/ml human β_2-microglobulin (β_2M) (Sigma, St. Louis, MO, USA).

HLA Class I Competition Binding Assays

Eight serial twofold dilutions of each competitor test peptide in PBS/BSA 0.5% were made (highest concentration 600 μM, sixfold assay concentration). In the assay, test peptides were tested from 100 μM to 0.8 μM. The Fl-labeled reference peptide was dissolved in PBS/BSA 0.5% at sixfold final assay concentration (see Table 2). In a well of a 96-well V-bottom plate, 25 μl of competitor peptide was mixed with 25 μl Fl-labeled reference peptide. Subsequently, the stripped B-LCLs were added at 4 × 10^4/well in 100 μl/well. After incubation for 24 h at 4 °C, cells were washed three times in PBS containing 1% BSA, fixed with 0.5% paraformaldehyde, and analyzed with FACScan flow cytometry (Becton Dickinson, San Jose, CA, USA) to measure the mean fluorescence (MF). The percentage inhibition of

Fl-labeled reference peptide binding was calculated using the following formula:

$$(1-(MF_{\text{reference + competitor peptide}} - MF_{\text{background}})/$$
$$(MF_{\text{reference peptide}} - MF_{\text{background}})) \times 100\%$$

The binding affinity of competitor peptide is expressed as the concentration that inhibits 50% binding of the Fl-labeled reference peptide (IC_{50}). IC_{50} was calculated applying nonlinear regression analysis (with software CurveExpert 1.3, SPSS Science Software, Erkrath, Germany). An $IC_{50} \leq 5$ μM was considered high-affinity binding, 5 μM $< IC_{50} \leq 15$ μM was considered intermediate-affinity binding, 15 μM $< IC_{50} \leq 100$ μM was judged low-affinity binding, and $IC_{50} > 100$ μM was regarded as no binding. These IC_{50} cutoff values are based on our experience with HLA-A2 and HLA-A3 binding ligands and CTL epitopes [27].

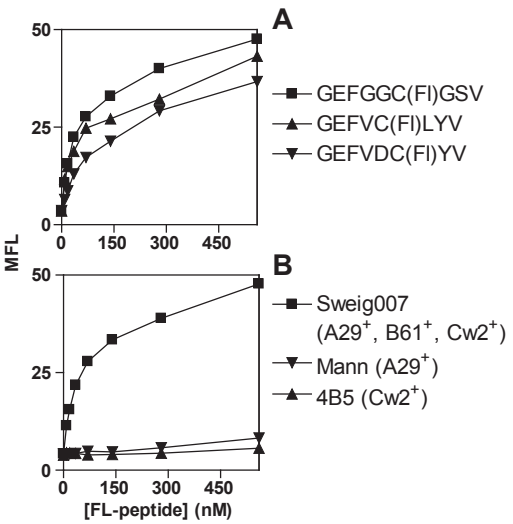

FIGURE 1 Determination of the optimal fluorescein (Fl)-labeled HLA-B61 binding reference peptide and exclusion of binding of the Fl-labeled peptide to alleles other than HLA-B61 that are expressed on B-LCL Swei007 (HLA I type: HLA-A29, -B61, and -Cw2). (A) Binding affinities of 3 Fl-labeled HLA-B61–binding reference peptides. The peptides were titrated at the indicated concentrations on B-LCL Swei007. After incubation for 24 hours at 4 °C fluorescence was measured with flow cytometry. (B) Exclusion of binding of the Fl-labeled reference peptide GEFGGC(Fl)GSV for HLA-B61 to coexpressed alleles on Swei007. The Fl-reference peptide was incubated for 24 hours at 4 °C with B-LCLs Swei007, Man (expressing HLA-A29), and 4B5 (expressing HLA-Cw2) and fluorescence was measured with flow cytometry at a FACScan. Results of one representative experiment of at least three performed are shown.

RESULTS

Selection of HLA Class I Alleles

The HLA-A2 and HLA-A3 alleles cover approximately 70% of the Caucasian population [27]. To enlarge the haplotype coverage, we chose to develop binding assays for 11 additional alleles (HLA-A1, -A11, -A24, -A68, -B7, -B8, -B14, -B35, -B60, -B61, and -B62) with high prevalence among most populations (Table 1). Together with HLA-A2 and HLA-A3, these alleles cover more than 95% of the Caucasian population, as calculated from a group of 1000 HLA-typed Dutch blood donors.

Selection of Optimal Fl-Labeled Reference Peptides

For each allele, one or two peptides to be used as Fl-labeled reference were derived from aa sequences shown to bind strongly to the particular allele. These peptides were originally identified as naturally presented class I ligand, CTL epitope, or consensus sequence, with the exception of the peptide for HLA-B7 (Table 2). For each peptide, several labeled variants were made by substituting at various positions a nonanchor residue for an Fl-conjugated cysteine. Fl-labeled reference peptides were titrated on B-LCL homozygously expressing the class I molecule of interest to identify for each allele the one that best retained its high binding capacity and to determine an optimal concentration of the Fl-labeled peptide to be used in the competition assay. As exemplified for HLA-B61, two peptides were selected: GEFGGFGSV (histone acetyltransferase 127-135 [36]), of which the phenylalanine at position 6 was substituted, rendering GEFGGC(Fl)GSV and GEFVDLYV (ribosomal protein S21 6-13 [36]), of which both GEFVC(Fl)LYV and GEFVDC(Fl)YV were tested (Figure 1A). Differences in binding capacity occurred depending on which original sequence was used and the particular residue that was substituted. The difference in binding capacity between the two variants of GEFVDLYV can be explained by altered contribution to overall binding affinity of the Fl-conjugated cysteine as compared with the original residue depending on the residue substituted and its position. The Fl-labeled reference peptide GEFGGC-(Fl)GSV, displaying highest binding capacity, was chosen as the labeled reference peptide for the assay (Figure 1A and Table 2). Optimal Fl-labeled reference peptides for the other alleles were likewise determined (Table 2). For each Fl-labeled peptide suboptimal saturating concentrations were used in the assay to optimally enable competition by the test peptides (Table 2). The maximal binding of Fl-labeled reference peptides at the chosen concentration after 24 h incubation at 4 °C resulted in a

MF of at least five times the background staining with PBS, as shown for HLA-B61 in Figures 1A and 1B.

Selection of HLA Class I Expressing Cell Lines

B-LCL homozygously expressing the allele of interest were used for the assays (Table 2). Control B-LCLs were tested to exclude binding of the Fl-labeled reference peptide to coexpressed class I molecules. As exemplified for the HLA-B61 binding assay, the Fl-labeled reference peptide GEFGGC(Fl)GSV did efficiently bind to B-LCL Sweig007 (HLA-A29, -B61, -Cw2), whereas binding to control B-LCL Mann and 4B5 expressing HLA-A29 and HLA-Cw2 respectively was absent (Figure 1B). This indicates that binding of the Fl-reference peptide to HLA-A29 and HLA-Cw2 can be excluded, and binding on Sweig007 was exclusively accomplished via binding to HLA-B61. B-LCL functioning optimally in assays for binding to HLA-A1, -A11, -A24, -A68, -B7, -B8, -B14, -B35, -B60, and -B62 were likewise found as listed in Table 2.

Determination of the Optimal Elution pH for Each Allele

We previously observed differences in the pH required for optimal elution of naturally bound peptides from HLA-A2 and HLA-A3 [27, 28]. Therefore, several pHs were tested for each new allele to find optimal conditions for removal of endogenous peptides, enabling efficient reconstitution of HLA class I–peptide complexes. For 8 of the 11 alleles for which novel assays were developed, elution at pH 3.1 produced the best results. However, for HLA-B14, -B35, and -B62, a different pH was chosen. For instance, elution of naturally presented peptides in HLA-B35 at pH 2.0 resulted in a considerable higher level of Fl-labeled reference peptide-binding than application of pH 2.4–4.0 (Figure 2). However, at pH lower than 2.8 cell viability decreased dramatically. We therefore chose pH 2.9 as an optimal compromise between these two phenomena. The optimal pH for every allele is listed in Table 2.

Optimization and Validation of the Competition Assays

A general improvement of the assay protocol was realized, compared with the published protocol [27], by adding FCS during incubation. Addition of 2% FCS improved binding of the Fl-labeled reference peptide (Figure 3) and increased cell viability from 30% to 90% after 24 hours (data not shown), which greatly enhanced cell recovery for FACS sample preparation. To validate each assay, the nonlabeled reference peptide or another positive control peptide, known from literature to be either a naturally presented ligand or CTL epitope, were tested in eight serial twofold dilutions

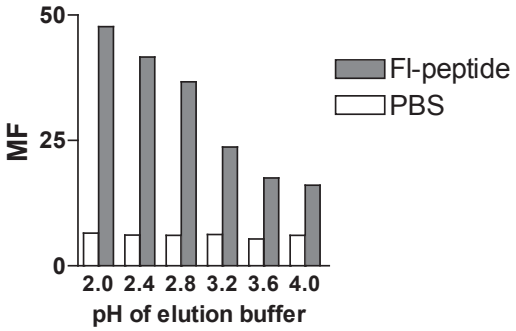

FIGURE 2 Determination of optimal pH of the elution buffer for HLA-B35. The elution buffer was adjusted to the various pHs and elution of naturally presented peptides from the surface of B-LCL BVR was performed at the indicated pHs as described in Material and Methods. Subsequently, B-LCL BVR was incubated with the HLA-B35 binding Fl-labeled reference peptide NPDIVC(Fl)YQY for 24 hours at 4 °C and fluorescence was measured with flow cytometry at a FACScan. Results of one representative experiment of at least three performed are shown.

(100–0.8 μM) for competition with the Fl-labeled peptide. The competition of binding of the HLA-B60 Fl-labeled reference peptide by the nonlabeled reference peptide KESTLHLVL is shown in Figure 4 as an example. Unmodified reference peptides and other positive control peptides were able to inhibit at least 50% of binding of the Fl-labeled reference peptide at concentrations lower than 5 μM ($IC_{50} < 5$ μM) (Table 3).

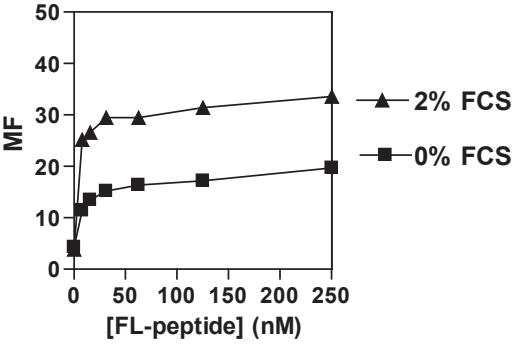

FIGURE 3 Influence of incubation without or with addition of 2% FCS on binding of the fluorescein (Fl)-labeled reference peptide. B-LCL JY expressing HLA-A*0201 was incubated with titrated amounts of the HLA-A2–binding Fl-labeled reference peptide FLPSDC(Fl)FPSV for 24 hours at 4 °C without or with addition of 2% FCS in the medium. Subsequently, fluorescence was measured with flow cytometry at a FACScan. Results of one representative experiment of at least three performed are shown.

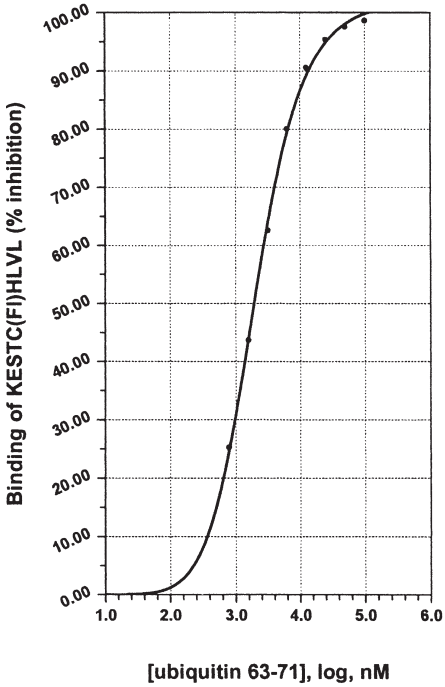

FIGURE 4 Competition of binding of the HLA-B60 fluorescein (Fl)-labeled reference peptide KESTC(Fl)HLVL by the nonlabeled original aa sequence KESTLHLVL to validate the HLA-B60 assay. The unlabeled peptide was titrated in 8 serial twofold dilutions (100 μM–0.8 μM) on B-LCL DKB (HLA-B60^{+}) together with the Fl-labeled peptide (125 nM) and was incubated for 24 hours at 4 °C. Fluorescence was measured with flow cytometry at a FACScan and the data were analyzed by regression analysis using software program CurveExpert 1.3 to determine the precise IC_{50} value expressed at a logarithmic scale. Results of one representative experiment of at least three performed are shown.

These results are in line with those obtained with high-affinity binding positive-control peptides in the published binding assays for HLA-A2 and HLA-A3 [27].

Identification of Novel HLA Class I Binding Peptides

The binding assays described herein were used for the identification of novel HLA class I–binding peptides derived from various protein sequences (HIV-1pol, p53, PRAME, mHag HA-1). For several alleles, candidate class I binding peptides were selected complying with the different HLA class I–binding motifs of interest, and their binding capacity was assessed. For these alleles, we successfully identified peptides binding with high or intermediate affinity (Table 4). Four peptides of HIV-1pol were found to bind with high affinity in HLA-A11 ($IC_{50} \leq 5$ μM), whereas one peptide displayed intermediate affinity (5 μM $< IC_{50} \leq 15$ μM). In HLA-A24, three peptides from HIV-1pol bound with high affinity, one peptide with intermediate affinity, and two with low affinity (15 μM $< IC_{50} \leq 100$ μM). Six peptides of p53 displayed high-affinity binding to HLA-B7. Seven of eight peptides derived from PRAME, predicted to bind in HLA-B35, displayed high binding affinity for this allele. Furthermore, we found four peptides of mHag HA-1 that bound with high affinity in HLA-B60 [37]. For the other alleles as well, several high-affinity binding peptides (derived from PRAME and BCR-ABL) were successfully identified by applying the present binding assays (manuscript in preparation). In summary, in all assays, peptides could be classified in the range from high-affinity binding to no observable binding affinity.

Correlation Between Peptide Binding Prediction and Peptide Binding Capacity

Although peptide binding prediction algorithms are extremely useful to select potential HLA class I–binding peptides, the currently prevailing view is that these predictions are not accurate enough to bypass binding measurements. We chose to analyze the binding prediction for HLA-A2 (-A*0201), because a refined binding motif is known for this extensively studied allele [10, 12, 18]. Previously, we identified 19 high- and 27 intermediate-affinity HLA-A2 binding peptides of tumor antigen PRAME (length 509 aa) of 65 nona- and 63 decamers selected [4] by using the BIMAS peptide-binding prediction algorithm [18]. Analysis of the data revealed that a relatively low prediction score did not necessarily exclude high-affinity binding. Examples of this group of peptides were decamers SLYSFPEPEA (PRAME 142-151) and FLKEGACDEL (PRAME 182-191) that ranked 35th and 46th in binding prediction for HLA-A2 (BIMAS algorithm), respectively (data not shown). Despite these low scores, SLYSFPEPEA bound second best (IC_{50} 1.9 μM), and FLKEGACDEL bound with high affinity as well (IC_{50}3 μM, ranking fifth for binding) [4]. Low prediction scores in these cases were caused by the lack of a canonical C-terminal anchor in SLYSFPEPEA and residues with a predicted deleterious effect on binding (E at P7 for SLYSFPEPEA and K at P3 for FLKEGACDEL). Conversely, a high prediction score for HLA-A2 did not necessarily correlate with high-affinity binding. Fifty percent of the predicted 16 best binding 9-mers and 18.7% of the 10-mers from the analogous group displayed only low or no binding affinity at all (Table 5). For instance, nonamer KMILKMVQL (PRAME 224-232) that ranked fifth in binding prediction for HLA-A2 actually failed to bind ($IC_{50} >$

TABLE 3 Positive control peptides used to validate binding assays

HLA class I allele	Positive control peptides[a]		
	Sequence	Source (ref.)	IC_{50} (μM)
HLA-A1	YLEPAIAKY	Consensus sequence (32)	
HLA-A2	FLPSDFFPSV	HBV cAg 18-27 (39)	0.5
	YIGEVLVSV	mHag HA-2 (45)	3.5
HLA-A3	KVFPCALINK	Consensus sequence (32)	0.7
	QVPLRPMTYK	HIV-1nef 73-82 (46)	0.2
HLA-A11	QVPLRPMTYK	HIV-1nef 73-82 (46)	2.0
	KQSSKALQR	BCR-ABL b3a2 (47)	5.7
HLA-A24	RYLKDQQLL	HIV-1env gp41 583-591 (40)	1.8
	AYIDNYKF	Consensus sequence (48)	0.6
HLA-A68	KTGGPIYKR	Influenza A NP 91-99 (41)	1.3
HLA-B7	APAPAPSWPL	Human p53 84-93 (not published)	0.5
	SPSVDKARAEL	Human SMCY 950-960 (mHag HY) (49)	0.7
HLA-B8	FLRGRAYGL	EBNA-3 339-347 (42)	0.2
	GFKQSSKAL	BCR-ABL b3a2 (47)	1.5
HLA-B14	ERYLKDQQL	HIV-1env gp41 584-592 (50)	7.5
HLA-B35	NPDIVIYQY	HIV-1 RT 330-338 (44)	1.2
HLA-B60	KESTLHLVL	Ubiquitin 63-71 (36)	1.9
HLA-B61	GEFGGFGSV	Histone acetyltransferase 127-135 (36)	0.2
	GEFVDLYV	40S ribosomal protein S21 6-13 (36)	0.3
HLA-B62	YLGEFSITY	40S ribosomal protein S15 114-122 (36)	0.6

[a] The unlabeled reference peptides were used as positive control peptide for all alleles except for HLA-A11 and -B13. For several alleles, additional positive control peptides were tested.

HLA = human leukocyte antigen.

100 μM) [4]. A possible explanation is that the strong deleterious effect on binding of glutamine at position 9 of the peptide [18] may also results from this aa in position 8 [12], but is not incorporated in the binding prediction score [18]. Taken together, we conclude that binding prediction for this particular set of peptides did not accurately correlate with binding affinities, confirming the need for actual peptide-binding assays.

DISCUSSION

Measurement of peptide HLA class I–binding affinity can be exploited for the identification of HLA class I–presented epitopes as is needed for, *e.g.*, vaccine development and insight in autoimmunity and graft-versus-host reactions. For these purposes especially HLA class I molecules with a prevalent distribution among different human populations are of interest (Table 1). The current report presents a concise summary of binding assays that were developed for 13 highly prevalent HLA class I molecules according to a competition-based strategy that uses a Fl-labeled class I–binding reference peptide and cell surface expressed HLA class I molecules. This type of binding assay has several advantages over molecular HLA class I–binding assays.

First, the assays are rapid and convenient, because there is no need for time-consuming production and purification of soluble HLA class I molecules. Furthermore, the readout is not dependent on either radioactive peptide labeling or conformation specific antibodies, of which the latter are not available for every allele, but instead on Fl-labeled reference peptides, the synthesis of which is straightforward. Finally, as equipment, a flow cytometer suffices.

We show that the concept of the assay can be adapted for basically every HLA class I allele of interest. Therefore, the present report can also be read as an instruction for the development of class I–binding assays that are still lacking. Several important allele-specific features need to be determined for each allele. Differences in binding capacity of the Fl-labeled reference peptides were observed depending on which residue was substituted for a Fl-labeled cysteine (Figure 1A). However, when a proper nonanchor residue was chosen for substitution (Figure 1A), the substitution did not appear detrimental for binding. Exceptionally, we met problems in finding a suitable Fl-labeled reference peptide. For example, we did not succeed thus far in obtaining a sufficiently binding Fl-labeled peptide for HLA-B44 (data not shown). We used B-LCL homozygously expressing HLA class I molecules of interest because B-LCLs are broadly available in the scientific community and can easily be generated from PBMC. The required exclusion of binding of the FL-labeled reference peptide to coexpressed alleles was accomplished with the use of properly selected nega-

TABLE 4 Identified HLA class I binding peptides

Allele	Sequence	Source	IC_{50} (μM)[a]
		HIV-1pol	
HLA-A11	AIKKKDSTK	221-229	4
	GIPHPAGLK	252-260	1
	QLDCTHLEGK	781-790	9
	AVFIHNFKP	898-946	2
	KIQNFRVYY	938-948	4
		HIV-1pol	
HLA-A24	FWEVQLGI	242-249	20
	RYQYNVLPQGW	298-309	1.3
	QYNVLPQGW	300-308	1
	PFLWMGYEL	381-389	1.2
	GYELHPDKW	386-394	20
	LWKGEGAVVI	957-966	6.5
		human p53	
HLA-B7	LPENNVLSPL	26-35	1.2
	SPALNKMFCQL	127-137	0.9
	RPILTIITL	249-257	0.2
	LPPGSTKRAL	299-308	0.2
	SPQPKKKPL	315-323	0.6
		PRAME	
HLA-B35	LPRELFPPL	48-56	0.7
	LPRRLFPPLF	48-57	1.6
	FPPLFMAAF	53-61	0.8
	RPRRWKLQV	113-121	>100
	IPVEVLVDLF	173-121	0.1
	LPTLAKFSPY	246-255	0.1
	CPHCGDRTFY	487–497	1.5
	EPILCPCFM	499-507	0.3
		mHag HA-1	
HLA-B60	KECVLHDDL	—	5.3
	KECVLRDDL	—	3.9
	KECVLHDDLL	—	1
	KECVLRDDLL	—	1.6

[a] Bindings affinity can be classified according to the following cutoffs. High affinity IC50 ≤ 5 μM; intermediate affinity 5 μM < IC50 ≤ 15 μM; low affinity; 15 μM < IC50 ≤ 100 μM; no binding IC50 > 100 μM.

HLA = human leukocyte antigen.

tive-control B-LCL (Figure 1B). As with in other competition-based assays, in our assays, the measured binding affinity of the test peptides is relative to the binding capacity of the Fl-labeled reference peptide. Therefore, we used well-defined HLA class I ligands or CTL epitopes as reference peptides (Table 2). As we have shown before for the HLA-A2 and HLA-A3 binding assays [27], the kinetics of peptide binding in our assays at 4 °C with an incubation time of 24 h followed the same pattern as those in assays applying soluble HLA molecules. Also, the ranking of peptides according to their IC_{50} was comparable to the ranking found in cell free-binding assays [27]. Validation of the newly developed assays with either the unlabeled reference peptide or other defined class I–binding peptides, showed IC_{50} values below or around 5 μM (Figure 3 and Table 3), which is in line with previously published results [27].

We were able to use the assays described for the identification of novel HLA class I–binding peptides as exemplified for HIVpol-derived peptides binding in HLA-A11 and HLA-A24, peptides of p53 with high affinity for HLA-B7, PRAME-derived peptides binding in HLA-B35, and peptides from mHag HA-1 with high affinity for HLA-B60 (Table 4). These peptides have been used for CTL inductions to identify new class I–presented epitopes [37].

An analysis of the motif-based peptide binding prediction in HLA-A2 revealed that rankings of the peptide-binding prediction and binding capacity (IC_{50}) did not accurately correlate (Table 5). This is caused by the incomplete knowledge of the contribution of each aa in every position of a peptide to HLA class I binding and,

TABLE 5 Accuracy of binding prediction in HLA-A*0201 of 128 peptides in PRAME

Binding prediction[a]		Binding affinity measured by HLA-A2 assay[c]			
Length	Ranking[b]	High	Intermediate	Low	No binding
9-mers	Ranked 1–16	6 (37.5%)	2 (12.5%)	6 (37.5%)	2 (12.4%)
	Ranked 17–32	1 (6.2%)	4 (25%)	10 (62.5%)	1 (6.3%)
	Ranked 33–48	1 (6.2%	4 (25%)	6 (37.5%)	5 (31.3%)
	Ranked 49-65	0 (0.0%)	0 (0.0%)	7 (41.0%)	10 (59.0%)
	Total (ranked 1–65)	8 (12.3%)	10 (15.4%)	29 (44.6%)	18 (27.7%)
10-mers	Ranked 1–16	6 (37.5%)	7 (43.7%)	3 (18.7%)	0 (0.0%)
	Ranked 17–32	1 (6.2%)	6 (37.5%)	7 (43.8%)	2 (12.5%)
	Ranked 33–48	4 (25%)	4 (25.0%)	5 (31.2%)	3 (18.7%)
	Ranked 491–63	0 (0.0%)	0 (0.0%)	6 (40.0%)	9 (60.0%)
	Total (ranked 1–63)	11 (17.5%)	17 (27.0%)	21 (33.3%)	14 (22.2%)

[a] Prediction by BIMAS algorithm, accessible via http://bimas.cit.nih.gov/molbio/hla_bind/ (ref.18).

[b] Ranking no. 1 is peptide with highest prediction score, which is predicted to bind best.

[c] Binding affinity classified according to the following cutoffs. High affinity: IC_{50} ≤ 6 μM; intermediate affinity; 6 μM < IC_{50} ≤ 15 μM; low affinity; 15 μM ≤ 100 μM; no binding IC_{50} > 100 μM.

HLA = human leukocyte antigen.

therefore, we consider actual peptide-binding assays compulsory for precise assessment of peptide-binding capacity to all HLA class I molecules. The currently presented peptide binding assays will be conveniently applicable for this purpose.

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CHAPTER 5

CHAPTER 2

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BCR-ABL fusion regions as a source of multiple leukemia-specific CD8$^+$ T-cell epitopes

JH Kessler[1], SA Bres-Vloemans[1], PA van Veelen[1,2], A de Ru[1,2], IJG Huijbers[1], M Camps[1], A Mulder[1], R Offringa[1], JW Drijfhout[1], OC Leeksma[1], F Ossendorp[1] and CJM Melief[1]

[1]*Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands and* [2]*Centre for Medical Systems Biology, Leiden, The Netherlands*

For immunotherapy of residual disease in patients with Philadelphia-positive leukemias, the BCR-ABL fusion regions are attractive disease-specific T-cell targets. We analyzed these regions for the prevalence of cytotoxic T lymphocyte (CTL) epitopes by an advanced reverse immunology procedure. Seventeen novel BCR-ABL fusion peptides were identified to bind efficiently to the human lymphocyte antigen (HLA)-A68, HLA-B51, HLA-B61 or HLA-Cw4 HLA class I molecules. Comprehensive enzymatic digestion analysis showed that 10 out of the 28 HLA class I binding fusion peptides were efficiently excised after their C-terminus by the proteasome, which is an essential requirement for efficient cell surface expression. Therefore, these peptides are prime vaccine candidates. The other peptides either completely lacked C-terminal liberation or were only inefficiently excised by the proteasome, rendering them inappropriate or less suitable for inclusion in a vaccine. CTL raised against the properly processed HLA-B61 epitope AEALQRPVA from the BCR-ABL e1a2 fusion region, expressed in acute lymphoblastic leukemia (ALL), specifically recognized ALL tumor cells, proving cell surface presentation of this epitope, its applicability for immunotherapy and underlining the accuracy of our epitope identification strategy. Our study provides a reliable basis for the selection of optimal peptides to be included in immunotherapeutic BCR-ABL vaccines against leukemia.

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Keywords: BCR-ABL fusion peptides; HLA class I peptide binding; proteasome; antigen processing; cytotoxic T lymphocyte epitopes

Introduction

Transfusions of donor lymphocytes (DLT) in relapsed chronic myeloid leukemia (CML) after allogeneic stem cell transplantation (SCT) induces lasting remissions in the majority of patients.[1] Proposed antigens involved in this graft-versus-leukemia effect include minor histocompatibility antigens,[2] BCR-ABL fusion proteins, proteinase 3,[3,4] Wilm's tumor protein 1[3] and the telomerase catalytic subunit (hTERT).[3] Treatment of CML with defined vaccines or T cells would enable bypassing SCT, which is often accompanied with serious graft-versus-host reactions. In the search for leukemia-specific antigens particularly the BCR-ABL fusion proteins have attracted much attention. The (9;22) chromosome translocation gives rise to the Philadelphia (Ph) chromosome and the BCR-ABL chimeric gene.[5] In CML, either the second or the third exon of the BCR gene is spliced to the second exon of the ABL gene creating b2a2 and b3a2 RNA messages, respectively. In Ph$^+$ acute lymphoblastic leukemia (ALL) a third BCR-ABL mRNA form is transcribed: the e1a2 variant.[6] The b3a2, b2a2 and e1a2 fusion proteins have abnormal tyrosine kinase activity and are critical to the leukemogenic process. The tyrosine-kinase inhibitor imatinib (Gleevec, Novartis, East Hanover, NJ, USA) can induce complete molecular remissions in chronic phase CML patients.[7–9] However, its effect in blast crisis CML and Ph$^+$ ALL is limited and resistance to this inhibitor owing to BCR-ABL kinase domain mutations is frequent.[9–11] For the eradication of residual disease, potent and specific immunotherapy is therefore still a worthy goal.

Peptides spanning the BCR-ABL fusion regions are strictly leukemia-specific antigens. Several studies have reported CD4$^+$ T-helper cell responses directed to BCR-ABL fusion peptides.[12,13] Although the role of CD4$^+$ T cells, either by directly targeting leukemic cells or in providing help for CD8$^+$ T cells via dendritic cell maturation, should not be underestimated, for massive and direct killing of leukemic cells, robust CD8$^+$ cytotoxic T lymphocyte (CTL) responses are required. Therefore, BCR-ABL fusion regions have been scrutinized as targets for CTL-mediated immunotherapy. Fusion peptides from the b3a2 and b2a2 regions were found to bind to the human lymphocyte antigen (HLA)-A3/A11,[14,15] HLA-B7,[16] HLA-B8,[14,17] HLA-B27,[16] HLA-B35[16] and HLA-B44[17] HLA class I molecules. Binding of fusion peptides to HLA-A2, by far the most prevalent molecule, is subject of conflicting data. Most studies[14,15,17–19] did not find binding of fusion peptides in HLA-A2, however, two others reported significant binding[16,20] (summarized in Table 2).

In vitro CTL responses were demonstrated against several of these HLA class I binding fusion peptides. Peptide specificity of CTL raised *in vitro* against b3a2^{HSATGFKQSSK} and b3a2^{KQSSKALQR} loaded in HLA-A3 was achieved in several studies[15,20–22] and CTL recognition of CML cells suggested their natural presentation.[20,22] HLA-B8 binding peptide b3a2^{GFKQSSKAL} was proven immunogenic *in vitro* and data supporting endogenous presentation of this peptide have been reported.[20,22] Finally, CTL raised against b3a2^{SSKALQRPV} in HLA-A2-killed HLA-matched CML cells,[20] although this peptide was not detected in peptides eluted from HLA-A2$^+$ CML cells.[23] To date, results – either biochemical or functional – unequivocally demonstrating natural HLA class I presented cell surface expression of BCR-ABL fusion peptides is mainly lacking. Only for b3a2^{KQSSKALQR} the natural expression was biochemically established because it was detected in peptides eluted from HLA-A3 expressed on CML cells.[24]

Based on these studies, phase 1 and 2 trials with a peptide vaccine containing five of the HLA class I binding b3a2 fusion peptides were nevertheless initiated by the group of Scheinberg.[25,26] In some patients CD8$^+$ T-cell reactivity was observed against two of the HLA-A3/A11 binding b3a2 peptides.

Correspondence: Dr JH Kessler, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Building 1: E3-Q, PO Box 9600, 2300 RC Leiden, The Netherlands.
E-mail: J.H.Kessler@lumc.nl
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Table 1 Newly identified HLA class I binding BCR-ABL fusion peptides

BCR-ABL peptide sequences[a]																*Binding capacity (IC_{50})*[b]			
																A68[c]	*B51*	*B61*	*Cw4*
b3a2																			
H	S	A	T	G	F	K	Q	S	S	**K**						**2**	—	—	—
	S	A	T	G	F	K	Q	S	S	**K**						**2.5**	—	—	—
		A	T	G	F	K	Q	S	S	**K**						**7**	—	—	—
			T	G	F	K	Q	S	S	**K**						32	—	—	—
		A	T	G	F	K	Q	S	S	**K**	A					>100	—	—	—
			T	G	F	K	Q	S	S	**K**	A					—	>100	—	—
		A	T	G	F	K	Q	S	S	**K**	A	L				>100	—	—	>100
			T	G	F	K	Q	S	S	**K**	A	L				—	>100	—	>100
				G	F	K	Q	S	S	**K**	A	L				—	—	—	95
					F	K	Q	S	S	**K**	A	L	Q	R		30	—	—	—
						K	Q	S	S	**K**	A	L	Q	R		98	—	—	—
							Q	S	S	**K**	A	L	Q	R	P	V	—	>100	—	—
b3b4 (BCR)																			
H	S	A	T	G	F	K	Q	S	S	N						>100	—	—	—
b2a2																			
		I	P	L	T	I	N	K	E	**E**	A					—	>100	—	—
				L	T	I	N	K	E	**E**	A	L	Q	R		**3.5**	—	—	—
					T	I	N	K	E	**E**	A	L	Q	R		**6.5**	—	—	—
						I	N	K	E	**E**	A	L	Q	R	P	V	—	—	**10**	—
							N	K	E	**E**	A	L	Q	R	P	V	—	—	21	—
								K	E	**E**	A	L	Q	R	P	V	—	—	**<1**	—
							N	K	E	**E**	A	L	Q	R	P	V	A	—	—	22	—
								K	E	**E**	A	L	Q	R	P	V	A	—	—	**<1**	—
									E	**E**	A	L	Q	R	P	V	A[d]	—	—	**<1**	
										E	A	L	Q	R	P	V	A[d]	—	—	**11.5**	—
e1a2																			
			G	A	F	H	G	D	A	**E**	A							—	>100	—	—
				A	F	H	G	D	A	**E**	A							—	—	—	>100
		E	G	A	F	H	G	D	A	**E**	A	L						—	—	—	>100
			G	A	F	H	G	D	A	**E**	A	L						—	>100	—	>100
				A	F	H	G	D	A	**E**	A	L						—	—	—	**15**
					F	H	G	D	A	**E**	A	L						—	—	—	>100
						H	G	D	A	**E**	A	L	Q	R	P	V		—	—	**3**	—
							G	D	A	**E**	A	L	Q	R	P	V		—	>100	**1**	—
								D	A	**E**	A	L	Q	R	P	V		—	**15**	**2.5**	—
									A	**E**	A	L	Q	R	P	V		—	—	**<1**	—
							G	D	A	**E**	A	L	Q	R	P	V	A	—	—	17	—
								D	A	**E**	A	L	Q	R	P	V	A	—	85	23.5	—
									A	**E**	A	L	Q	R	P	V	A	—	—	**<1**	—
										E	A	L	Q	R	P	V	A[d]	—	—	**11.5**	—
a1a2 (ABL)																			
								L	E	E	A	L	Q	R	P	V	A	—	—	**<1**	—

HLA, human lymphocyte antigen.
[a]The aa. encoded by the BCR-ABL fusion DNA-triplet is printed bold and underlined.
[b]Binding affinity classified according to the following cutoffs:
High affinity (bold printed): $IC_{50} \leqslant 5\ \mu M$; intermediate affinity (bold printed): $5\ \mu M < IC_{50} \leqslant 5\ \mu M$; low affinity: $15\ \mu M < IC_{50} \leqslant 100\ \mu M$; no binding $IC_{50} > 100\ \mu M$. Dash, not tested.
[c]The HLA class I molecule that was tested.
[d]BCR-ABL fusion-peptides that are identical to ABL a1a2-derived peptides.

Recently, a similar peptide vaccination trial was conducted by Bocchia *et al.*[27] in patients who were treated with either imatinib or interferon-α and resulted in several complete cytogenetic remissions.

A systematic study of the proteasomal processing of BCR-ABL breakpoint peptides binding to HLA class I molecules has not been reported although our previous work[28,29] and that of others[30–32] have unequivocally shown that C-terminal precise excision by the proteasome is a major requirement for cell surface representation of any CTL epitope (reviewed by Kloetzel and Ossendorp[33]), whereas N-terminal extentions of the epitope can be trimmed by aminopeptidases.[34] As a consequence of inappropriate processing, on average, only less than one third of all HLA class I binding peptides is generated by the intracellular processing machinery.[35]

The current study, therefore, evaluates the BCR-ABL fusion regions as source for naturally expressed CTL targets by a systematic reverse immunology approach involving both HLA binding and proteasomal processing studies.[28] The ability to be processed and thereby appear at the cell surface of BCR-ABL fusion peptides has not been comprehensively tested before. Furthermore, many HLA class I molecules have not yet been tested for their capacity to harbor fusion peptides and binding of the e1a2 fusion region derived peptides to HLA class I was neglected until now. We first searched for BCR-ABL fusion peptides binding in novel HLA class I molecules to enable a

BCR-ABL-targeted immunotherapy covering all haplotypes. Subsequently, the C-terminal generation of both the already reported and the newly identified HLA class I binding fusion peptides was assessed by *in vitro* proteasome digestions. In our previous work,[28] and thereafter in studies of other groups,[36–38] this combination of HLA class I binding studies with proteasome digestion analysis resulted in the efficient and accurate identification of CTL epitopes. Here, the analysis of the enzymatic generation of all HLA class I binding BCR-ABL fusion peptides led to the identification of multiple novel predicted BCR-ABL CTL epitopes. Against one HLA-B61 presented epitope functional T cells were generated that recognized leukemic cells expressing the relevant fusion protein, thereby underscoring the reliability of our approach. Importantly, we show that processing constraints preclude the efficient endogenous generation of more than half of HLA class I binding fusion peptides, including two peptides that have previously been considered suitable as vaccine peptides. Our study strongly facilitates the selection of appropriate vaccine peptides for improved BCR-ABL breakpoint-directed CML and ALL immunotherapy.

Materials and methods

Cell lines

For HLA-A2, HLA-A68, HLA-B51, HLA-B61 and HLA-Cw4 binding assays, respectively Epstein–Barr virus transformed B-lymphoblastoid cell lines (B-LCL) JY (A*0201, B*0702, CW*0702), A68HI (A*6801, B*4402, Cw*0704), C1R-B*5101 (C1R transfected with HLA-B*5101[39]), Sweig007 (A*2902, B*4002 (B61), Cw*0202) and the C1R cell line (HLA-Cw*0401 positive), were chosen. For T-cell recognition the ALL cell line TOM1 (HLA type: HLA-A*11, -A*26, -B*46, -B*54, -Cw*01) was used and stably transduced with either plasmid pcDNA3.1-HLA-B*4002 (encoding HLA-B61) or, as control, the empty vector. The neomycin resistant population was enriched for high HLA-B61 expression by flow cytometric sorting using human Moab JOK3H5 against HLA-B61. Cell lines were cultured in complete culture medium Iscove's modified Dulbecco's medium ((IMDM); Biowithaker, Verviers, Belgium) supplemented with 8% fetal calf serum ((FCS); Gibco BRL, Carlsbad, CA, USA) and 2 mM L-glutamine.

Peptides

Short 8–11-mer peptides and long 27-mer polypeptides were synthesized as described before.[28]

HLA class I peptide-binding prediction and HLA class I molecule selection

The b3a2, b2a2 and e1a2 fusion regions were screened with HLA peptide-binding prediction software of BIMAS (NIH, USA; http://bimas.cit.nih.gov/molbio/hla_bind/)[40] and SYFPHEITI (University of Tuebingen, Germany; www.syfpeithi.de).[41] For prediction of peptide binding in HLA-A*0201 the MOTIF (MTF) algorithm was used as well.[42] Octameric, nonameric and decameric fusion peptides harboring two primary anchor residues, which for that reason scored high in peptide-binding prediction, were selected. HLA class I molecules were selected for which such peptides were found and that were not studied before. For these molecules, the set of test peptides was extended with the length and shift variant peptides that harbored only one major anchor residue.

Cellular binding assays for HLA-A2, HLA-A68, HLA-B51, HLA-B61 and HLA-Cw4

Affinity of peptides for HLA-A2 (A*0201), HLA-A68 (A*6801), HLA-B51 (B*5101), HLA-B61 (B*4002) and HLA-Cw4 (Cw*0401) was measured using competition-based cellular peptide-binding assays developed at our department as described.[43,44] The capacity of test peptides to compete with the cell surface expressed HLA class I binding of a fluorescein (Fl)-labeled reference peptide that is known to bind with high affinity in the HLA molecule of interest was tested. As Fl-labeled reference peptides were used: FLPSDC(Fl)FPSV (derived from hepatitis B virus (HBV) nucleocapsid 18–27) and YMLDLC(Fl)-PETT (human papilloma virus (HPV) E7 11–20) for HLA-A*0201, KTGGPIC(Fl)KR for HLA-A*6801, TGYLNTC(Fl)TV[45] for HLA-B*5101, GEFGGC(Fl)GSV for HLA-B61 (B*4002) and SFNC(Fl)GGEFF[46] for HLA-Cw*0401.[43,44] The percentage inhibition of Fl-labeled reference peptide binding by the competitor test peptide was calculated using the following formula:

$$(1 - (MF_{\text{reference and competitor peptide}} - MF_{\text{no reference peptide}})/(MF_{\text{reference peptide}} - MF_{\text{no reference peptide}})) \times 100\%.$$

The binding affinity of competitor peptide is expressed as the concentration needed to inhibit 50% binding of the Fl-labeled reference peptide (IC_{50}). An $IC_{50} \leqslant 5\,\mu M$ was considered high-affinity binding, $5\,\mu M < IC_{50} \leqslant 15\,\mu M$ was considered intermediate-affinity binding, $15\,\mu M < IC_{50} \leqslant 100\,\mu M$ was judged low-affinity binding and $IC_{50} > 100\,\mu M$ was considered as absence of binding.

HLA-A2-peptide assembly and dissociation assay

Stability of peptides in HLA-A*0201 was measured as described.[47] In short, JY cells (HLA-A*0201$^+$) were treated with 10^{-4} M emetine (Sigma, St Louis, MO, USA) for 1 h at 37°C to stop synthesis of major histocompatibility complex (MHC) class I molecules. Naturally bound peptides in HLA-A*0201 were removed by acid treatment and reconstituted with the peptide of interest at 200 μM in 2% FCS/IMDM containing 2 μg/ml human β_2-microglobulin for 1.5 h at room temperature. At this time point HLA-A2 – peptide assembly was determined by staining with the Moab BB7.2 and GAM-Fitc followed by flow cytometric analysis. Peptide assembly is expressed as fluorescence index which is calculated as

$$(MF_{\text{sample}} - MF_{\text{background}})/MF_{\text{background}}.$$

Remaining cells were washed twice to totally remove free peptide and incubated at 37°C for another 2 h and subsequently the residual HLA-A2 – peptide complexes were again stained and analyzed to determine the peptide dissociation rate. The HLA-A*0201-peptide stability (dissociation) is expressed as percentage of remaining complexes after 2 h relative to the amount of assembled complexes at 0 h. As positive control the HBV nucleocapsid 18–27 epitope was used.

Subunit composition of 20S proteasome preparations

Proteasomes (20S) were purified as described[48] from HeLa cells, a B-LCL cell line and CML cells that were isolated from a patient in blast crisis. B-LCL are known to contain mainly immunoproteasomes[49] and HeLa mainly the constitutive proteasome subunits. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis to separate proteins and Western blotting was

performed according to standard protocols. For staining of the immunosubunits polyclonal antibodies raised against human LMP2 and MECL-1 peptides were used[50] and the constitutive subunits were stained with antibody MCP421 against human Delta and antibody P93250 for human MB1.[51,52]

In vitro *proteasome-mediated digestions and mass spectrometrical analysis*

In vitro 20S proteasome-mediated digestions of 27-mer BCR-ABL polypeptides were performed as described.[28] Briefly, peptides (3 nmol) were incubated with 1 μg of purified proteasome at 37°C for 1, 4 and 24 h in 300 μl proteasome digestion buffer. Trifluoroacetic acid was added to stop the digestion and samples were stored at −20°C. Electrospray ionization mass spectrometry was performed on a hybrid quadrupole time-of-flight mass (Q-TOF) spectrometer (Micromass) as described.[28] The peaks in the mass spectrum were searched in the digested precursor peptide using the Biolynx software (Micromass). The intensity of the peaks was used to establish the relative amounts of peptides digested. The relative amounts of the digestion fragments are given as the percentage of the total mass-peak intensities of digested 27-mer at 1 h incubation time, because this time point best reflects physiologically relevant enzymatic activity.[28]

In vitro *CTL response inductions against putative HLA-A68 and HLA-B61 CTL epitopes*

In vitro $CD8^+$ CTL inductions against synthetic peptides were performed as described.[28] Peripheral blood mononuclear cells of an HLA-B61-positive healthy donor (for inductions against $b2a2^{12\text{-}21}$ and $e1a2^{13\text{-}21}$) and an HLA-A68-positive (for induction against $b2a2^{8\text{-}18}$) healthy donor were used for the generation of antigen presenting cells and autologous $CD8^+$ T cells. Peptide specificity of bulk CTL cultures was tested at day 28 in a standard ^{51}Cr cytotoxicity assay. The highly peptide-specific T-cell culture against $e1a2^{13\text{-}21}$ (AEALQRPVA) was cloned by limiting dilution at day 29. Recognition of tumor cells by peptide-specific CTL clones was tested in an interferon-γ enzyme-linked immunosorbent assay (ELISA): 20.000 CTL/well were coincubated with 20 000 tumor cells/well in a 96-well plate and supernatants were harvested after 14 h.

Results

Identification of novel HLA class I binding BCR-ABL fusion peptides

To date, 12 BCR-ABL fusion peptides have been reported to bind to several HLA class I molecules (HLA-A2, -A3/A11, -B7, -B8, -B27, -B35 or HLA-B44) with a least intermediate affinity (listed in Table 3). To expand this set, enabling immunotherapy for more haplotypes thereby covering a greater percentage of the population, the BCR-ABL fusion regions were screened, with the help of two freely accessible HLA class I peptide-binding predictions algorithms, for the presence of binding motifs of all HLA class I molecules that were not evaluated before. Fusion peptides containing two primary anchors, a prerequisite for efficient binding, were found for HLA-A68, HLA-B51, HLA-B61 and HLA-Cw4, motifs for all other HLA class molecules were not found. In general, HLA class I binding predictions are reliably selecting peptides with actual binding capacity,[43] but false negative predictions cannot be completely excluded. Therefore, we extended the set of fusion peptides that were predicted to have binding capacity with length and shift variants containing only one primary anchor, because in particular these peptides may have binding capacity despite not predicted to bind strongly. These peptides were synthesized and tested for their actual binding capacity in competition-based cellular-binding assays which we developed for these HLA molecules.[43,44]

Ten peptides from the b3a2 and b2a2 breakpoint regions that contained an anchor residue at position 2 (Val, Thr or Ser) and/or the C-terminal anchor (Arg or Lys)[41] were tested for binding to HLA-A68 (A*6801). Five peptides bound with high ($IC_{50} \leqslant 5\ \mu M$) or intermediate affinity ($5\ \mu M < IC_{50} \leqslant 15\ \mu M$). The other peptides displayed low ($15\ \mu M < IC_{50} \leqslant 100\ \mu M$) or undetectable binding capacity ($IC_{50} > 100\ \mu M$). Because the three high-affinity binding b3a2 peptides differ from the non-leukemic BCR b3b4 region only at their C-terminal lysine (encoded by the fusion-triplet codon), we additionally tested the BCR b3b4 peptide HSATGFKQSSN. Importantly, this peptide, lacking the C-terminal HLA-A68 anchor, did not display any binding in HLA-A68 (Table 1), indicating that the binding of the b3a2 peptides is strictly BCR-ABL-specific and cross-reactivity of $CD8^+$ T cells against the b3a2 peptides with normal BCR peptides will not occur.

Ten peptides harboring at least one anchor complying with the HLA-B51 (B*5101) binding motif (Ala, Pro or Gly at P2; Phe, Ile or Val at the C-terminus)[41] were tested. Only $e1a2^{DAEALQRPV}$, which is the peptide that scored best in binding prediction, showed a significant binding affinity (IC_{50} 15 μM).

Fourteen b2a2 and e1a2 fusion peptides were tested for binding to HLA-B61 (B*4002). Three b2a2 peptides and two e1a2 peptides that contained anchors at both the P2 (Glu) and the C-terminal position (Val, Ala or Ile)[53] displayed high-binding affinity ($IC_{50} \leqslant 5\ \mu M$). Furthermore, three e1a2 peptides (HGDAEALQRPV, GDAEALQRPV and DAEALQRPV) that harbored only one anchor bound with high affinity as well. For GDAEALQRPV this can be explained by the presence of the glycine at P1 and the glutamine at P4, which at these positions are preferred binding residues for HLA-B61. The other six peptides displayed intermediate or low-binding capacity for HLA-B61 ($IC_{50} > 5\ \mu M$). It is important to note that, although harboring the fusion DNA triplet encoded aa., HLA-B61 binding $b2a2^{EEALQRPVA}$ and $b2a2/e1a2^{EALQRPVA}$ are identical to normal ABL a1a2 sequences. Furthermore, $b2a2^{KEEALQRPV(A)}$ and $e1a2^{AEALQRPV(A)}$ differ only in one residue from their corresponding ABL a1a2 peptides ((L)EEALQRPVA) which displayed equally high-binding capacity ($IC_{50} < 1\ \mu M$). This implicates that cross-reactivity of T cells specific for those b2a2 and e1a2 peptides may occur towards normal a1a2 ABL peptides.

Finally, eight fusion peptides from b3a2 and e1a2 that contained at least one anchor residue for HLA-Cw4 were tested for binding. Only $e1a2^{AFHGDAEAL}$ that contained both anchors (Tyr, Pro or Phe at P2; Leu, Phe or Met at the C-terminus)[41] bound efficiently (IC_{50} 15 μM).

In summary, we identified five fusion peptides for HLA-A68, one for HLA-B51, 10 fusion peptides for HLA-B61 and one for HLA-Cw0401 with high or intermediate class I binding capacity ($IC_{50} \leqslant 15$) (Table 1).

*Reevaluation of binding and stability of fusion peptides in HLA-A*0201*

Binding of fusion peptides in HLA-A2, with a frequency of 44% in the Caucasian population, is subject of conflicting data (see Table 2). Most studies[14,15,17–19] did not find significant binding of BCR-ABL fusion peptides in HLA-A2. In contrast, four

Table 2 Binding and stability of BCR-ABL fusion peptides in HLA-A2 (A*0201)

BCR-ABL fusion-peptide sequences[a] and positive control peptide	Length	Competition assay: Pred.[b] MTF	Competition assay: Pred.[b] SYFP.	Competition assay: IC_{50}[c]	Stabilization assay: Assembly[d]	Stabilization assay: Dissociation[e]	Published binding[f]: Efficient Refs.	Published binding[f]: No bind. Refs.
F L P S D F F P S V (HBV 18–27)	10	67	24	<1	1.53	74%		
B3A2								
A T G F K Q S S **K** A L	11	27	—	>100	—	—	—	14;16
T G F K Q S S **K** A L	10	27	11	>100	—	—	—	16
G F K Q S S **K** A L	9	27	10	nt	—	—	—	18;19;15;16
K Q S S **K** A L Q R P V	11	32	—	17.5	0.37	3%	20	—
Q S S **K** A L Q R P V	10	27	10	>100	—	—	20	—
S S **K** A L Q R P V	9	31	12	>100	0.01	0%	20	18;19;15
S **K** A L Q R P V	8	30	0	nt	—	—	20	—
B2A2								
P L T I N K E **E** A	9	49	12	>100	—	—	—	18;19;14;15
I P L T I N K E **E** A L	11	29	—	>100	—	—	—	—
P L T I N K E **E** A L	10	62	17	>100	—	—	—	—
L T I N K E **E** A L	9	47	20	29	0.45	3.1%	16	18;19;14;15
T I N K E **E** A L	8	52	0	>100	—	—	—	14
P L T I N K E **E** A L Q	11	36	—	>100	—	—	—	—
K E **E** A L Q R P V	9	35	11	nt	—	—	—	18;19;15
E1A2								
E G A F H G D A **E** A L	11	30	—	>100	—	—	—	—
G A F H G D A **E** A L	10	30	17	79	0.22	2.9%	—	—
H G D A **E** A L Q R P V	11	34	—	>100	—	—	—	—
G D A **E** A L Q R P V	10	30	15	>100	—	—	—	—
D A **E** A L Q R P V	9	26	13	>100	—	—	—	—
A **E** A L Q R P V	8	30	0	>100	—	—	—	—

HBV, hepatitis B virus; HLA, human lymphocyte antigen; MTF, MOTIF algorithm; Pred., predicts; Refs., references.
[a]All fusion peptides containing at least one canonical HLA-A*0201 anchor are listed. The BCR-ABL fusion aa is printed bold; primary anchors for HLA-A*0201 are underlined.
[b]Peptide binding prediction by MTF- and SYFPEITHI algorithms (see Materials and methods). A higher score predicts better binding.
[c]IC_{50} in μM; nt, not tested. Fl-labeled HPV E7 11–20 was used as reference peptide.
[d]Assembly expressed as fluorescence index (see Materials and methods) after 1.5 h loading with 200 μM peptide. Dash, not tested.
[e]Dissociation: % complexes left after 2 h incubation in peptide-free condition. Dash, not tested.
[f]Overview of published HLA-A2 binding capacities of the listed peptides. Dash, no publications.

HLA-A2 binding b3a2 peptides have been identified by Yotnda *et al.*[20] and b2a2^{LTINKEEAL} has been found by Berke *et al.*[16] to bind to HLA-A2. Therefore, we re-evaluated the binding to HLA-A2 (A*0201). All fusion peptides harboring at least one canonical HLA-A2 binding anchor (Leu or Met at P2; Val or Leu at the C-terminus)[41] were tested. As data obtained with our competition assay are relative to the binding capacity of the reference peptide, we performed the assay with two reference peptides.

Using the high-affinity binding Fl-labeled HBV cAg 18–27 peptide we did not observe binding of the selected fusion peptides (data not shown). In contrast, using Fl-labeled HPV E7 11–20 as reference peptide, which has lower affinity for HLA-A2 than HBV cAg 18–27,[44] two BCR-ABL peptides displayed significant binding for HLA-A2 (Table 2). The 11-mer b3a2^{KQSSKALQRPV} bound with IC_{50} of 17.5 μM, confirming the binding reported by Yotnda *et al.*[20] In contrast, nonamer b3a2^{SSKALQRPV} that has been observed in the latter study to bind in HLA-A2 as well and was suggested to be naturally presented,[20] showed hardly any binding (Table 2). It only inhibited the binding of the Fl-labeled reference peptide for 6% at the highest concentration of 100 uM tested (data not shown), which is in line with its absence of HLA-A2 binding in other studies.[15,18,19] Nonamer b2a2^{LTINKEEAL} was the second peptide that bound with moderate capacity (Table 2, IC_{50} of 29 μM). This confirmed its binding in HLA-A2 as reported by Berke *et al.*,[16] but is in contrast to earlier studies reporting lack of binding.[14,15,18,19] The only peptide containing both the genuine P2 and C-terminal anchors for HLA-A2, namely decamer b2a2^{PLTINKEEAL}, did not bind (IC_{50} > 100 μM) (Table 2), which has also been reported in other studies.[14,16] This can be attributed to a deleterious effect of Pro at position 1 for binding capacity.[40] Of the e1a2 fusion peptides, only decamer GAFHGDAEAL bound with low affinity (IC_{50} > 79 μM) (Table 2).

To more precisely define binding characteristics of the four peptides of primary interest, HLA-A2 – peptide complex stability was assessed by first measuring assembly after 1.5 h peptide loading and subsequently determining the dissociation rate of HLA-A2 – peptide complexes (off-rate of the peptide) after 2 h incubation at 37°C in peptide-free condition[47] (Table 2). In this assay as well, peptide b3a2^{SSKALQRPV} did not show appreciable assembly of HLA-A2 molecules. The three peptides that exhibited binding capacity in our competition assay also induced HLA-A2 assembly. However, this binding was very unstable because these peptides showed high dissociation rates from HLA-A2: <5% of HLA-A2-peptide complexes were detectable after 2 h of incubation. We conclude that stable binding of BCR-ABL junctional peptides in HLA-A2 did not occur.

In vitro *proteasome-mediated digestions of 27-mer BCR-ABL fusion regions*

In vitro proteasome digestions are known to reliably yield MHC class I ligands from polypeptides[54–57] and thus cleavage pattern analysis has been applied for the successful identification of CTL

Table 3 Proteasome digestions of BCR-ABL fusion regions in relation to HLA class I binding fusion peptides.

A **Digestion b3a2 fusion region**[a]

HLA class I binding b3a2 peptides[b]

	C-term[c]	HLA[d]	Ref.[e]
H S A T G F K Q S S **K**	Minor	A3, A11, A68	14; Curr.
S A T G F K Q S S **K**	Minor	A68	Curr.
A T G F K Q S S **K**	Minor	A3, A11, A68	14; Curr.
A T G F K Q S S **K** A L	No	B7	16
G F K Q S S **K** A L	No	B8	14
F K Q S S **K** A L Q	No	B8	17
F K Q S S **K** A L Q R	Major	B27	16
K Q S S **K** A L Q R	Major	A3, A11	14
K Q S S **K** A L Q R P V	No	A2	Curr.
S S **K** A L Q R P V	No	A2	20

Digest. sites[f]

27-mer V I V H S A T G F K Q S S **K** A L Q R P V A S D F E P Q

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Fragments[g]

Fragment[g] Start	End	Intensity, 1 h (%)[k] B-LCL	Hela	CML
1	18	9.9	9.7	9.5
1	21	15.9	32.2	41.6
5	21	11.5	0,0	0,0
5	27	14.9	4.1	15.9
6	27	3.7	3.5	0,0
7	27	5.6	20.7	6.2
10	27	5.8	0,0	0,0
15	27	1.4	1.6	4.8
19	27	14.4	10.4	7.9

B **Digestion b2a2 fusion region**

HLA class I binding b2a2 peptides

	C-term	HLA	Ref.
I P L T I N K E **E** A L	Minor	B7, B35	16
P L T I N K E **E** A L	Minor	B7	16
L T I N K E **E** A L	Minor	B8, B7, A2	17; 16; Curr.
L T I N K E **E** A L Q R	Major	A68	Curr.
T I N K E **E** A L Q R	Major	A68	Curr.
I N K E **E** A L Q R P V	No	B61	Curr.
K E **E** A L Q R P V	No	B44, B61	17; Curr.
K E **E** A L Q R P V A	Major	B61	Curr.
E **E** A L Q R P V A	Major	B61	Curr.
E A L Q R P V A	Major	B61	Curr.

Digest. sites

27-mer Q T V H S I P L T I N K E **E** A L Q R P V A S D F E P Q

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Fragments

Fragment Start	End	Intensity, 1 h (%) B-LCL	Hela	CML
9	10	4,0	4.1	3.5
1	13	4.5	10.9	4.8
1	16	5.3	4.1	4.9
1	18	12.5	12.8	12.3
1	21	32.1	30.9	32.1
5	21	6.2	3.6	5.1
7	21	5,0	7.3	11.5
8	25	6.1	5.7	5.9
11	27	5.1	2.6	4.2
19	27	7.7	5.6	4.8

C **Digestion e1a2 fusion region**

HLA class I binding e1a2 peptides

	C-term	HLA	Ref.
A F H G D A **E** A L	Minor	C4	Curr.
H G D A **E** A L Q R P V	No	B61	Curr.
G D A **E** A L Q R P V	No	B61	Curr.
D A **E** A L Q R P V	No	B61, B51	Curr.
A **E** A L Q R P V	No	B61	Curr.
G D A **E** A L Q R P V A	Major	B61	Curr.
D A **E** A L Q R P V A	Major	B61	Curr.
A **E** A L Q R P V A	Major	B61	Curr.
E A L Q R P V A	Major	B61	Curr.

Digest. sites

27-mer W P N D G E G A F H G D A **E** A L Q R P V A S D F E P Q

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Fragments

Fragment Start	End	Intensity, 1 h (%) B-LCL	Hela	CML
1	11	4.2	5.5	6.6
1	12	8.3	17.4	8.6
1	16	2.9	3.9	3.5
11	17	9.5	7.4	12.0
1	18	6.7	8.3	8.2
1	21	42.1	21.8	27.4
5	21	3.5	7.2	2.8
13	27	9.3	9.5	11.7
17	27	2.8	4.8	3.4
19	27	5.5	10.1	7.1

D **Digestion ABL, a1a2 region**

Digest. sites

27-mer K K G L S S S S S C Y L E **E** A L Q R P V A S D F E P Q

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Fragments

Fragment Start	End	Intensity, 1 h (%) B-LCL	Hela	CML
1	12	26.0	10.9	25.1
1	21	8.2	26.2	14.5
11	21	2.5	2.3	0.0
13	21	4.2	2.6	0.0
13	27	18.4	5.3	18.9
14	27	6.0	7.4	16.6
17	27	4.5	6.9	0.0
15	27	7.8	15.3	24.9
19	27	0.0	9.9	0.0

a For digestions, 27-mer peptides were used with the breakpoint aa. at position 14 (printed bold)
b All efficiently class I binding fusion peptides (from literature and this study) are listed (breakpoint aa. bold)
c Liberation of C-terminal aa. of HLA class I binding peptide by minor or major cleavage site. No; no cleavage after C-term
d HLA class I molecule to which the indicated fusion peptide can bind efficiently.
e Literature reference (first publication) of indicated peptide with respect to HLA class I binding. Curr.; current study.
f Major site: bold arrow: fragments containing as C-terminus the residue N-terminal from the cleavage, or complements, are present for ≥ 5% at 1 h incubation for all proteasome types. Minor site: thin arrow: fragments containing as C-terminus the residue N-terminal from the cleavage, or complements, are present for < 5% at 1 h incubation.
g Digestion fragments are ordered according to their C-terminus. Some low abundant irrelevant fragments have been ommited
k Intensity is expressed as % of total summed mass-peak intensities of digested 27-mer at 1 h digestion.

Table 4 Predicted cell surface expressed HLA class I – BCR-ABL fusion peptide complexes

	BCR-ABL fustion peptide[a]																C-terminal processing[b]	HLA class I binding			Candidate CTL epitope[f]
																		HLA[c]	IC_{50}[d]	Ref.[e]	
b3a2	H	S	A	T	G	F	K	Q	S	S	**K**						Minor	A3/A11	int./high	14	+
	H	S	A	T	G	F	K	Q	S	S	**K**						Minor	A68	2	Curr.	+
		S	A	T	G	F	K	Q	S	S	**K**						Minor	A68	2.5	Curr.	+
			A	T	G	F	K	Q	S	S	**K**						Minor	A3/A11	int./high	14	+
			A	T	G	F	K	Q	S	S	**K**						Minor	A68	7	Curr.	+
						F	K	Q	S	S	**K**	A	L	Q	R		Major	B27	high	16	++
							K	Q	S	S	**K**	A	L	Q	R		Major	A3/A11	high	14	++
b2a2			I	P	L	T	I	N	K	E	**E**	A	L				Minor	B7	high	16	+
			I	P	L	T	I	N	K	E	**E**	A	L				Minor	B35	high	16	+
				P	L	T	I	N	K	E	**E**	A	L				Minor	B7	int.	16	+
					L	T	I	N	K	E	**E**	A	L				Minor	B8	Int.	17	+
					L	T	I	N	K	E	**E**	A	L				Minor	B7	high	16	+
					L	T	I	N	K	E	**E**	A	L				Minor	A2	29	Curr.	+
					L	T	I	N	K	E	**E**	A	L	Q	R		Major	A68	3.5	Curr.	++
						T	I	N	K	E	**E**	A	L	Q	R		Major	A68	6.5	Curr.	++
									K	E	**E**	A	L	Q	R	P V A	Major	B61	<1	Curr.	++
										E	**E**	A	L	Q	R	P V A[g]	Major	B61	<1	Curr.	++
e1a2					A	F	H	G	D	A	**E**	A	L				Minor	C4	15	Curr.	+
								G	D	A	**E**	A	L	Q	R	P V A	Major	B61	17	Curr.	+
									D	A	**E**	A	L	Q	R	P V A	Major	B61	23,5	Curr.	+
										A	**E**	A	L	Q	R	P V A	Major	B61	<1	Curr.	++
											E	A	L	Q	R	P V A[g]	Major	B61	11,5	Curr.	++

Curr.; current study; CTL, cytotoxic T lymphocyte; HLA, human lymphocyte antigen; ref., reference.
[a]Fusion peptides with signficicant HLA class I binding capacity that are excised after their C-term are listed (breakpoint aa. bold and underlined).
[b]Efficiency of C-terminal liberation: see by major site: fragments containing this C-terminus together with their complements are present for ⩾5%. By minor site: fragments containing this C-terminus together with their complements are present for <5% (at 1 h digestion).
[c]HLA class I molecule for which the indicated fusion peptide has efficient binding capacity.
[d]Binding capacity in IC_{50} (μM) from current study or according to literature (classified in: intermediate (int.) or high-affinity binding).
[e]Literature reference (first publication) of indicated peptide with respect to HLA class I binding.
[f]Epitope prediction. Classification: (++) high-density epitope: major digestion site and high-affinity binding; (+) low abundant epitope: minor site and/or intermediate-affinity binding.
[g]BCR-ABL fusion-peptides that are identical to ABL a1a2-derived peptides (the EALQRPVA peptide is both in e1a2 and b2a2).

epitopes among HLA class I binding peptides.[28,36–38,58] We digested polypeptides encompassing the b3a2, b2a2 and e1a2 fusion regions *in vitro* with purified proteasome complexes to assess the C-terminal liberation of HLA class I binding fusion peptides, which is considered to be mediated solely by the proteasome. The digestion pattern after 1 h incubation was analyzed because this best reveals physiological cleavages.[28]

The proteasome complex can exist mainly in two forms: constitutive proteasomes expressing β1 (delta), β2 (Z) and β5 (MB1) catalytic subunits and immunoproteasomes in which these units are replaced for β1i (LMP2), β2i (MECL-1) and β5i (LMP7), respectively.[50] Both types of proteasomes are expressed dependent on differentiation by interferons and source tissue[59] and have overlapping but qualitatively and/or quantitatively different enzymatic activity.[60,61] Thus for immunotherapy it is essential to know which proteasome type is expressed in the leukemic cells.[62] Western blotting with subunit-specific antibodies revealed that purified 20S proteasomes isolated from blast crisis CML cells consisted exclusively of the immunotype (Figure 1).

The leukemic proteasomes, proteasomes derived from HeLa cells, known to express mainly constitutive proteasomes, and proteasomes from B-LCL, containing preferentially immunoproteasomes[49] were incubated with fusion region spanning 27-mers and digestion mixtures were analyzed by mass spectrometry.

Table 3A shows the digestions of the 27-mer encompassing the b3a2 fusion region, containing 10 HLA class I binding fusion

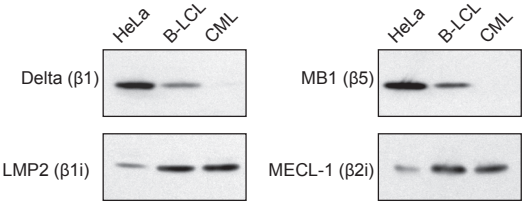

Figure 1 Western blot analysis of proteasome composition. Expression of constitutive proteasome subunits (Delta and MB1 proteins) versus immunoproteasome subunits (LMP2 and MECL-1 proteins) was analyzed by Western blotting. Proteasomes were isolated from HeLa, B-LCL and CML cells from two patients in blast phase, here we show a representative example.

peptides (reported in literature or identified in this study as indicated). Abundant cleavage sites were present after Arg-18 and especially after Ala-21. This indicates that HLA-B27 binding b3a2$^{9–18}$ and HLA-A3 binding b3a2$^{10–18}$ (KQSSKALQR), which share Arg-18 as C-terminus, are likely efficiently produced by the proteasome *in vivo*. Cleavages after Leu-16, Gln-17 and Val-20 were not observed, indicating that peptides with these C-termini, which include HLA-B8 binding b3a2$^{8–16}$ (ref. 19) and HLA-A2 binding b3a2$^{10–20}$ (this study) and b3a2$^{12–20}$ (SSKALQRPV),[20] are very unlikely to be naturally presented.

The HLA class I binding peptides with Lys-14 as C-terminus, including two HLA-A3/A11 binding peptides ((HS)ATGFKQSSK),[14] are not expected to be cell surface presented at a density needed for immunogenicity because only a low abundant complementary digestion fragment (aa 15–27) was found.

Table 3B depicts digestions of the b2a2 fusion region containing nine HLA class I binding peptides. Abundant cleavage sites were again produced after Arg-18 and Ala-21, indicating that both HLA-A68 peptides ($b2a2^{8/9\text{-}18}$) and three HLA-B61 binding peptides ($b2a2^{12/13/14\text{-}21}$), which contain these residues as C-terminal anchors respectively, are expected to be efficiently liberated C-terminally and expressed on living cells. A much less abundant cleavage site was observed after Leu-16, rendering it possible that the HLA class I binding peptides ending at Leu-16 will be produced although in low abundance. Cleavage after Val-20 did not take place, which precludes intracellular generation of 11-mer $b2a2^{10\text{-}20}$ (HLA-B61) and nonamer $b2a2^{12\text{-}20}$ (HLA-B44, -B61).

Table 3C shows the digestions of the e1a2 fusion region containing nine HLA class I binding fusion peptides. The C-terminal Ala-21 of four HLA-B61 binding N-terminal length variants was efficiently liberated. Therefore, these peptides are expected to be efficiently liberated and cell surface expressed. Because an abundant cleavage site was also observed after Asp-12, which will generate the N-terminus, this points to especially $e1a2^{AEALQRPVA}$ as a putative abundantly expressed CTL epitope. In contrast, the four HLA-B61 binding peptides and one HLA-B51 binding peptide that share Val-20 as C-terminus are not predicted to be endogenously produced, because of the absence of cleavage after Val-20. HLA-Cw4 binding peptide $e1a2^{8\text{-}16}$ may be naturally presented, however, probably in low abundance, because only a minor cleavage was observed after Leu-16.

We observed abundant cleavages after Arg-18 and Ala-21 in both the b3a2, b2a2 and e1a2 polypeptides (Table 3A–C). This similar cleavage pattern in the ABL-derived a2 region, irrespective of the joining BCR region, fits with the current knowledge that proteasome specificity is mainly dependent on residues directly adjacent to the cleavage site. However, residues further up- or downstream may influence the specificity as well.[56] Because two HLA-B61 binding fusion peptides ($b2a2^{13\text{-}21}$ and $b2a2/e1a2^{14\text{-}21}$) equaled non-leukemic ABL peptides and four others differed only at their N-terminus (Tables 1 and 3), we studied the digestion of the non-leukemic ABL a1a2 polypeptide. As shown in Table 3D, analysis of cleavages in the a2 region showed a different pattern when contained in the a1a2 polypeptide that was proteasome type dependent. Digested with CML or B-LCL-derived immunoproteasomes, the cleavage after Arg-18 was absent and the cleavage efficiency after Ala-21 was strongly reduced (e.g., only 14.5% of the digested a1a2 polypeptide in the CML digestion) when compared to this cleavage in the BCR-ABL fusion regions (41.6, 48.7 and 30.3% of the digested b3a2, b2a2 and e1a2 polypeptides, respectively, in the CML digestion). The reduced cleavage efficiencies in a1a2 after Arg-18 and Ala-21 by immunoproteasome preparations were accompanied by an abundant cleavage after Leu-12 upon digestion by this proteasome type: fragment 1–12 and its complements added up to 48.6 and 44% for B-LCL and CML proteasome digestions respectively. In contrast, in the a1a2 HeLa digestion those fragments added up to only 18.8%. Enhanced cleavage efficiency by immunoproteasomes after leucine residues is reported in the literature.[60] In the digestion with (constitutive) HeLa proteasomes, the less abundant cleavage after Leu-12 correlated with a quantitative recurrence of the cleavages after Arg-18 and Ala-21 (Table 3D). These results indicate that $b2a2^{13\text{-}21}$ and $b2a2/e1a2^{14\text{-}21}$ will be much less abundantly generated from the normal ABL a1a2 region in professional antigen presenting cells (containing immunoproteasomes).

CTL response against HLA-B61 binding e1a2 peptide AEALQRPVA

The combined strength of our reverse immunology approach enabled the identification of six novel predicted CTL epitopes expected to be abundantly expressed at the cells surface. These include two HLA-A68 binding fusion peptides and two HLA-B61 binding peptides from breakpoint region b2a2, and from e1a2 two HLA-B61 peptides. All of these peptides have both high binding capacity and are efficiently liberated after their C-terminus by the proteasome.

To demonstrate the accuracy of our approach and to prove natural presentation of these novel predicted CTL epitopes, $CD8^+$ T cells from healthy donors were induced against synthetic peptides. Specific responses against HLA-B61 binding peptides $b2a2^{12\text{-}21}$ and $e1a2^{13\text{-}21}$ and HLA-A68 binding peptide $b2a2^{8\text{-}18}$ were generated, indicating their immunogenicity (data not shown). The T-cell culture against $e1a2^{13\text{-}21}$ (AEALQRPVA) was highly peptide-specific and was cloned by limiting dilution. Several CTL clones efficiently lyzed peptide-pulsed HLA-$B61^+$ B-LCL Sweig007 (Figure 2a). To test endogenous production and natural presentation of $e1a2^{AEALQRPVA}$ in HLA-B61, cell line TOM1, derived from ALL and expressing e1a2, was transfected with HLA-B61 (TOM1-B61). The peptide specific CTL clones recognized TOM1-B61 efficiently, whereas recognition of TOM1 expressing the empty vector was only at background levels (Figure 2b), indicating that $e1a2^{AEALQRPVA}$ is endogenously produced and expressed on the surface of leukemic cells and underscoring the relevance of our epitope prediction procedure.

Because $e1a2^{AEALQRPVA}$ differs only at its N-terminus from normal ABL a1a2 sequence EEALQRPVA, which may be expressed on normal cells, the recognition of the latter peptide, loaded at the same concentration, was tested. All CTL clones displayed only very low cross-reactivity with this peptide (Figure 2a), implying the e1a2 specificity of the CTL response. Absent recognition of B-LCL Sweig007 (HLA-$B61^+$ and expressing normal ABL), either non-pulsed or pulsed with an irrelevant peptide (Figure 2a and b), also confirmed the lack of an ABL-a1a2-directed CTL response. Thus the applicability of $e1a2^{AEALQRPVA}$ as immunotherapeutic target in HLA-$B61^+$ ALL patients is warranted.

Discussion

This comprehensive analysis of the BCR-ABL fusion regions as a source of CTL epitopes yielded 17 b3a2, b2a2 and e1a2 fusion peptides that were shown for the first time to bind efficiently in HLA-A68, HLA-B51, HLA-B61 or HLA-Cw4 (Table 1, IC_{50} $<15\,\mu M$), thereby more than doubling the 12 fusion peptides shown previously to bind in other HLA class I molecules.[14–17,20] Motif-based peptide-binding predictions, which are needed to limit the amount of peptides to be tested, are adequately pré-selecting peptides with binding capacity.[3,43] Despite that, binding affinities have to be determined experimentally because the ranking of prediction scores mostly differs substantially from the ranking of measured values as we have shown previously.[43] Predicted absence of binding is only occasionaly contradicted by actual positive binding measurements.[43] Despite that, by

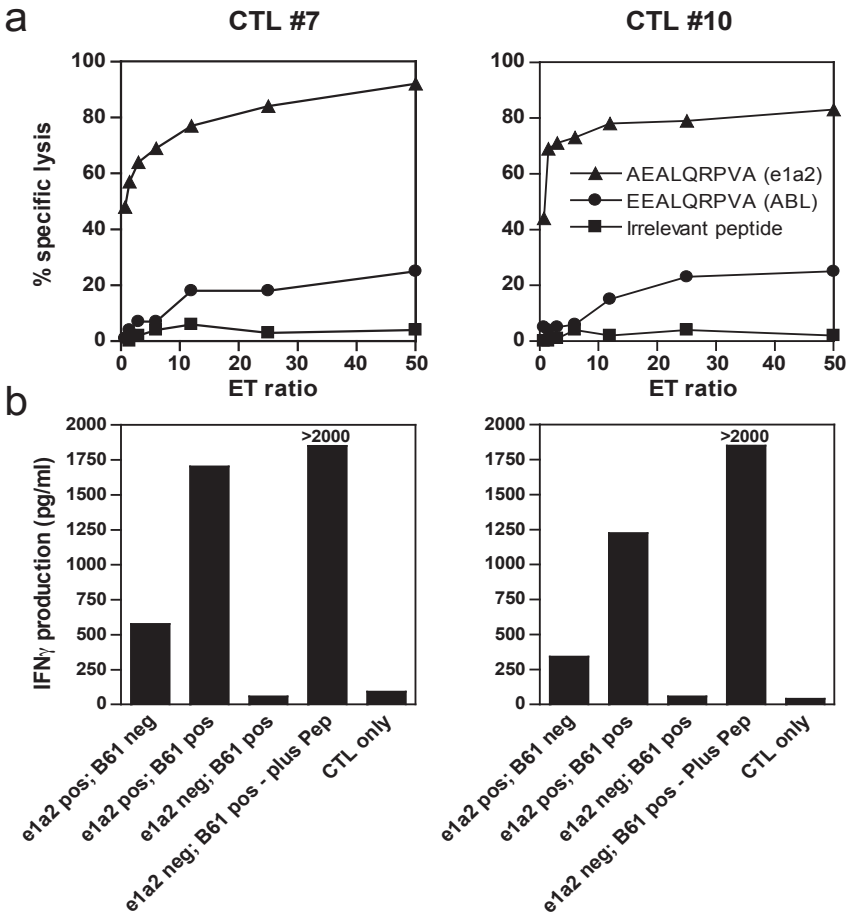

Figure 2 HLA-B61 restricted recognition of the endogenously processed e1a2 epitope AEALQRPVA on ALL cell line TOM1 by two CTL clones. Panel (**a**): lysis by CTL clones #7 and #10 anti-e1a2^{AEALQRPVA} of ^{51}Cr-labeled B-LCL Sweig007 (homozygously expressing HLA-B61) loaded with 5 μM of either the relevant peptide (▲), an irrelevant HLA-AB61 binding peptide (■) or ABL a1a2 peptide EEALQRPVA (●) at ET ratios ranging from 50 to 0.75. Panel (**b**): recognition by CTL clones #7 and #10 anti-e1a2^{AEALQRPVA} of ALL-derived TOM1 cell line expressing HLA-B61 (e1a2 pos; HLA-B61 pos), TOM1 transfected with the empty vector (e1a2 pos; HLA-B61 neg), and B-LCL Sweig007 (e1a2 neg; HLA-B61 pos) without peptide or Sweig007 loaded with EEALQRPVA. Readout is interferon-γ production by CTL as measured with ELISA. Results of one representative experiment out of three performed are shown.

including negatively scoring length- and shift variants of predicted binders that harbor only one anchor residue in our assays, we reduced the risk of missing fusion peptides with binding capacity. Therefore, and because we screened the fusion regions for binding motifs of all HLA class I molecules, it is unlikely that a significant number of class I binding BCR-ABL fusion peptides remains to be identified.

Apart from HLA class I binding and TAP translocation, of which the latter is less restrictive,[63] the third and foremost important event defining a CTL epitope is its intracellular enzymatic generation. As discussed in recent reviews, N-terminal extensions of epitope precursors can be trimmed by a diversity of aminopeptidases, but, owing to the absence of carboxypeptidases in the cytosol and endoplasmic reticulum, the precise C-terminal excision by the proteasome is an established sine qua non for the far majority of CTL epitopes.[33,34,64] To date, only one defined exception to this rule has been reported.[65] Our proteasome digestion analysis enabled an appraisal of the endogenous generation of all already published and newly identified HLA class I binding BCR-ABL fusion peptides and showed that the 28 different peptides fall into three major groups (Tables 3 and 4).

The first group consisted of 11 peptides that lacked enzymatic C-terminal excision by the proteasome, which precludes their natural presentation.[28,33] Among these peptides are b3a2^{SSKALQRPV} (in HLA-A2)[20] and b3a2^{GFKQSSKAL} (in HLA-B8)[22] that were already included in the BCR-ABL peptide vaccines.[25–27] The absence of proteasomal excision of these peptides, however, is hard to reconcile with their natural presentation and, therefore, we score them as inappropriate for immunotherapeutic targeting.

The second group included seven fusion peptides that are excised after their C-terminus by a minor cleavage site. Therefore, although likely produced intracellularly, their cell

surface density is expected to be low (Table 4). In this category especially the b3a2 peptides HSATGFKQSSK and ATGFKQSSK, binding in HLA-A3/A11[14] and now also demonstrated to bind to HLA-A68, are important because they were included in published vaccines.[25–27] Efficient induction and full activation of CTL responses in patients vaccinated with these peptides may enable subsequent lysis of leukemic HLA-A3/A11/A68$^+$ target cells expressing those peptides at low densities, because for the elicitation of CTL effector functions a much lower epitope density (100- to 1000-fold lower) than for full T-cell activation and proliferation is required.[66–68] A distinct disadvantage, however, of immunotherapeutic targeting of low-density epitopes is that either lower cytotoxic levels are reached[69] or that for equally efficient killing a higher T-cell receptor affinity is required than for targeting abundantly expressed epitopes,[70] which limits the availability of T cells with a sufficient affinity.

The third group consisted of the remaining 10 peptides (34%) that were efficiently liberated from their C-terminal flanking region, because major cleavage sites were observed after their C-terminal residues Arg-18 or Ala-21 (Table 3). Consequently, these 10 peptides are expected to be abundantly produced in living cells. In this group, especially the eight peptides with high or intermediate HLA class I binding affinity, namely b3a2$^{10\text{-}20}$ (KQSSKALQR), binding in HLA-A3[14,24] and b3a2$^{9\text{-}18}$ binding in HLA-B27[16] and six of the newly identified HLA-A68 and HLA-B61 binding peptides (from b2a2 and e1a2), are predicted to be high-density cell surface expressed CTL epitopes (Table 4). For that reason, these eight peptides are to date the most favored vaccine candidates.

Unequivocal evidence for the natural cell surface presentation of BCR-ABL fusion peptides until now has only been reported for b3a2^{KQSSKALQR}, because it has been identified by Clark *et al.*[24] in peptides eluted from HLA-A3 expressed on CML cells. This is in line with the abundant proteasome-mediated cleavage after its C-terminus (Arg-18; Table 3) and warrants its application in vaccine trials.[25–27] We now demonstrate by CTL recognition of leukemic cells that e1a2^{AEALQRPVA} is a second proven BCR-ABL fusion region derived CTL epitope presented in HLA-B61 (Figure 2).

Future biochemical and T-cell studies have to confirm cell surface expression and immunogenicity of the other predicted CTL epitopes (Table 4). This will help to further establish the suitability of these epitopes for peptide vaccines. Importantly, in our previous work, using the same strategy, four out of four predicted CTL epitopes that were C-terminally excised by a major proteasomal cleavage were found to be both immunogenic and cell surface expressed.[28] The reliability of the current extended reverse immunology strategy has thereafter been further validated by other groups;[36–38] for example, six out of six predicted CTL epitopes from human proinsulin were proved to be immunogenic and cell surface expressed,[37] implying a high degree of accuracy in epitope prediction.

We ré-evaluated binding of BCR-ABL fusion peptides in HLA-A*0201 (Table 2). The near absence of binding capacity of b3a2^{SSKALQRPV} in HLA-A2 in our hands was in concordance with earlier studies[15,18,19] but in contradiction with Yotnda *et al.*[20] The latter study used the same competitor peptide as we did (HBV cAg 18–27), but tested b3a2^{SSKALQRPV} at 20 mM, whereas our highest concentration was 100 μM, which may explain the discrepancy. We only found intermediate, but unstable, binding capacity of b3a2$^{10\text{-}20}$ and b2a2$^{8\text{-}16}$ for HLA-A2 and low affinity, unstable binding of e1a2^{GAFHGDAEAL}. Recently, Pinilla-Ibarz *et al.*[71] designed analogue HLA-A2 binding BCR-ABL fusion peptides with improved binding capacity that were found to efficiently induce CTL cross-reacting with the native peptide. According to our proteasome digestions, showing a cleavage site after Leu-16, the b2a2 analog YLINKEEAL may induce T cells that recognize the naturally expressed native b2a2$^{8\text{-}16}$ sequence (LTINKEEAL, IC_{50} 29 μM; Table 2) which is therefore a candidate vaccine peptide. The unstable binding of LTINKEEAL (Table 2) in conjunction with its C-terminal excision by only a minor proteasomal cleavage site (Table 3), will certainly negatively influence its cell surface density, but it still may be a cell surface expressed epitope that can be used for immunotherapeutic targeting. Unstable binding in principle may not preclude elicitation of T-cell cytotoxic effector functions, because killing can already be triggered by short time engagement with the target cell.[66,68] In contrast, full T-cell activation and proliferation is only induced after sustained interactions with epitopes at higher density,[68] and, therefore, unstable low-affinity binding peptides are not suitable as vaccine peptides; analogues with improved binding capacity indeed might serve as the solution for this problem.[71]

The cleavage site in b3a2, b2a2 and e1a2 that predominates occurs after Ala-21. Therefore, the five fusion peptides from b2a2 and e1a2 that bind avidly in HLA-B61 and share Ala-21 as their C-terminus are expected to be efficiently expressed on leukemic cells (Table 4). However, in this group, b2a2$^{EEALQRPVA\text{-}21}$ and b2a2/e1a2$^{EALQRPVA\text{-}21}$ equal ABL a1a2 sequences, and the other peptides (b2a2$^{12\text{-}21}$, e1a2$^{11\text{-}21}$ and e1a2$^{13\text{-}21}$) differ only N-terminally from normal ABL peptides that have equally good binding capacities (Table 1). Thus, immunotargeting of these peptides requires either absence of cross-reactivity of reactive T cells towards the corresponding ABL peptides or, alternatively, differential antigen processing leading to preferential presentation of these peptides when excised from b2a2 and e1a2. The CTL induced against e1a2$^{13\text{-}21}$ (AEALQRPVA) showed near absence of cross-reaction with ABL a1a2 sequence EEALQRPVA (Figure 2a). Importantly, our study also revealed a differential processing effect that was dependent on the proteasome subtype (Table 3). In the a1a2 (ABL) region, the cleavage after Arg-18 was absent and the cleavage after Ala-21 was strongly reduced, when digested with leukemic immunoproteasomes. This will lead to a leukemia specific high-density presentation of the HLA-B61 binding peptides when excised from b2a2 and e1a2 and thus their applicability in immunotherapy. Moreover, as discussed by Sun *et al.*,[72] in leukemic cells the BCR-ABL protein is expressed in addition to the ABL and BCR products, which will also contribute to the preferential expression of these peptides on leukemia cells.

We are the first to define HLA class I binding of peptides in the e1a2 region. Results of DLT after SCT in ALL are in general disappointing.[1] However, the specific targeting of CD8$^+$ T-cell epitopes in the e1a2 fusion region may contribute to successful immunotherapy of ALL (e.g. by vaccination or adoptive T-cell transfer). The identification of e1a2^{AEALQRPVA} as an HLA-B61 presented CTL epitope now enables such endeavors.

Negative associations of HLA-A3[73] and recently also HLA-A68 and HLA-B61 (HLA-B*40)[74] with BCR-ABL transcripts have been reported. This suggests natural immunity and CTL-mediated immunosurveillance against BCR-ABL peptides presented in these HLA class I molecules. The reported natural presentation of b3a2^{KQSSKALQR} in HLA-A3[24] is in line with the protection effect of HLA-A3. Importantly, our current data identifying e1a2^{AEALQRPVA} as an HLA-B61 presented CTL epitope (Figure 2) and several other b2a2 and e1a2 fusion peptides as predicted HLA-B61 and HLA-A68 presented epitopes with high cell surface density (Tables 1, 3 and 4) are

now also providing a basis for the protection against Ph^+ leukemia conferred by HLA-A68 and HLA-B61.[74]

In the last decade, numerous studies have extensively elaborated immunity against BCR-ABL fusion regions. This previous work in conjunction with the current study, applying a reliable and systematic extended reverse immunology strategy, renders it unlikely that a significant number of candidate CTL epitopes in the BCR-ABL fusion regions still remains to be identified, particularly when taking into account their short length with only two major proteasomal cleavage sites in the a2-exon encoded region.

We conclude that the patient population coverage of (predicted) CTL epitopes in BCR-ABL fusion regions is not complete owing to the only moderate cumulative prevalence of the presenting HLA class I molecules in combination with the different BCR-ABL variants (Table 4). The relevance of our study lies, therefore, not only in the identification of the best candidate vaccine peptides among the combined set of old and novel HLA class I binding BCR-ABL fusion peptides, but also in the conclusion that a defined and broadly applicable immunotherapeutic vaccine for Ph^+ leukemia will benefit from the identification of CTL epitopes in other leukemia specific antigens, next to those identified in BCR-ABL fusion regions.

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CHAPTER 6

CHAPTER 3

Submitted for publication

Novel antigen-processing pathways for cytotoxic T cell recognition

Jan H. Kessler[1], Selina Khan[1], Ulrike Seifert[2], Sylvie Le Gall[3], K. Martin Chow[4], Sandra A. Bres-Vloemans[1], Arnoud de Ru[1], Nadine van Montfoort[1], Willemien E. Benckhuijsen[1], Jill M. Brooks[5], Thorbald van Hall[6], Kallol Ray[4], Ilias I.N. Doxiadis[1], Paul F. van Swieten[7], Hermen S. Overkleeft[7], Annik Prat[8], Birgitta Tomkinson[9], Jacques Neefjes[10], Peter M. Kloetzel[2], David W. Rodgers[4], Louis B. Hersh[4], Jan W. Drijfhout[1], Peter A. van Veelen[1#], Ferry Ossendorp[1#], Cornelis J. M. Melief[1#]

[1] Dept. of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands. [2] Institut für Biochemie-Charité, Medical Faculty of the Humboldt University Berlin, Berlin, Germany. [3] Partners AIDS Research Center, Massachusetts General Hospital, Harvard Medical School, Boston, USA. [4] Dept. of Molecular and Cellular Biochemistry, and Center for Structural Biology, University of Kentucky, Lexington, USA. [5] Cancer Research UK Institute for Cancer Studies, University of Birmingham, Birmingham, United Kingdom. [6] Dept. of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands. [7] Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands. [8] Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montreal (IRCM), Montreal, Canada. [9] Dept. of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden. [10] Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands. [#] Contributed equally to this study.

ABSTRACT

Cytotoxic T lymphocytes (CTL) kill cells through recognition of microbial or cancer-associated antigenic peptides presented by HLA class I molecules on the cell surface. For binding to class I molecules these peptides need to have an appropriate C-terminal anchor residue, which is classically generated by the proteasome complex.

Here we identify two cytosolic endopeptidases that complement the proteasome. Nardilysin contributes not only to C-terminal but also to N-terminal generation of HLA class I presented peptides. Thimet oligopeptidase (TOP) acts as a C-terminal trimming endopeptidase in antigen processing. We define a novel mechanism by which these peptidases cooperatively produce the C-terminus of a tumor-specific CTL epitope.

The actions of nardilysin and TOP broaden the antigenic peptide repertoire by shaping non-functional protein degradation products into class I ligands, thereby strengthening the immune defense against intracellular pathogens and cancer.

INTRODUCTION

Short peptides (8–12 aa) presented by HLA class I molecules, called CTL epitopes when recognized by $CD8^+$ cytotoxic T lymphocytes (CTL), are produced during the continuous turnover in the cytosol of full length and misfolded proteins into peptides and eventually free amino acids [1-3]. This degradation process is accomplished by the multicatalytic proteasome complex and by cytosolic peptidases [1,3]. Tripeptidyl peptidase II (TPPII) [4], TOP [5,6] and aminopeptidases [1,2] have been implicated in degradation, but the contribution of other (endo)peptidases has remained elusive. A few intermediate length degradation products (on average 8–16 aa) escape further cytosolic destruction through translocation via the TAP transporter into the endoplasmic reticulum (ER), where they can be trimmed N-terminally by ERAAP/ERAP1 [1,2] and assemble with HLA class I heterodimers before being routed to the cell surface.

The absence of C-terminal excision of CTL epitopes in proteasome-inhibited cells [7,8], together with a failure to detect C-terminal trimming activities in the cytosol or ER, has led to the current notion that solely the proteasome liberates the exact C-terminus [1,2]. Cytosolic endopeptidases, however, may also produce peptides with a C-terminus fit for class I binding from protein degradation products lacking such an anchor. Indeed, cells treated with proteasome inhibitors still express significant levels of peptide-loaded HLA class I molecules [9-13]. Although these compounds are known to not inhibit completely, this suggests the existence of a partially proteasome-independent pathway of class I ligand generation. So far only TPPII reportedly produces the C-terminus of a defined CTL epitope (from HIV Nef) [14], but a broad role of TPPII in the generation of class I ligands is considered unlikely [15]. Other peptidases liberating the C-terminus of HLA class I ligands are not known. To define such peptidases, we began an in-depth investigation of the generation of a CTL epitope with an unambiguously proteasome-independent C-terminus.

RESULTS

Cleavage C-terminally to a lysine is not readily accomplished by proteasomes [16]. In line with this, expression of HLA-A3, a class I molecule that binds peptides with a C-terminal lysine, is particularly insensitive to proteasome inhibition [9,12]. Therefore, we searched for a CTL epitope with a proteasome-independent C-terminus presented in HLA-A3. The nonamer ELFSYLIEK ($PRA^{190-198}$) from tumor-associated protein PRAME [17] was selected by binding prediction and bound with high affinity to HLA-A3 (Suppl. Fig. 1). Digestion of 27-mer $PRA^{182-208}$ with purified 20S proteasomes showed no cleavage after the epitope's C-terminal Lys-198 or in the region flanking the C-terminus. In contrast, its N-terminus was liberated by an efficient proteasomal cleavage before Glu-190 (Fig. 1A). Tandem mass spectrometry (MS/MS) identified the ELFSYLIEK peptide in peptides eluted from HLA-A3 on the surface of cell line K562-A3 (K562, $PRAME^+$, transfected with HLA-A3) (Suppl. Fig. 2). Also, the CTL clone raised against the $PRA^{190-198}$ epitope exogenously loaded on HLA-A3, efficiently recognized K562-A3 and other tumor cell lines expressing PRAME and HLA-A3 (Fig. 1B) confirming natural presentation of the epitope. The CTL also recognized the exogenously loaded 11-, 12- and 13-meric C-terminally extended variants of the epitope (ELFSYLIEKVK/R/K), but these peptides – while possessing HLA-A3 binding affinity (Suppl. Fig. 1) – are not naturally presented (Suppl. Fig. 2). We used this feature of the CTL

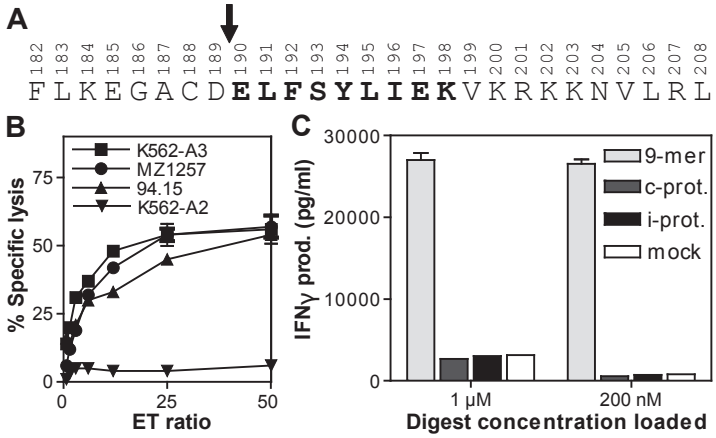

Figure 1. PRA$^{190-198}$ is an HLA-A3 presented CTL epitope with proteasome-independent C-terminus.

A: Proteasomal digestion of 27-mer PRA$^{182-208}$ (ELFSYLIEK, bold). Digestion (1 h incubation) by immuno- or constitutive human 20S proteasomes gave comparable results. Bold arrow indicates the efficient cleavage before Glu-190. Fragments containing Asp-189 at the C-terminus or complementary fragments added up to >7% of the digested material (1 h digest) as determined by mass spectrometrical analysis. Digestions were repeated three times with similar results.
B: Recognition by CTL anti-ELFSYLIEK of erythroleukemia cell line K562-A3 and renal cell carcinoma (RCC) lines MZ1257 and 94.15 (all PRAME$^+$ and HLA-A3$^+$). K562-A2 (HLA-A2$^+$) was not recognized.
C: Absence of recognition of 25-mer PRA$^{190-214}$ (ELFSYLIEKVKRKKNVLRLCCKKLK, epitope at N-terminus) digested for 1 h with immuno- or constitutive proteasomes (SEM of triplicate wells). Digests were titrated, loaded on EKR cells (HLA-A3$^+$) and co-incubated with CTL anti-ELFSYLIEK. Background recognition is 25-mer substrate incubated at 37°C without enzyme (mock digest), positive control is the equally titrated ELFSYLIEK peptide (9-mer).

to monitor the generation of the epitope and its C-terminally extended variants in digests of longer PRAME peptides. The proteasomal digests of 25-mer PRA$^{190-214}$ (ELFSYLIEK at its N-terminus) loaded onto PRAME-negative HLA-A3$^+$ cells were not recognized by the CTL (Fig. 1C), confirming proteasome-independence of the epitope's C-terminus.

Identification of nardilysin

Therefore, we searched for the peptidase(s) generating the C-terminus of the ELFSYLIEK epitope. First, CTL recognition was strongly inhibited when K562-A3 cells were treated with the metallopeptidase inhibitor o-phenanthroline, whereas other inhibitors had no effect (Fig. 2A). Since the TPPII inhibitor butabindide can be inactivated by serum [4], we further excluded the potential involvement of TPPII [14,18] by digestion analysis and RNA interference (RNAi) (Suppl. Fig. 3).
Second, in order to identify the peptidase, we exposed the internally quenched fluorescent 15-mer substrate **ELFSYL(-dab)IEK**VKRC(-FL)KN (PRA$^{190-204}$, epitope in bold) to digestion with cytosolic fractions (separated by anion exchange chromatography) of K562-A3 cells. Fraction #37 cleaved, in a phenanthroline-sensitive fashion, between the quencher dabcyl at Lys-195 and the fluorescein (FL) group at position 202, thereby releasing fluorescence (Fig. 2B). This fraction contained nardilysin as the only peptidase among five proteins identified by MS/MS (Fig. 2B, Suppl. Fig. 4). Nardi-

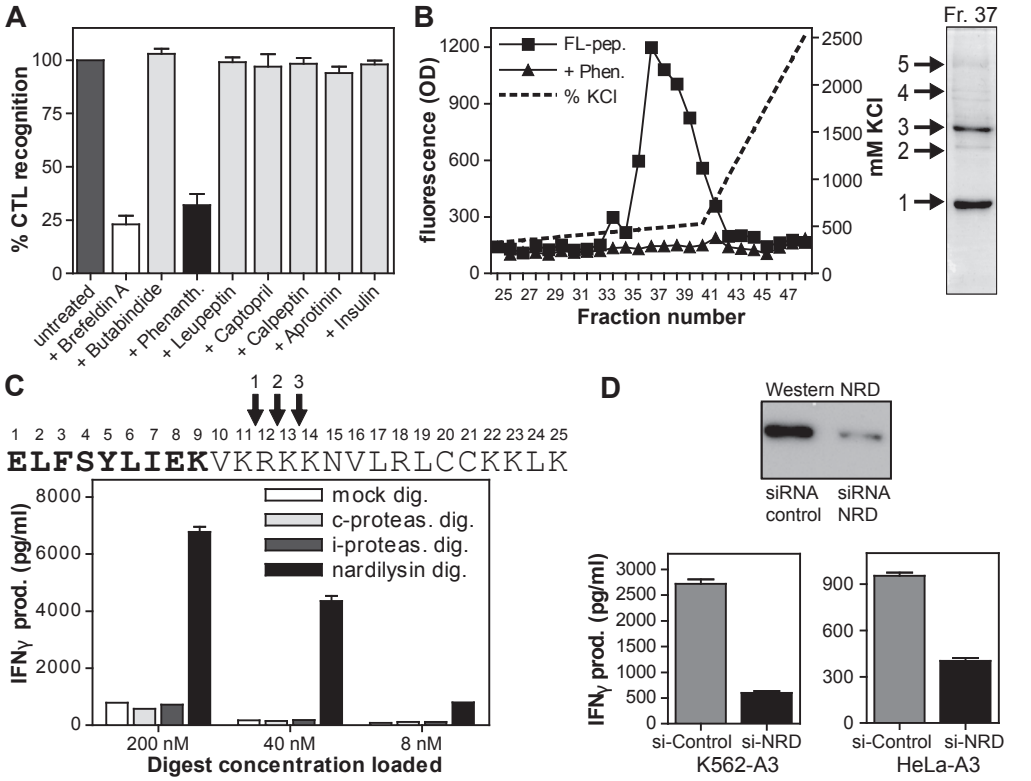

Figure 2. Nardilysin produces C-terminal extended precursors of the PRA$^{190-198}$ epitope.

A: K562-A3 was stripped and untreated (100% recognition) or treated with indicated inhibitors (concentration and activity, see methods section) and co-incubated with the CTL clone (SEM of three experiments).

B: Cytosolic fractions eluted from an anion exchange column (KCl-gradient indicated; see Methods for details) of K562-A3 were co-incubated for 30 min with fluorogenic substrate ELFSYL(-dabcyl)IEKVKRC(-FL)KN in the absence (square symbol) or presence (triangle) of phenanthroline (1 mM). The same substrate with the first three aa being D-aa's to block N-terminal trimming showed only slightly reduced fluorescence (data not shown). Fraction no. 37 was separated by SDS-PAGE and the bands were isolated. Proteins were identified by MS/MS as: (band 1) high mobility protein, (2) keratin, (3) α- & β-tubulin, (4) no matches and (5) nardilysin (see Suppl. Fig. 4).

C: Digestion of 25-mer PRA$^{190-214}$ (epitope, bold) with purified nardilysin, immuno-proteasomes or constitutive proteasomes for 30 min. Nardilysin cleavages are shown: efficient cleavage behind the 11-mer was present in 33.9% of digestion-fragments (arrow 1), behind the 12-mer in 41.2% of the fragments (arrow 2) and behind the 13-mer in 16.9% of the fragments (arrow 3). Digests (nardilysin, proteasome, mock) were titrated, loaded on HLA-A3$^+$ cells and co-incubated with the CTL to assess generation of epitope-precursors.

D: CTL recognition of K562-A3 and HeLa-A3 cells stably transfected to express siRNA suppressing nardilysin (NRD) or non-targeting control siRNA (SEM of triplicate wells of representative experiment). Immunoblot with nardilysin antibody of K562-A3 transfectants is shown.

lysin, hitherto not implicated in class I antigen processing, is a cytosolic endopeptidase of the pitrilysin family of zinc-metalloproteases and is ubiquitiously expressed (see ref. 19 and the UniGene database at: http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.584782).

Third, the digest of 25-mer PRA$^{190-214}$ (**ELFSYLIEK**VKRKKNVLRLCCKKLK) with purified nardilysin showed highly efficient production of C-terminally extended 11-, 12- and 13-mer variants of the epitope by efficient cleavages after Lys-200, Arg-201 and Lys-202 (Fig. 2C), in accordance with the cleavage specificity of nardilysin before or between two basic residues [20,21] in substrates up to ~30 aa in length. Indeed, the CTL efficiently recognized the nardilysin-digest, due to the presence of the ELFSYLIEKVK/R/K fragments, whereas the proteasomal digest was not recognized (Fig. 2C). Fourth, nardilysin was required in cells for epitope generation because small inhibitory RNA (siRNA)-mediated suppression of nardilysin in K562-A3 and HeLa-A3 (HeLa, PRAME$^+$, with HLA-A3) strongly reduced their recognition by the CTL (Fig. 2D), and overexpression of nardilysin in HeLa-A3 cells enhanced CTL recognition (Suppl. Fig. 5). Together, these findings implicate nardilysin as indispensable for the endogenous C-terminal pre-processing of the PRA$^{190-198}$ epitope by producing cleavages after Lys-200, Arg-201 and Lys-202, but it does not excise the exact C-terminus (Lys-198).

Identification of thimet oligopeptidase

Next we pursued identification of the peptidase that generates the correct C-terminus of the epitope. Because of their cytosolic location, ubiquitous expression and specificity, prime candidates were TOP, neurolysin and insulin-degrading enzyme. Only the RNAi-mediated suppression of TOP in K562-A3 strongly reduced CTL recognition, whereas suppression of the other peptidases had no effect (Fig. 3A), indicating TOP's involvement in the endogenous generation of ELFYSLIEK. Moreover, purified TOP cleaved efficiently directly behind the epitope's C-terminal Lys-198 in the nardilysin-dependent 12- and 13-mer precursors (PRA$^{190-201/202}$; ELFSYLIEKVKR/K). Thus, TOP released three or four C-terminal residues, in accordance with its cleavage preference [22,23], thereby efficiently producing the exact nonameric epitope (Fig. 3B). Indeed, the TOP-digest of 12-mer PRA$^{190-201}$ loaded on target cells was recognized almost as efficiently as the minimal epitope (Fig. 3C). In line with its preference for peptides 6 to 17 aa in length [24], longer peptides were not cleaved by TOP (Suppl. Fig. 6B, and data not shown), indicating that pre-processing by nardilysin into the 12-mer and 13-mer is required to make epitope-precursors susceptible to TOP. This was confirmed in the co-digestion of 25-mer PRA$^{190-214}$ with nardilysin and TOP, which resulted in the production of ELFSYLIEK (identified by mass spectrometry, data not shown). Accordingly, the double-digest was recognized more efficiently by the CTL – due to the presence of the epitope – than the digest with nardilysin alone which contained only the precursors (Suppl. Fig. 6B). Thus, the C-terminus of the ELFSYLIEK epitope is released through sequential cleavages by nardilysin and TOP.

Generation of the N-terminus of the PRA$^{190-198}$-epitope

The N-terminus of the epitope is generated by an efficient caspase-like proteasomal cleavage before Glu-190 (Fig. 1A). This cleavage was completely blocked by the specific inhibitor AcAlaProNleLeuVSOH [25] of the caspase-like proteasome activity as shown in a digestion of

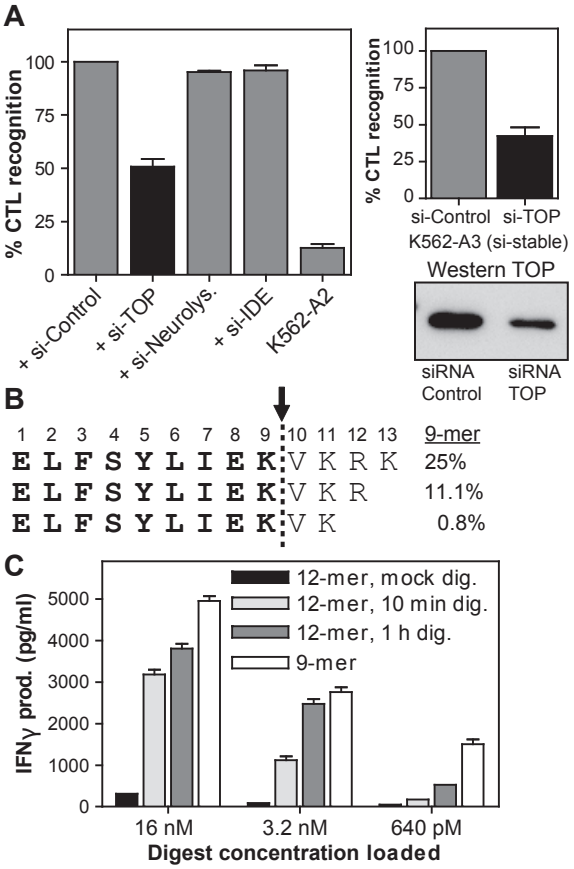

Figure 3. TOP produces the C-terminus Lys-198 of the epitope.

A: Left: CTL recognition of K562-A3 transiently transfected with pools of siRNA duplexes suppressing TOP, neurolysin and insuline degrading enzyme (IDE) or non-targeting control siRNA (100% reference recognition). Right: CTL recognition of K562-A3 stably transfected to express siRNA suppressing TOP or non-targeting control siRNA. SEM of three or four experiments. Immunoblot with TOP antibody of stably transfected K562-A3 variants is shown.

B: Digestion of 13-, 12- and 11-mer substrates PRA$^{190\text{-}202/201/200}$ (ELFSYLIEKVK/R/K, epitope bold) with purified TOP; the substrates are shown. Bold arrow indicates the efficient cleavage behind Lys-198. From each substrate the % of fragments in the digest (including non-digested substrate) that match the ELFSYLIEK-epitope after 30 min digestion are listed.

C: CTL recognition of 12-mer PRA$^{190\text{-}201}$ (ELFSYLIEKVKR) digested by TOP for 10 min or 1 h. The digests were titrated and loaded on HLA-A3^{+} EKR cells. The non-digested 12-mer (mock dig.) is not recognized at these concentrations. Recognition of equally titrated ELFSYLIEK peptide (9-mer) is the positive reference.

27-mer PRA$^{172\text{-}198}$ (Suppl. Fig. 7A,B). Indeed, CTL recognition of cells treated with this inhibitor was strongly reduced (Suppl. Fig. 7C). The general proteasome inhibitor epoxomycin only partially inhibited cleavage before the N-terminus (Suppl. Fig. 7A,B). Interestingly, when cells were treated with epoxomycin the presentation of ELFSYLIEK was markedly enhanced (Suppl. Fig. 7C), likely due to reduced competition with epoxomycin-sensitive HLA-A3 ligands.

TAP-dependence of PRA$^{190\text{-}198}$

Thus, together, the proteasome, nardilysin and TOP produce the minimal epitope in the cytosol (schematic in Suppl. Fig. 8A). As expected, presentation of the epitope was completely dependent on transport into the ER by TAP (Suppl. Fig. 9A). The ELFSYLIEK peptide was efficiently transported by TAP into the ER, whereas the 12-, and 13-mer epitope-precursors were less efficiently translocated (Suppl. Fig. 9B). Therefore, these nardilysin-produced precursors are available in the cytosol for processing by TOP, whereas the minimal epitope – after its production – can rapidly escape from cytosolic destruction by translocation into the ER.

General roles of nardilysin and TOP in class I antigen processing

This is the first evidence implicating nardilysin and TOP in CTL epitope production. Do these enzymes, either apart or together, have a more general role in class I antigen processing?

Nardilysin in principle can support the generation of both the C-terminus and the N-terminus of epitopes (Suppl. Fig. 8B). A bioinformatics analysis of all 1620 published ligands of the most prevalent class I molecules with their flanking regions revealed the dibasic cleavage motif of nardilysin [20,21] directly at the N-terminus or C-terminus in 15% of the ligands (Table 1). The motif is especially frequent directly at the (basic) C-terminus of ligands presented by the HLA-A3 supertype (-A3, -A11, -A68) and at the N-terminus of HLA-B27 ligands (Tables 1 and 2). We then examined whether purified nardilysin actually excised prototypical HLA-A3 and -B27 ligands at the motif sites. Indeed, the C-termini of two HLA-A3-presented overlapping immunodominant epitopes from HIV-1 Gag p17 [26] were efficiently produced (Fig. 4A) whereas the proteasome failed to do so (data not shown). Importantly, over 35% of all HLA-B27 ligands contain a basic residue at position 1 (P1) next to their dominant class I binding Arg-anchor at P2, together constituting a nardilysin cleavage-site (Tables 1 and 2). Nardilysin efficiently cleaved directly before the N-terminus of eight out of eight HLA-B27 ligands tested and in three cases produced the epitope's C-terminus; among these are immunodominant viral epitopes from HIV-1 gag p24 and EBNA3C [27] (Fig. 4B,C). Noteworthy, four of these ligands were identified previously to be proteasome-independent as studied with proteasome inhibitors (the others were not tested) [13].

To demonstrate nardilysin-dependence of these epitopes during processing in cells, nardilysin was suppressed by RNAi in EBNA3C-positive target cells resulting in strongly reduced recognition by CTL against the HLA-B2705-presented EBNA3C$^{258\text{-}266}$ (RRIYDLIEL) epitope [27] (Fig. 4D). Endogenous processing of the minimal epitope with its N-terminal flanking region confirmed the nardilysin-dependence to reside in the N-terminal epitope processing (Fig. 4D). Thus, although several aminoeptidases exist in the cytosol and ER, nardilysin is required for the N-terminal liberation of the EBNA3C$^{258\text{-}266}$ epitope. Our results help to explain the strong over-representation of Arg and Lys at P1 in HLA-B27 ligands, residues that contribute at that position only modestly to binding capacity but enforce peptide stability in the cytosol [28].When nardilysin cleaves before a basic doublet, it may also liberate ligands with a non-basic C-terminus presented by e.g. HLA-A1, -A2, -B8 and HLA-B35 (Suppl. Fig. 8B). In addition, the dibasic motif is often present within 4 aa of the N- or C-terminus (Table 1), suggesting a multi-step mechanism of epitope-production. The motif is present directly at or within 4 aa of the N- or C-terminus in 26% of the 1620 ligands analyzed (Table 1). Because nardilysin can cleave sometimes before mono-basic sites [21] or at atypical sites (see e.g. Fig. 4A: ASFDKAKLK-epitope), its influence may reach beyond the dibasic motif. Nardilysin may also destroy certain potential class I binding peptides. In vaccine design, the deliberate insertion of the nardilysin cleavage motif between CTL epitopes in poly-epitope sequences may be useful to promote vaccine efficacy.

TOP has been considered to destroy epitopes [6], thereby limiting class I antigen presentation [5] and raising the question whether the excision of ELFSYLIEK by TOP is an exception. The preferred action of TOP is the release of three to five C-terminal residues [22,23] with a broad sequence specificity [24,29] from substrates up to 17 aa long [24], endowing TOP with potential C-terminus generating capacity. Furthermore, an undefined epitope-promoting effect of TOP has been reported [30].

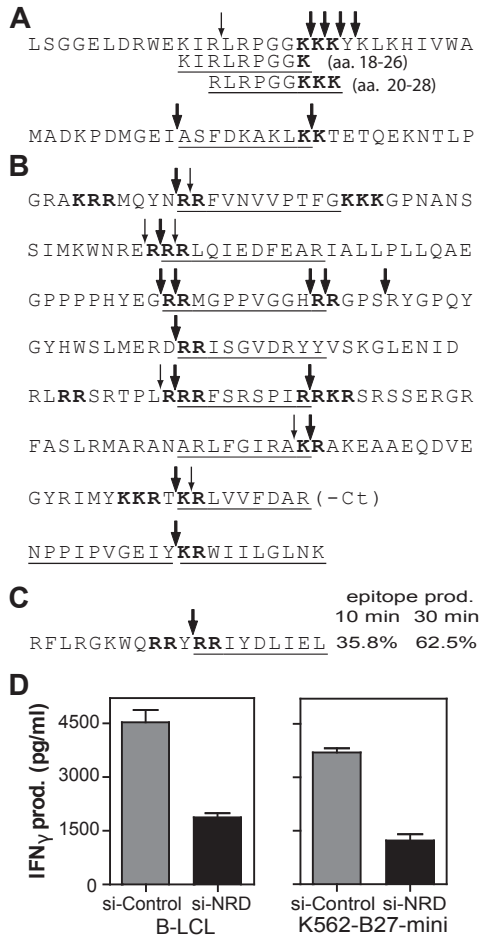

Figure 4. The role of nardilysin in HLA class I antigen processing.

Digestions by purified nardilysin of prototypical HLA-A3, -A11 and -B27 presented peptides. Bold residues constitute nardilysin-cleavage motif. Digestion time was 30 min. Bold arrow, cleavage present in >5% of the digestion-fragments; thin arrow, cleavage present in ≤ 5% of fragments.
A: Digestion by purified nardilysin of (upper) 30-mer HIV-1 gag p17 aa 8-37 encompassing two overlapping HLA-A3-presented CTL epitopes (separately depicted, underlined) and (lower) 30-mer thymosin-β aa 1-30 encompassing HLA-A11-presented ligand ASFDKAKLK (underlined). These examples were taken from the SYFPEITHI database (www.syfpeithi.de).
B: Digestion by purified nardilysin (30 min) of long peptides encompassing eight published HLA-B2705 presented ligands (underlined) from the SYFPEITHI database in their natural flanking regions. Ligands are (top to bottom): 40S ribosomal protein S30 aa 40-51; Cell death-regulatory protein GRIM19 aa 57-67; Ribonucleoprotein aa 312-322; NADH-ubiquinone oxidoreductase aa 52-61; Son3 DNA-binding protein BASS 1 aa 2026-2034; 60S ribosomal protein L13 aa 188-196; DNA-directed RNA polymerases 7 kDa polypeptide aa 50-58; and HIV-1 gag p24 aa 265-274, which is an immunodominant CTL epitope presented in HLA-B2705 (KRWIILGLNK) adjacent to the HLA-B35 presented CTL epitope NPPIPVGEIY (at the left side).
C: Digestion by purified nardilysin of 20-mer RFLRGKWQRRYRRIYDLIEL (EBNA3C aa 246-266, HLA-B2705 presented epitope underlined). Production of the epitope after 10 and 30 min incubation is indicated.
D: Left panel: recognition by CTL anti-RRIYDLIEL of autologous B-LCL (HLA-B2705$^+$) expressing EBNA3C, stably expressing siRNA suppressing nardilysin or control siRNA. Right panel: CTL recognition of K562-B27-mini cell line, which is K562 expressing HLA-B2705 and minigene MFLRGKWQRRYRRIYDLIEL (EBNA3C aa 247-266, epitope underlined), with or without suppressed nardilysin by stable siRNA expression.

Therefore, we assessed the flexibility of TOP in producing different potential epitopes by systematically substituting the positions (P1 and P1') surrounding the site of TOP's cleavage creating the C-terminus of ELFSYLIEK. P1'-substitutions affected cleavage efficiency, but in all cases except for Pro at P1' which prevented digestion, production of the epitope (ELFSYLIEK) was >5% after 10 min of digestion (Suppl. Fig. 10A). Partial destruction by a subsequent cleavage in the middle of the epitope occurred, but did not prevent epitope-production (Suppl. Fig. 10A) as also shown by efficient CTL recognition of these digests (Suppl. Fig. 10B). Digestions of the P1-substituted variants,

on the other hand, resulted in generation of the 'epitope' (ELFSYLIE**X**) when P1 is either Lys (wild type sequence), Arg or Glu (Suppl. Fig. 10A), but not for other residues at this position. This was caused by inefficient cleavage after ELFSYLIE**X** for some P1-residues (e.g. Val, Ile, Asp) and/or by rapid efficient cleavage in the middle of the epitope (for e.g. Phe, Trp, Cys, Tyr). The necessity for a Lys, Arg or Glu at the P1-position, however, is not absolute. When we changed the epitope-sequence completely, TOP produced epitopes with e.g. a C-terminal Ala or Leu (SLYSFPEPEA and VLDGLDVLL [17]) (Suppl. Fig. 10C). These data indicate that TOP can produce epitopes from a wide array of C-terminally extended precursors.

Therefore, TOP's role in class I antigen processing is likely balanced between its anti-epitopic and pro-epitopic actions: on the one hand limiting presentation – by partial destruction – of epitopes whose correct C-terminus has already been made by (e.g.) the proteasome (like SIINFEKL [5,7]), and, on the other hand, producing correct C-termini fit for class I binding by trimming C-terminally extended epitope precursors that lack such an anchor. Importantly, this new model is in accordance with a recent analysis of cytosolic substrates and products of TOP, demonstrating that TOP both destroys and generates peptides of the length of class I ligands [31]. This study also showed, in line with our results, that many TOP-products have a Lys at the C-terminus.

The general implication of our study is that protein degradation products that are not fit for antigenic presentation due to lack of an appropriate C-terminal anchor residue can be processed by nardilysin and/or TOP to suit the binding requirements of HLA class I molecules. Because of the substrate length constraints of nardilysin and TOP, 30 aa and 17 aa respectively, prior to their action, protein hydrolysis by the proteasome or other proteolytic systems is likely required in most cases. The proportions of class I ligands that are dependent on nardilysin and TOP are not definitively determined, but our data indicate that both enzymes are capable of shaping and broadening the antigenic peptide repertoire, thereby expanding the options for successful immune responses to intracellular pathogens and cancer.

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TABLES

TABLE 1. Proportion of HLA class I ligands potentially excised by nardilysin.

HLA class I	Ligands[a]	C-dir.[b](%)	C-ind.[c](%)	N-dir.[d](%)	N-ind.[e](%)	Total[f](%)
HLA-A1	86	5.8	7.0	0.0	3.5	16.3
HLA-A2	515	1.7	5.0	2.5	4.5	13.7
HLA-A3	121	10.7	5.8	4.1	5.0	25.6
HLA-A11	71	11.3	2.8	1.4	7.0	22.5
HLA-A68	46	21.7	17.4	2.2	0.0	41.3
Cumulative A3-type[g]	***(238)***	***13.0***	***7.1***	***2.9***	***4.6***	***27.6***
HLA-A24	51	2.0	3.9	5.9	9.8	21.6
HLA-B7	68	2.9	7.4	1.5	4.4	16.2
HLA-B8	52	0.0	5.8	0.0	3.8	9.6
HLA-B2701	8	0.0	12.5	37.5	25.0	75.0
HLA-B2702	18	0.0	5.6	61.1	5.6	72.3
HLA-B2703	29	3.4	3.4	62.1	0.0	68.9
HLA-B2704	52	5.8	3.8	38.5	3.8	51.9
HLA-B2705	185	7.6	5.9	31.4	4.3	49.2
HLA-B2706	38	5.3	7.9	26.3	10.5	50.0
HLA-B2707	3	0.0	0.0	66.7	0.0	66.7
HLA-B2709	56	1.8	7.1	26.8	3.6	39.3
Cumulative B27[h]	***(389)***	***5.4***	***5.9***	***35.2***	***4.9***	***51.4***
HLA-B35	11	9.1	9.1	9.1	9.1	36.4
HLA-B3501	20	0.0	5.0	0.0	5.0	10.0
HLA-B44	53	3.8	1.9	1.9	15.1	22.7
HLA-B60 (B4001)	34	2.9	5.9	0.0	8.8	17.6
HLA-B61 (B4002)	7	0.0	14.3	0.0	0.0	14.3
HLA-B62 (B1501)	96	4.2	5.2	5.2	5.2	19.8
Cumulative	**1620**	**4.8**	**5.7**	**10.4**	**5.2**	**26.1**

[a] Number of ligands per class I molecule. For each allele, all ligands in the SYFPEITHI database were analysed for presence of NRD-motifs in the ligand and its N-term. and C-term. flanking regions.

[b] C-dir.; NRD-motif present leading to direct production of the C-terminus.

[c] C-ind.; indirect production of C-terminus: NRD-motif present within 4 aa of the C-terminus.

[d] N-dir.; NRD-motif present leading to direct production of the N-terminus.

[c] N-ind.; indirect production of N-terminus: NRD-motif present within 4 aa of the N-terminus.

[f] Percentage of ligands per allele that may be either N-term. or C-term. dependent on NRD cleavage.

[g] Cumulative percentages of NRD-motif containing ligands for HLA-A3, HLA-A11 and HLA-A68.

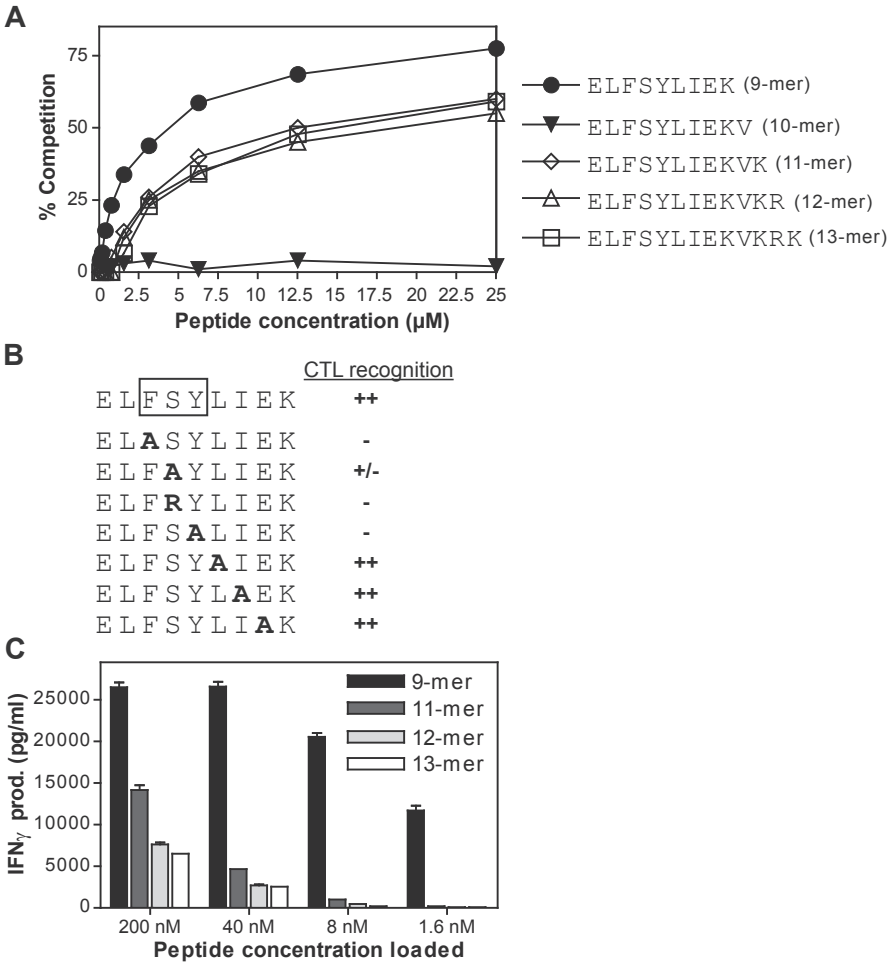
[h] Cumulative percentages of NRD-motif containing ligands for HLA-B27 subtypes.

TABLE 2. HLA-B27 ligands and nardilysin-cleavage motifs, exemplified for HLA-B2703.

Ligands in flanking regions and dibasic-motifs[a]	Source protein and position of ligand
KLQEEERE**RR**DNYVPEVSALDQEIIE	40S ribosomal protein S17 aa.79-87
VAEVDKVTGRFNGQFKTYAICGAI**RR**	40S ribosomal protein S21 aa.44-53
GLIKLVSKHRAQVIYTRNTKGGDAPA	40S ribosomal protein S25 aa.103-111
KNYDPQKD**KR**FSGTVRLKSTPRPKFS	60S ribosomal protein L10A (Csa-19) aa.46-54
NNLKARNSFRYNGLIH**RK**TVGVEPAA	60S ribosomal protein L28 aa.37-45
LFESWCTD**KR**NGVIIAGYCVEGTLAK	Cleavage and polyadenylation specif. Fact. 73kDa aa.348-357
AIRPQIDL**KR**YAVPSAGLRLFALHAS	DNA dep. protein kinase catal. subunit Q13 aa.263-272
DIPHALRE**KR**TTVVAQLKQLQAETEP	EIF-3 protein 6 aa.73-81
IPDVITYLSRLRNQSVFNFCAIPQVM	Farnesyl-diphosphate farneysltransferase 1 aa.278-286
VDLVRFTE**KR**YKSIVKYKTAFYSFYL	Farnesyl pyrophosphate synthetase aa.191-199
LEKIIQVGNRIKFVI**KR**PELLTHSTT	General transcription factor II aa.532-540
GTVALREI**RR**YQKSTELLI**RK**LPFQR	Histone H3.3 aa.52-60
EVECATQL**RR**FGDKLNFRQKLLNLIS	Immediate early response protein APR aa.30-38
VGEKIALS**RR**VEKHWRLIGWGQI**RR**G	Initiation factor eIF-2 gamma aa.444-452
VHFKDSQN**KR**IDIIHNLKLDRTYTGL	KIAA1197 aa.399-407
IWQLSSSL**KR**FDDKYTLKLTFISGRT	Microsomal sign. peptidase 25kDa Su aa.164-172
HWSLMERD**RR**ISGVDRYYVSKGLENI	NADH-ubiquinone oxidoreductase aa.52-60
SPDDKYSRHRITI**KKR**FKVLMTQQPR	Nop10p aa.44-52
DFDWNLKHGRVFIIKSYSEDDIHRSI	Ny-Ren antigen aa.410-418
PLLLTEEE**KR**TLIAEGYPIPTKLPLT	OASIS protein aa.267-275
MLSTILYS**RR**FFPYYVYNIIGGLDEE	Proteasome Su C5 aa.99-107
VKGPRGTL**RR**DFNHINVELSLLG**KKK**	Ribosomal protein L9 aa.35-43
SLVKGGLC**RR**VLVQVSYAIGVSHPLS	S-adenosylmethionine synthetase gamma aa.312-320
RGDSVIVVLRNPLIAGK *(*C-term. protein)	Small nuclear ribonucleoprotein SM D2 aa.110-118
KDSKTDRL**KR**FGPNVPALLEAIDDAY	SMC6 protein aa.478-487
PRLAILYA**KR**ASVFVKLQKPNAAIRD	ST13-like tumor suppressor aa.149-157
KLEAINELIRFDHIYTKPLVLEIPSE	XBP1 aa.281-289

[a] All HLA-B2703 ligands in the SYFPEITHI database (at: www.syfpeithi.de) are listed and were analysed for the presence of dibasic nardilysin cleavage motifs. Ligand is underlined and nardilysin cleavage motifs are printed bold.

SUPPLEMENTARY FIGURES

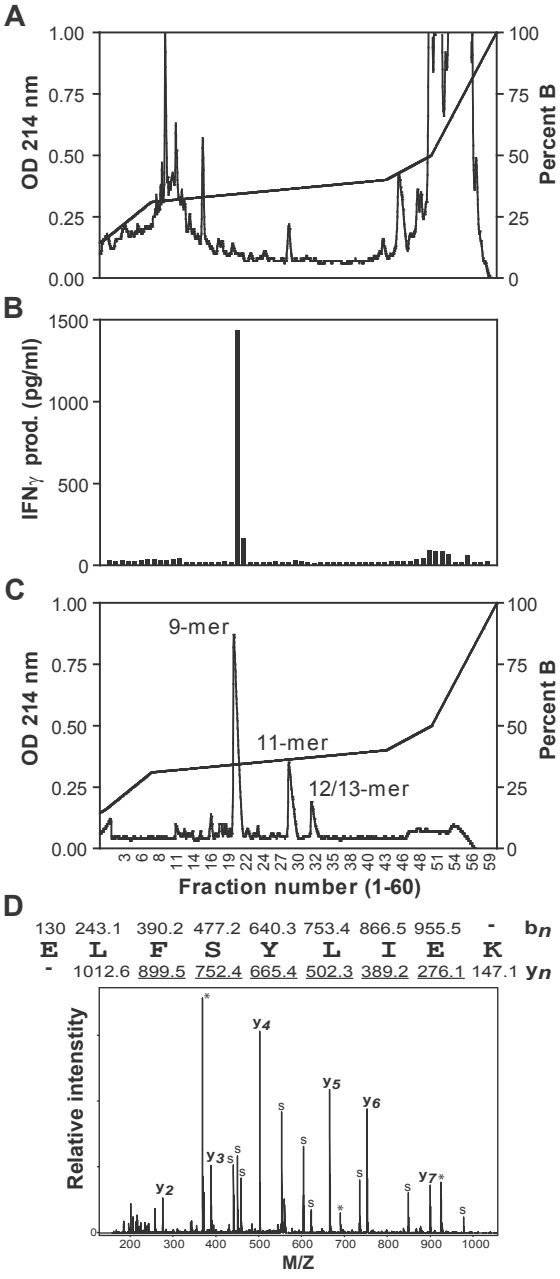


Suppl. Figure 1. HLA-A3 binding and CTL recognition of the ELFSYLIEK epitope and its C-terminally extended precursors.

A: Binding affinity for HLA-A3 of the nonameric (PRA$^{190-198}$) ELFSYLIEK epitope and its C-terminally elongated 10-, 11-, 12- and 13-meric variants as determined in a competition-based binding assay. As shown, the 10-mer has no binding capacity due to its lack of a C-terminal HLA-A3 binding anchor.

B: Alanine-scan to identify T cell receptor (TCR)-contact residues in the ELFSYLIEK peptide. Substitution-variants of the ELFSYLIEK peptide, each with a single amino acid replaced by Ala, were loaded on B-LCL EKR (HLA-A3$^+$) and co-incubated with the CTL clone that was raised against the exogenously-loaded ELFSYLIEK peptide. T cell recognition of the variants is indicated as (++), (+/-) or (-) (all variants did bind to HLA-A3; data not shown). The results imply that the ^{192}Phe-Ser-Tyr^{194} sequence (indicated as box) is required for TCR recognition, allowing the recognition of C-terminally extended variants of ELFSYLIEK by the CTL clone.

C: CTL recognition of C-terminally extended variants of the ELFSYLIEK peptide. The 11-mer, 12-mer and 13-mer PRA$^{190-200/201/202}$ (ELFSYLIEKVK/R/K) were titrated and separately loaded on B-LCL EKR cells (HLA-A3$^+$). The targets were co-incubated with the CTL for 14 h and the IFNγ-production was measured.



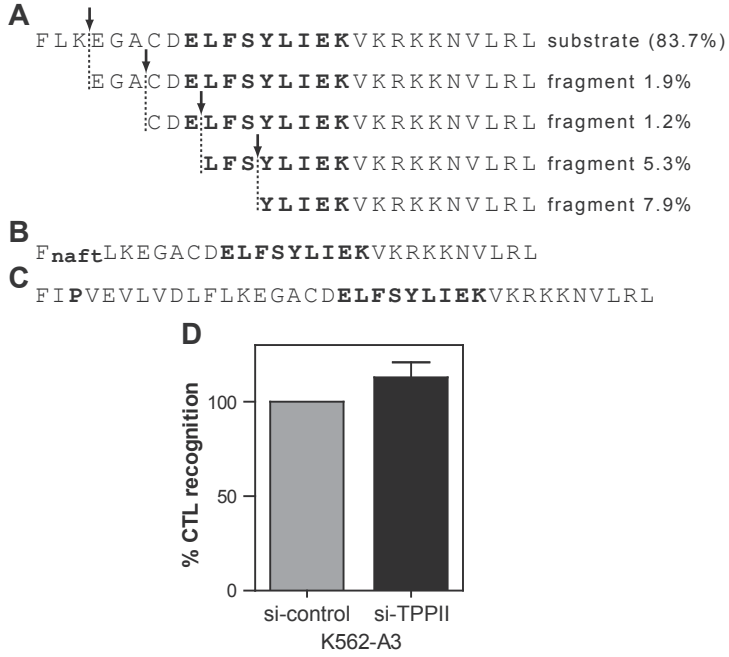
Suppl. Figure 2. Identification of PRA$^{190-198}$ as HLA-A3 presented peptide.

A: HPLC-profile of HLA-A3 presented peptides purified from K562-A3 cells (2x10^{10}). HLA-A3 molecules were immunoaffinity purified from the cell lysate, and acid-eluted peptides were separated in 60 fractions by reverse-phase HPLC, applying a gradient (depicted) of 31% to 40% buffer B in 36 min (left axis, OD214; right axis, % B).

B: Recognition by CTL anti-ELFSYLIEK of 60 HPLC-fractions loaded on B-LCL EKR (HLA-A3$^+$) to detect the fraction containing the eluted peptide-epitope. Fraction no. 21 sensitized the CTL.

C: HPLC-profile of the separation of a mixture of the synthetic 9-mer ELFSYLIEK peptide and its C-terminal extended 11-, 12-, and 13-mer variants ELFSYLIEKVK/R/K (PRA$^{190-200/201/202}$). The separation was conducted identical to the separation of cell surface expressed peptides (in panel A). The 9-mer, 11-mer, 12-mer and 13-mer peptides eluted in fractions 21, 29, and 32, respectively (confirmed by mass spectrometry; not shown).

D: The MS/MS spectrum of m/z 571.3 (2+) present in fraction 21 of the eluted peptides was identified as ELFSYLIEK; matching b- an y-ions are underlined. The MS/MS spectrum of the eluted peptide was identical to the MS/MS spectrum of the synthetic ELFSYLIEK peptide (not shown). Peaks indicated with 'S' could not be explained by 'regular' peptide fragment-ions, however, were also present in the MS/MS spectrum of the synthetic peptide. The peaks indicated with an asterisk are unrelated background peaks.



Suppl. Figure 3. TPPII does not generate the ELFSYLIEK epitope.

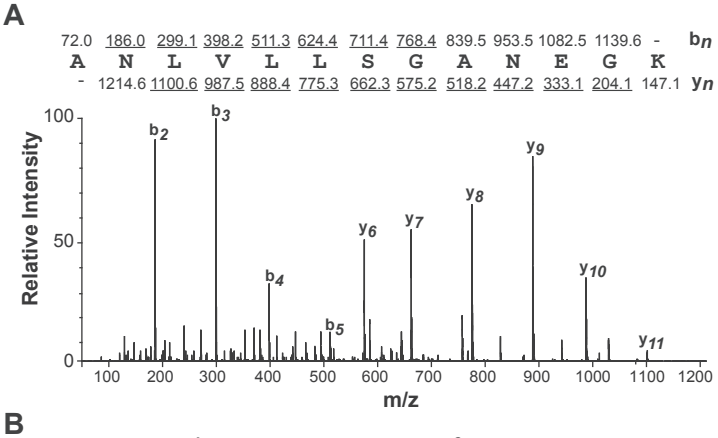
A: In vitro digestion with purified TPPII of 27-mer PRA$^{182-208}$ containing the ELFSYLIEK peptide (bold) in the middle. The fragments obtained after 1 h digestion are shown and quantified as the percentage of the total summed intensities measured by mass spectrometry. Arrows indicate cleavage-sites in the substrate and in the subsequently generated fragments.

B: A similar digestion with purified TPPII of 27-mer PRA$^{182-208}$, however with a modified substrate. At the N-terminal phenylalanine a 2-naphylsulfonyl group is attached to protect the substrate from N-terminal exo-peptidase activity. No cleavage products were detected (1 h digestion).

C: In vitro digestion with purified TPPII of 37-mer PRA$^{172-208}$. The third position in this peptide is a proline which is known to block TPPII-mediated N-terminal exo-peptidase activity when present at this position. No cleavage products were detected (1 h digestion). Together, the results point to a sequential removal of 3 aa from the N-terminus of the non-modified PRA$^{182-208}$ substrate and its subsequently generated fragments, which is in accordance with the known exo-peptidase trimming-activity of TPPII.

D: CTL recognition of K562-A3 stably transfected to express siRNA suppressing TPPII or non-targeting control siRNA (SEM of triplicate wells of representative experiment).

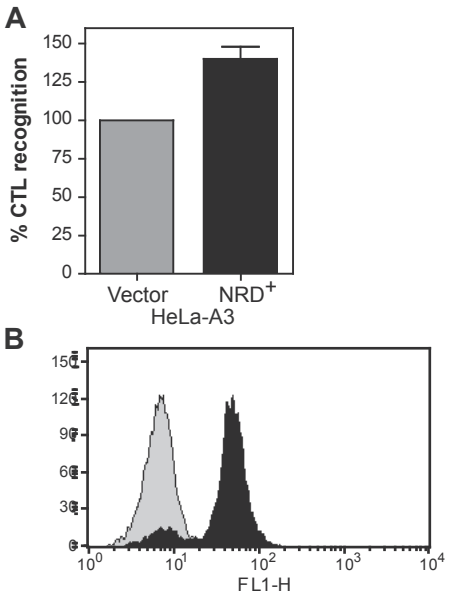
A



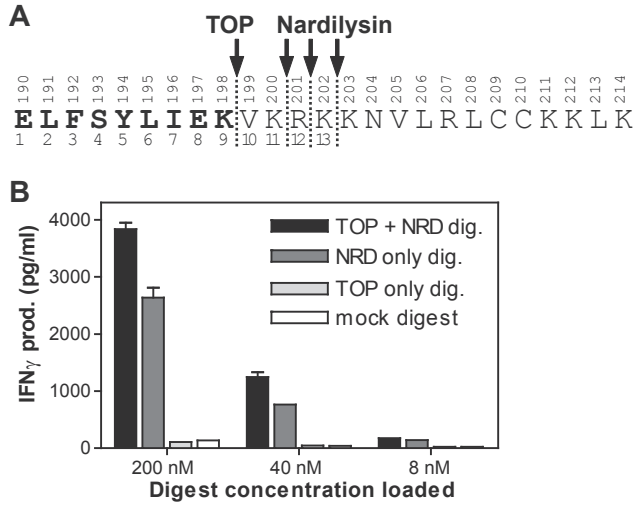
B

Band[a]	MS/MS[b]	Sequence identified[c]	Source protein
1	507.6 (3+)	IKGEHPGLSIGDVAK	High mobility group protein
	531.6 (3+)	KHPDASVNFSEFSK	High mobility group protein
	796.9 (2+)	KHPDASVNFSEFSK	High mobility group protein
2	536.3 (2+)	LAGLEEALQK	Keratin[d]
	739.8 (2+)	DSLENTLTETEAR	Keratin
	426.2 (2+)	GLTGGFGSR	Keratin
	813.4 (2+)	LNVEVDAAPTVDLNR	Keratin
	519.7 (2+)	AQYDDIASR	Keratin
3	514.6 (2+)	TAVCDIPPR	β-tubulin
	508.3 (2+)	DVNAAIATIK	α-tubulin
	912.9 (2+)	VGINYQPPTVVPGGDLAK	α-tubulin
	470.9 (3+)	QLFHPEQLITGK	α-tubulin
4	no precursors found		
5	643.3 (2+)	ANLVLLSGANEGK	Nardilysin[e]

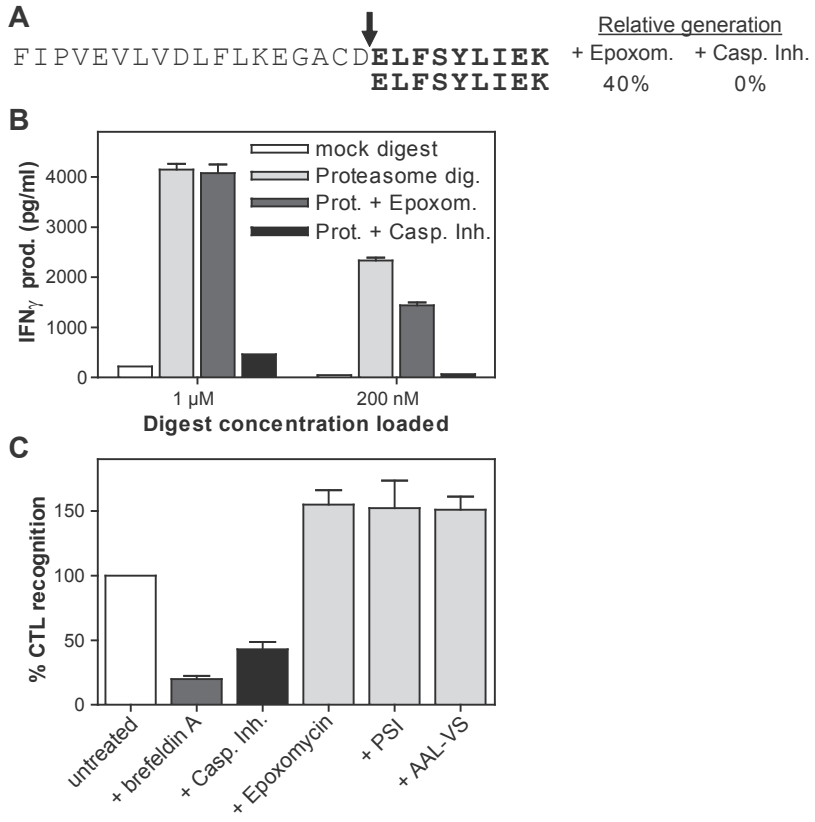
Suppl. Figure 4. Identification of nardilysin in cytosolic fraction no. 37 of K562-A3 cells.
Anion-exchange fraction no. 37 of K562-A3 cell lysate, which showed the highest digestive capacity of fluorogenic substrate ELFSYL(-dabcyl)IEKVKRC(-FL)KN, was separated by SDS-PAGE and protein bands were isolated (as shown in Fig. 2B). The proteins were identified by tandem mass spectrometry (MS/MS).
A: The MS/MS spectrum of m/z 643.3 (2+) that was found in band 5 (see panel B) is depicted. It was identified as peptide ANLVLLSGANEGK from nardilysin; matching b- and y-ions are underlined.
B: Identification by MS/MS of proteins in bands 1–3 as high mobility protein (band 1), keratin (band 2), and α- & β-tubulin (band 3). Notes: (a) Protein bands isolated from SDS-PAGE gel; (b) Mass (charge) that was chosen for MS/MS; (c) aa sequence as identified by MS/MS; (d) keratin is a contamination that is often found is this kind of samples; (e) from band 5, nardilysin was further confirmed by mass only of fragments 536.27 (2+), being aa seq. YIATTDFTLK and 679.32 (2+) which is aa seq. IEEFLSS-FEEK (data not shown).



Suppl. Figure 5. CTL recognition of nardilysin over-expressing HeLa-A3 cells.
A: Recognition by CTL anti-ELFSYLIEK of HeLa-A3 cells over-expressing nardilysin (HeLa-A3-NRD$^+$) as measured by IFNγ ELISA. Nardilysin over-expressing HeLa-A3 cells were selected by high EGFP expression. The population with low EGFP expression with natural nardilysin expression was used as reference population. Mean ± SEM of four experiments is shown.
B: Overlay histogram of HeLa-A3 cells transfected with pIRES-NRD-EGFP and sorted after transfection for either EGFP-low or EGFP-high expression, the latter population over-expressing nardilysin.



Suppl. Figure 6. CTL recognition of PRA$^{190-214}$ co-digested with nardilysin and TOP.
A: Substrate 25-mer PRA$^{190-214}$ (ELFSYLIEKVKRKKNVLRLCCKKLK, epitope boldfaced) with cleavage sites of nardilysin and TOP indicated as arrows. In the co-digest with nardilysin and TOP (2 h), approximately 3% of the digestion fragments were the minimal nonameric ELFSYLIEK epitope, as analysed by mass spectrometry.
B: CTL recognition of 25-mer PRA$^{190-214}$ digested for 2 h by nardilysin (NRD), TOP, or both peptidases together. SEM of triplicate wells of a representative experiment is indicated.
Importantly, the 25-mer incubated with TOP alone did not result in the minimal epitope (or other digestion fragments), as is shown here from the absence of CTL recognition. A similar digest of 17-mer PRA$^{190-206}$ with TOP also showed absence of (C-terminal) epitope generation (data not shown).

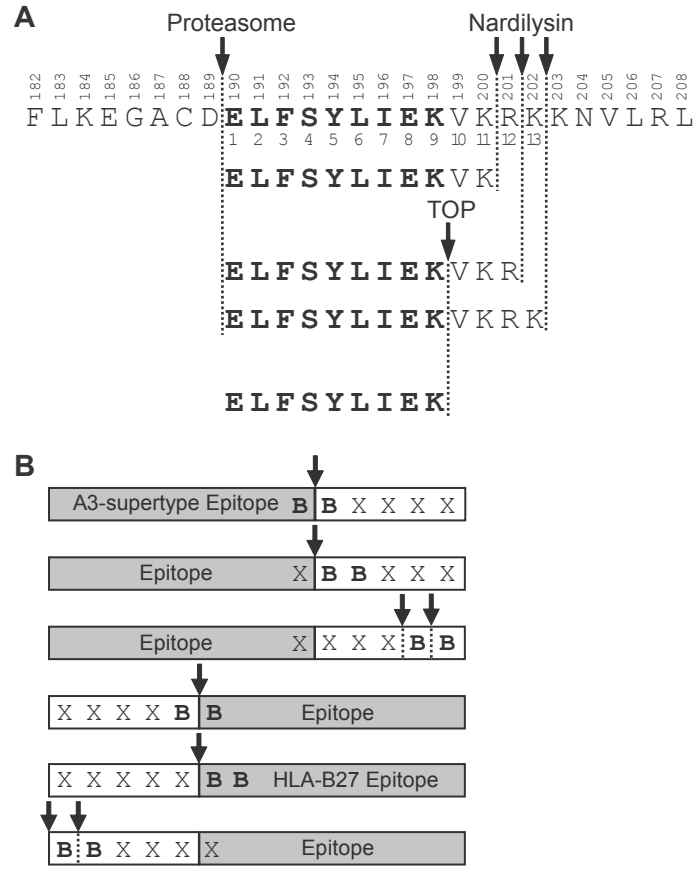


Suppl. Figure 7. The N-terminus of ELFSYLIEK is produced by the caspase-like cleavage activity of the proteasome.

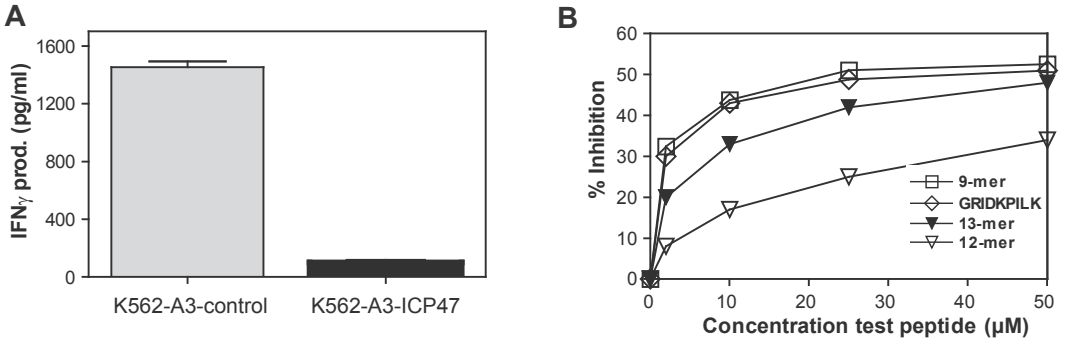
A: Proteasomal cleavages in 27-mer PRA$^{172-198}$ (epitope at C-terminus). Digestions were performed with immuno- and constitutive human 20S proteasomes for 1 h in the absence of inhibitors or in the presence of epoxomycin (10 µM) or AcAlaProNleLeuVSOH (Casp. Inh., used at 50 µM), a specific inhibitor of the caspase-like proteasomal activity. Arrow indicates the abundant cleavage before Glu-190 (liberating the epitope): the precise epitope-fragment and complementary fragments (containing Asp-189 as C-terminus) added up to >5% of the digested material after 1 h. Results for immuno- and constitutive proteasomes were comparable. The efficiency of epitope generation in the presence of the inhibitors is indicated as percentage of the epitope generated without inhibitor. The experiment was repeated three times with similar results.

B: CTL recognition of the digests of 27-mer PRA$^{172-198}$. The substrate was digested with constitutive proteasomes (1 h) in the absence of inhibitors or in the presence of epoxomycin or AcAlaProNleLeuVSOH (Casp. Inh.). Digests were titrated, loaded on HLA-A3^{+} cells and co-incubated with the CTL anti-ELFSYLIEK. Negative control is the 27-mer incubated at 37°C without proteasome (mock digest).

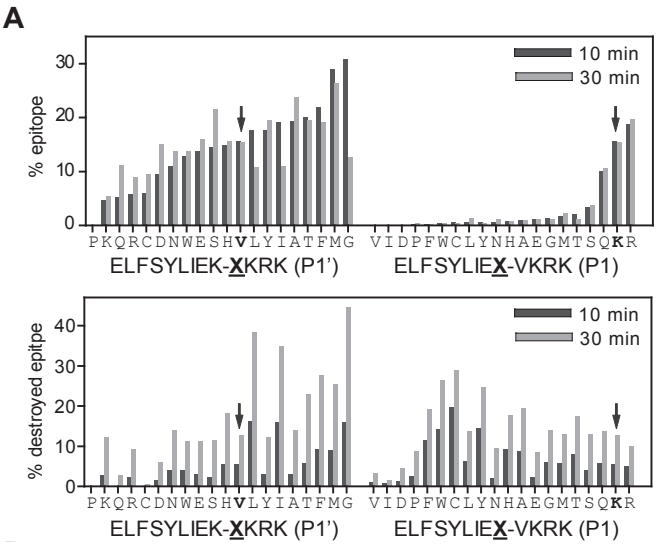
C: Relative CTL recognition of K562-A3 cells treated with general proteasome inhibitors epoxomycin (1 µM), PSI (10 µM) and AdaAhx3L3VS (AAL-VS; 50 µM) or the inhibitor of the caspase activity AcAlaProNleLeuVSOH (Casp. Inh., at 50 µM) as measured with intracellular IFNγ-staining. Reference 100% recognition is K562-A3 stripped (see methods), but untreated; background recognition is K562-A3 stripped and immediately treated with brefeldin A to block class I re-expression. SEM of three experiments are indicated.



Suppl. Figure 8. Mechanism of epitope generation and role of nardilysin in class I processing.
A: The proteasome, nardilysin and TOP co-operate in the generation of the PRA$^{190-198}$ epitope. The arrows depict the efficient cleavages of (1) the proteasome before Glu-190, (2) of nardilysin behind Lys-200, Arg-201 and Lys-202 and (3) the cleavage of TOP behind Lys-198 in the nardilysin-dependent 12- and 13-mer precursors together generating the epitope's C-terminus.
B: Sites where nardilysin can aid in the generation of HLA class I presented peptides depending on the position of the dibasic motif relative to the epitope (B, basic aa: Arg or Lys).



Suppl. Figure 9. TAP-dependency and translocation of PRA$^{190-198}$ epitope and its precursors.
A: TAP-dependent presentation of the ELFSYLIEK epitope. K562-A3 cells were transduced with the viral TAP-inhibitor ICP47 (K562-A3-ICP47) to block TAP-mediated peptide translocation, and used as target for the CTL anti-ELFSYLIEK. Reference K562-A3 cells were transduced with the empty construct (K562-A3-control). SEM of triplicate wells of representative experiment.
B: Efficiency of TAP translocation of 9-mer ELFSYLIEK and its 12-mer and 13-meric C-terminal extended length variants (ELFSYLIEKVKR/K). The peptides were tested as competitors in a TAP-translocation assay with a fluorescently labeled reference peptide that is known to be translocated efficiently. Peptide GRIDKPILK was used as positive control; this peptide is an HLA-B7 presented ligand also known to be efficiently translocated by TAP.



Suppl. Figure 10. The epitope-generating capacity of TOP.
A: Digestion by TOP of variants of 13-mer ELFSYLIEKVKRK (PRA$^{190-202}$), where the residues at the P1- and P1'-positions surrounding the TOP cleavage site (ELFSYLIEP1-P1'KRK) were substituted for all possible residues. The wild type 13-mer sequence is included two times (at both the P1'- and P1- substitutions) and is indicated by an arrow and bold printed residue. Upper panel: epitope-production (ELFSYLIEK or ELFSYLIEX for P1'- and P1-substitutions, respectively) after 10 min and 30 min is indicated and quantified as its percentage of the total summed intensities measured by mass spectrometry. Substitutions are ordered according to efficiency of epitope-production at 10 min. Lower panel: epitope-destruction (i.e. peptides in the digest constituting fragments of the epitope) after 10 min and 30 min is indicated as percentage of total summed intensities.
B: CTL recognition of TOP-digested 13-mer variants of PRA$^{190-202}$ with substitutions at P1' (ELFSYLIEK-P1'KRK). The digests were titrated, loaded on HLA-A3^{+} EKR target cells, co-incubated with the CTL anti-ELFSYLIEK and IFNγ-release was measured. Recognition of the wild type 13-mer sequence is indicated by an arrow and bold printed residue.
C: Capacity of purified TOP to generate the C-terminus of different CTL epitopes from precursors with two artificial C-terminal extended regions. Epitopes tested are SLYSFPEPEA (PRA$^{142-151}$), VLDGLDVLL (PRA$^{100-108}$) with flanking sequence VKRK or RRFV and ELFSYLIEK (PRA$^{190-198}$) with flanking sequence RRFV. Epitope production after 10, 30 and 60 min incubation of the substrate with TOP is indicated and expressed as percentage of the total summed intensities measured by mass spectrometry.

METHODS

Cell lines

Cell lines used were erythroleukemia cell line K562 (naturally expressing PRAME), cervical carcinoma cell line HeLa (PRAME$^+$), renal cell carcinoma (RCC) lines MZ1257, Le-94.15 (both lines, PRAME$^+$ and HLA-A3$^+$) and MZ1851 (HLA-A3$^+$, PRAME-negative) (RCC lines provided by Dr. E. Verdegaal, LUMC, The Netherlands) and B-LCL EKR (HLA-A3$^+$ and PRAME-negative). PRAME-expression was assessed by rtPCR. K562, which is naturally class I negative, and HeLa cell lines were transduced retrovirally to express HLA-A3, HLA-A2 or HLA-B2705 (designated K562-A3, K562-A2, K562-B27 and HeLa-A3) using HLA-A2 and -A3 encoding retroviral LZRS plasmids [32] (provided by dr. M. Heemskerk, dept. Experimental Hematology, Leiden University Medical Center) or an HLA-B2705 encoding plasmid. Cell surface expression of HLA-A3, -A2 and -B2705 was measured by flow cytometry using monoclonal antibodies GAP-A3, BB7.2 and W6.32 respectively (data not shown). HeLa-A3 cells overexpressing nardilysin were made by transfection with the pIRES2-EGFP vector containing rat NRD1 cDNA (pIRES2-NRD1-EGFP) (rat NRD1 has similar cleavage specificity as human NRD1). After transfection cells were sorted in EGFP-low and EGFP-high populations, the latter population over-expressing nardilysin. K562-A3 cells expressing viral TAP inhibitor ICP47 and control cells (K562-A3-ICP47 and K562-A3-vector) were made by retroviral transduction using the pLZRS-IRES-eGFP plasmid with or without the ICP47 insert. K562-B2705 was stably transfected with a plasmid encoding the minigene MFLRGKWQRRY**RRIYDLIEL** (EBNA3C aa 247-266, with an extra N-terminal methionine; CTL epitope in bold), transfected cells were selected with hygromycine. All cell lines were cultured in complete culture medium consisting of IMDM (Biowithaker, Verviers, Belgium) supplemented with 8% fetal calf serum (FCS; PAA, Linz, Austria), 100 IU/ml penicillin and 2 mM L-glutamine.

Synthetic peptides

Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Syro II, MultiSyntech, Witten, Germany) using Fmoc-chemistry. For fluorescent peptides, fluorescein was covalently coupled to the cysteine residue using 5-(iodoacetamido)fluorescein (Fluka Chemie AG, Buchs, Switzerland). The quencher residue, Fmoc-L-Lys(Dabcyl)-OH was obtained from Neosystems (France). For blocking of N-terminal trimming in digestion experiments, the N-terminus of the peptide was blocked with a 2-naphylsulfonyl group. All peptides were HPLC-purified and validated by mass spectrometry.

Isolation and identification of nardilysin in cytosolic fractions

K562-A3 cells (5×10^9) were homogenized in lysis buffer (10 mM Tris-HCl, 1 mM DTT, 1 mM NaN_3, 25 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 2 mM ATP, 50 mM NaF, 0.1 mM Na_8VO_4, pH 7.5) using a Dounce glass homogenizer. The homogenate was centrifuged for 20 min at 10,000 × *g*, and the supernatant was centrifuged for 6 h at 100,000 × *g* thereby removing proteasomes and TPPII. The clarified lysate was filtered over a 0.2 µm filter. The proteins in the lysate were separated (60 fractions of 0.5 ml) by anion exhange chromatography using a mono Q column (HR 5/5, Amersham) with a linear gradient (20 ml) from 0 to 500 mM KCl in lysis buffer at a flow rate of 1 ml/min. Specific peptidase activity in the fractions was assessed with in-

ternally quenched fluorogenic 15-mer substrate ELFSYL(-dab)IEKVKRC(-FL)KN ($PRA^{190-204}$). For technical reasons the fluorescein (FL)-group was attached to a cysteine in stead of the natural lysine at this position. The assay was performed with substrate at a final concentration of 0.5 µM in substrate buffer (30 mM Tris, 10 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5) either in the absence or presence of 1 mM phenathroline. Appearance of fluorescence, due to spatial separation of the quencher from the fluorophore, was measured using 360 nm extinction and 460 nm emission settings. Fractions (20 µg) containing peak proteolytic activity (fraction #36 to 40) were run on 12% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue according to standard procedure. Selected protein bands were isolated from fraction #37, subjected to digestion with trypsin as described [33] and analysed using mass spectrometry.

HLA-A3 peptide binding assay

Affinity of peptides for HLA-A3 was measured with a competition-based cellular peptide binding assays as described previously [34]. In this assay the capacity of test peptides to compete with the cell surface expressed HLA class I binding of a fluorescein (FL)-labeled reference peptide that is known to bind with high affinity in the HLA molecule of interest was tested. The percentage inhibition of fluoresceine labeled reference peptide binding by the competitor test peptide was calculated using the following formula: $(1-(MF_{\text{reference and competitor peptide}} - MF_{\text{no reference peptide}}) / (MF_{\text{reference peptide}} - MF_{\text{no reference peptide}})) \times 100\%$. The binding affinity of the test peptide is expressed as the concentration needed to inhibit 50% binding of the FL-labeled reference peptide (IC_{50}). An $IC_{50} \leq 5$ mM was considered high affinity binding and 5 mM < $IC_{50} \leq 15$ mM was considered intermediate affinity binding. The FL-labeled reference peptide used for HLA-A3 was KVFPC(FL)ALINK [34].

RNA interference mediated suppression of peptidases

RNA interference mediated suppression of peptidase expression in K562-A3, K562-B27 and HeLa-A3 was established by transient transfection with pools of four interfering oligonucleotide RNA duplexes for each peptidase (siGENOME SMARTpool from Dharmacon) and/or by stable transfection of the pSUPER-puro vector (a gift from dr. R. Bernards, Netherlands Cancer Institute) encoding siRNA sequences. Transfection of pSUPER-puro plasmids in K562-A3 and K562-B27 was performed by electroporation, and in HeLa-A3 with reagent FuGENE (Roche Molecular Biochemicals, Indianapolis) according to the instructions of the manufacturer. Cells stably expressing siRNA were obtained by culturing in the presence of puromycine (2 µg/ml for K562-A3 and –B27 and 0.2 µg/ml for HeLa-A3). Transient transfection of siRNA duplexes in K562-A3 was performed with HiPerFect (Qiagen) and transfection in HeLa-A3 with DharmaFECT 1 (Dharmacon), according to instructions of the manufacturers. Stable suppression of TPPII was targeted at GCAGTATTCACATCGCCAC (from ref. 14). The target sequence chosen for stable suppression of nardilysin was AGCAGACCCTTGGGTACCA (resulting in the best suppression from five newly designed sequences tested). Suppression of TOP by stable expression was targeted at CCTCAACGAGGACACCACC (from ref. 35) and the transiently transfected pool of duplexes was directed at: (1) TAGATGAGCTGGCGCAGAATT, (2) TCAAACGCATCAAGAAGAATT, (3) GCAAGGTTGGCATGGATTATT, and (4) AGACCAAGCGCGTGTAT-

GATT (in accession number NM_003249). Suppression of Neurolysin by the pool of duplexes was targeted at: (1) GGATAAAGCTACAGGAGAA, (2) GATAATGAATCCAGAGGTT, (3) CAATTGAGGTGGTCACTGA, and (4) GAACTCAAGTATTCCATAG (in accession number NM_020726). Suppression of insulin degrading enzyme by the pool of duplexes was directed at: (1) TCAAAGGGCTGGGTTAATA, (2) ACACTGAGGTTGCATATTT, (3) GAACAAAGAAATACCCTAA, and (4) GTGGAGAGCATACCAATTA (in accession number NM_004969). As non-silencing control for the SMARTpool duplexes we used the si*CONTROL* Non-Targeting siRNA pool (Dharmacon). The non-silencing control for the pSUPER-puro expressed siRNA's was a non-targeting scrambled sequence. The level of suppression of the peptidases was assessed by quantitative RT-PCR and western blotting. For detection of TOP by immunoblotting, we used monoclonal antibody IVD6 (Upstate), and Western blotting of nardilysin was performed with an affinity-purified rabbit antiserum.

Peptide translocation

TAP translocation of PRAME peptide was tested using microsomes that were prepared from a B-LCL cell line as described previously [36]. Microsomes were thawed from frozen aliquots and resuspended in transport buffer (5 mM Hepes-pH 7.3, 130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$). Translocation efficiency of test peptides was measured by competition for translocation with a fluorescein (FL)-labeled reference peptide that contains a consensus site for N-linked glycosylation in the ER. Briefly, samples (60 µl) were pepared containing titrated amounts of the competing test peptide (50, 25, 10 and 2 µM – final concentrations), 0.5 µM FL-labeled reference peptide C(-FL)VNKTERAY [37] and 10 mM ATP in transport buffer. Per sample, 40 µl of microsomes (containing 1.3×10^6 cell equivalents) was added and samples were incubated for 10 min at 37°C. Peptide translocation was stopped by adding 1 ml ice cold transport buffer with 10 mM EDTA, samples were washed, pellets resuspended in lysis buffer (50 mM Tris-pH 7.5, 500 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100) and rotated for 30 min at 4°C. Debris was removed by centrifugation (10 min, 13000 rpm at 4°C) and the glycosylated FL-reference peptide was recovered by incubation for 2 h at 4°C with 100 µl packed concanavalin A-Sepharose 4B (GE Healthcare, Uppsala, Sweden). Con A-Sepharose was then washed three times in lysis buffer and glycosylated FL-reference peptide was released in the supernatant by incubation for 1 h at RT on a shaker in 200 ul elution buffer (50 mM Tris-HCl pH 8.0, 500 mM mannopyranoside, 10 mM EDTA). Fluorescence in the supernatant was measured using excitation 485 nm and emission 535 nm settings. Maximal fluorescence was obtained from a sample containing no competitor peptide, minimal fluorescence from a sample without ATP and containing 0.5 M EDTA. The GRIDKPILK positive control competitor peptide is a reported efficiently translocated peptide [38].

Identification of the ELFSYLIEK peptide on the cell surface in HLA-A3

K562-A3 cells (30×10^9) were lysed in lysis buffer, 20 mM Tris/pH 8, 5 mM $MgCl_2$ containing 0.5% Zwittergent-12 (Calbiochem). Membrane fragments were removed by ultracentrifugation and the supernatant was precleared with sepharose CL-4B (Amersham Biosciences). Immunoaffinity purification of HLA-A3 molecules was performed with pan-HLA class I antibody W6.32 coupled to protein A beads. Peptides were eluted with 10% HAc, filtered

over a 10 kD filter (Amicon), and separated in 60 fractions (100 µl) using reverse-phase micro C2C18 HPLC (Smart System, Amersham). Buffer A was 0.1% heptafluorobutyric acid (HFBA) in water, buffer B 0.1% HFBA in acetonitrile and a gradient of 31% to 40% buffer B in 36 min was applied. Fractions were lyophilized to remove acetonitrile, dissolved in water (100 µl end volume) and 10 µl was used for testing CTL reactivity and 90 µl for mass spectrometry (see mass spectrometry). For CTL assays, the fractions were loaded on EKR cells (20000/well, 96 well plate) and co-incubated with CTL (25000/well). EKR cells were pre-treated with mild acid buffer to remove cell surface expressed peptides, and resuspended in IMDM containing 2 µg/ml human β_2-microglublin (Sigma). CTL reactivity was measured with IFNγ ELISA.

CTL assays and functional peptidase inhibitor studies

CTL recognition was measured by the ^{51}Cr-release assay according to standard procedures [17] or by IFNγ ELISA (Sanquin Reagents, Amsterdam, The Netherlands) according to manufacturer's instruction. In case of peptidase inhibitor studies, intracellulair IFNγ-staining of CD8$^+$ T cells was used as readout. Briefly, target K562-A3 cells were first treated with the indicated inhibitors for 1.5 h at 37°C then stripped by mild acid treatment (300 mM glycine, pH 2.9, 1% BSA) for 90 sec to remove cell surface class I presented peptides, washed, and treated with the same inhibitor for 5 h at 37°C. Background epitope expression was measured by treating a sample with brefeldin A (10 µg/ml) immediately after stripping, and the maximal epitope expression sample was stripped but left untreated to allow full reconstitution of class I molecules. After inhibitor treatment, the samples were washed in PBS, plated as target cells in triplicate wells of a 96-well plate (30000/well) and co-incubated with the CTL clone (30000/well) for 6 h. After 30 min co-incubation brefeldin A (Sigma) was added at 5 µg/ml. The co-cultures were harvested, washed, fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA) according the manufacturer's instructions and T cells were stained intracellularly with CD8-APC (Dako) and IFNγ-PE (BD Pharmingen) to measure T cell activation by flow cytometry. Inhibitors used were: Brefeldin A (Sigma), used at 10 µg/ml; Phenanthroline (Sigma), inhibitor of metallopeptidases used at 400 µM (in cellular assays); Insulin (Sigma), competitive inhibitor of insulin degrading enzyme used at 50 µM; Captopril (Sigma), inhibitor of angiotensin converting enzyme used at 50 µM; Leupeptin (Calbiochem) inhibitor of trypsin-like proteases and cysteine proteinases used at 100 µM; Calpeptin (Calbiochem) inhibitor of calpains used at 30 µM; Aprotinin (Calbiochem) a serine proteases inhibitor used at 5 µM; and Butabindide (Tocris), inhibitor of TPPII used at 200 µM. For inhibition of the proteasome we used PSI, epoxomycine (both Calbiochem), AdaAhx3L3VS [39] and AcAla-ProNleLeuVSOH [25].

In vitro induction of CTL

The in vitro CD8$^+$ T cell induction against the exogenously loaded synthetic PRA$^{190-198}$ peptide (ELFSYLIEK) was performed as described previously [17]. Briefly, PBMC of an HLA-A3 positive healthy donor were used for the generation of antigen presenting cells (DC's and activated B cells) and autologous CD8$^+$ T cells. T cells were stimulated with peptide-pulsed DC's and restimulated three times with peptide-pulsed activated B cells. Peptide specificity of the bulk CTL culture was tested at day 28 and the culture was cloned by limiting dilution

at day 29 to obtain CTL clones specifically recognizing the ELFSYLIEK peptide. One specific CTL clone was selected for functional CTL assays based on T cell sensitivity and growth characteristics. The CTL clone specific for the HLA-B2705 presented immunodominant CTL epitope RRIYDLIEL from EBNA-3C (aa 258-266) was generated as reported previously [27].

Digestions with proteasomes, TPPII, nardilysin and TOP and digest recognition assays

Proteasomes (20S) were purified as described [40] from HeLa cells, which contain mainly constitutive proteasomes, and B-LCL line JY that expresses mainly immunoproteasomes. Proteasome composition was confirmed by Western blotting (data not shown). TPPII was purified to homogeneity from erythrocyte concentrates as described [41] with minor modifications [42]. Purified TPPII (0.05 mg/ml) in 100 mM potassium phosphate buffer, pH 7.5, containing 30% glycerol and 1 mM DTT was stored at –80°C before usage. Recombinant mouse nardilysin was prepared as previously described [21] using the Bac-to-Bac system (Invitrogen, Carlsbad, Calif.) and purified using Ni-resin (Qiagen) affinity chromatography. The nardilysin preparation was homogeneous as judged by SDS-PAGE [21]. Long peptide digestions were performed with purified mouse nardilysin and with cytosolic fraction #37 of K562 cells containing human nardilysin as enzyme (see for details the section *'Isolation and identification of nardilysin in cytosolic fractions'*) with similar results. Mouse and human nardilysin have identical cleavage specificities [43]. TOP was produced by overexpression in Escherichia coli and purified using Ni-resin (Qiagen) affinity chromatography as previously described [44].

Digestions were performed in a volume of 300 µl with mostly 10 µM peptide and 4.3 nM enzyme at 37°C. TPPII digestions were performed with 1 µg enzyme (MW $>10^6$) and 2 nmol peptide in 100 µl. A mock digestion of the substrate at 37°C without enzyme was always included. For proteasome and nardilysin, the digestion buffer was 30 mM Tris (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT. For TOP digestions the buffer was 50 mM Hepes (pH 7.6), 2 mM $MgCl_2$, 0.1 mM DTT. TPPII digestions were performed in a buffer of 20 mM Hepes, 2 mM $Mg(Ac)_2$, 2 mM DTT, containing 5% glycerol. At different time points (dependent on the digest at 10 min, 30 min, 1 h, 2 h, and 4 h) samples of 50 µl or 75 µl were drawn and the enzymatic reaction was stopped by addition of 5% (v/v) acetic acid, or when digest were used for CTL recognition assays by addition of 1 mM phenanthroline. Samples were diluted four times before measuring with mass spectrometry. For functional recognition assays the digest-samples were first titrated and then 10 µl digest was loaded onto 90 µl B-LCL EKR cells (HLA-$A3^+$, PRAME-negative) in triplicate wells of a 96-well plate (17000 cells/well) for 2 h at 4°C. Synthetic reference PRAME peptides were titrated at equal concentrations and loaded similarly. Prior to loading, to improve loading-efficiency, EKR cells were stripped from naturally expressed peptides by acid treatment and resuspended in IMDM containing 2 µg/ml human β_2-microglublin (Sigma). After loading, CTL (25000) were added in 50 µl/well, co-incubated for 12 h, and IFNγ in the supernatant was measured by ELISA.

Mass spectrometry

Electrospray ionization mass spectrometry was performed on a Q-TOF1 (Micromass), equipped with an on-line nanoelectrospray interface, approx. flow rate of 250 nL/min.

This flow was obtained by splitting of the 0.4 ml/min flow of a conventional high pressure gradient system, using an Acurate flow splitter (LC Packings). Injections were done with a dedicated micro/nano HPLC autosampler, the FAMOS (LC Packings). Digestion solutions were diluted five times in water-acetonitrile-formic acid (95:3:1, v/v/v), and 1 µl was trapped on a precolumn (MCA-300-05-C18; LC Packings) in water-acetonitrile-formic acid (95:3:1, v/v/v). The precolumn was washed for 3 min to remove the buffers in the digests and the trapped analytes were eluted with a steep gradient from 70% B to 90% B in 10 min, with a flow of 250 nl/min (A: water-acetonitrile-formic acid (95:3:1, v/v/v); B: water-acetonitrile-formic acid (10:90:1, v/v/v)). Mass spectra were recorded from mass 50-2000 Da. In MS/MS mode ions were selected with a window of 3 Da. The collision gas was argon (4×10^{5} mbar), and the collision voltage 30V.

For proteasome, nardilysin, TOP and TPPII digestions peaks in the mass spectra were searched in source substrate peptides using Biolynx/proteins software (Micromass). The intensity of the peaks was used for the relative quantification of the digestion fragments as percentage of the total amount of peptide digested at indicated time points. For identification of the proteins in fraction no. 37 of the K562-A3 cytosolic preparation, MS/MS spectra were interpreted against the SwissProt database using the PeptideSearch software.

For identification of the $PRA^{190-198}$ epitope, peptides present in the HPLC-fractions of eluted peptides were sequenced by tandem mass spectrometry. To this end, the fractions were first lyophilized, resuspended in 95/3/0.1 v/v/v water/acetonitrile/formic acid (solvent A) and analyzed by online nanoHPLC-MS. The nanoHPLC system consisted of a conventional gradient HPLC system (Agilent 1100), the flow of which was reduced to 300 nl/min by an in-house constructed splitter. Two-microliter injections were done onto a precolumn (10 mm × 100 µm; AQUA-C18, 5-µm particle size (Phenomenex)) and eluted via an analytical nano-HPLC column (15 cm × 75 um; AQUA-C18, 5-µm particle size. HPLC columns were packed in-house. The gradient was run from 0 to 50% solvent B (10/90/0.1 v/v/v water/acetonitrile/formic acid) in 90 min. The mass spectrometer was an HCT^{plus} (Bruker Daltonics), which was run in data-dependent MS/MS mode during peptide elution. The ELFSYLIEK peptide was identified by its mass and MS/MS spectrum.

Supplementary references in methods

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CHAPTER 7

СНАЧАЛА

Discussion

Cytotoxic T cell immunity is at the basis of the majority of immunotherapeutic approaches for cancer, in particular adoptive T cell transfer and various vaccination strategies (summarized in chapter 1). Next to their capacity to specifically recognize and kill tumor cells, $CD8^+$ cytotoxic T lymphocytes (CTL) are prominently involved in the eradication of cells that are infected with pathogens. Therefore, mounting an effective CTL response is the main goal of therapeutic vaccinations not only for cancer but also for infectious diseases. Basically, the success of any T cell-based immunotherapy depends heavily upon the quantity and quality of the CTL that are working in the patient. To induce and/or maintain a successful therapeutic CTL response, next to an optimal immunostimulatory context of the therapy, the proper selection with respect to quantity and quality of the targeted tumor-associated or pathogen-derived antigens, and, more precisely, the T cell epitopes contained in these proteins is of crucial importance. Success rates in T cell based immunotherapy for cancer are still low [1], and efficacious therapeutic vaccines against diseases like HIV/AIDS and tuberculosis are yet to be developed [2,3], but our growing understanding of immune activation and antigen processing will likely help to improve the efficacy of immunotherapy in the near future.

In this thesis, new insights in the processing of antigens for their presentation by HLA class I molecules are revealed (chapter 6), the reverse immunology strategy of CTL epitope identification is improved and applied to identify epitopes from PRAME and BCR-ABL (chapter 2, 4 and 5) and responses towards identified PRAME epitopes are analyzed (chapter 3). Here, the relevance of these results is discussed in the context of immunotherapy for cancer and infectious diseases.

Efficiency and accuracy of CTL epitope identification by reverse immunology

The presentation of a peptide by an HLA class I molecule is the end result of various events in the so-called antigen processing pathway. It is only since the early 1990s that our knowledge concerning the three predominant processing events has evolved to the level that these events can be experimentally tested, allowing a prediction of the peptides that end up on the cell surface. In the first years of epitope prediction, only class I binding was taken into account, but, obviously, the accuracy of such a prediction is enhanced when proteolytic mechanisms are also considered. It has been estimated that, on average, less than one third of all possible HLA class I binding peptides in a protein are actually generated by intracellular proteolysis [4]. This was demonstrated in chapter 2, where proteasomal excision of HLA-A0201-binding peptides at their C-terminus was used as extra selection criterion to predict CTL epitopes in tumor associated antigen (TAA) PRAME. The accuracy of epitope prediction was enhanced, strongly limiting the number of peptides of which natural presentation had to be tested (validated) by CTL recognition (in the validation phase; see chapter 1, fig. 2). The experimental verification of proteasomal excision, being much less laborious than CTL inductions, thereby enhanced the overall efficiency of the identification procedure (as defined in man-hours work).

For the three main processing events, being (1) proteasomal processing, (2) TAP translocation, and (3) class I binding, nowadays screening algorithms are available that allow the in silico prediction of class I ligands in known proteins. In silico assessment of class I binding and proteasomal cleavages (and TAP translocation, which is much less selective) consumes only a little amount of time, but is also much less accurate than experimental in vitro assessments. Upon comparison of class I peptide binding prediction algorithms, significant differences in the predictions often occur. As an example, we calculated percentages of overlap between predictions by the BIMAS and SYFPEITHI algorithms (which are most often used, see chapter 1) of the best 20, 50 and 100 predicted peptides from full length PRAME (509 aa) for three prevalent HLA class I molecules. The overlap in the best 20 predicted binders may be as low as 25% for certain class I molecules

Table 1. Overlap between predictions by BIMAS and SYFPEITHI algorithms.

		best 20 ranked[a]	best 50 ranked	best 100 ranked
HLA-A1	9-mer	40%	56%	64%
	10-mer	50%	60%	62%
HLA-A2	9-mer	50%	64%	68%
	10-mer	70%	68%	68%
HLA-A3	9-mer	50%	54%	59%
	10-mer	25%	34%	54%

[a] Peptides with identical scores of those at position 20, 50 or 100 were included.

(Table 1). Also, the ranking of peptides by in silico predicted binding does not perfectly correlate with the actual binding measurements and false positive prediction of binding occurs. For instance, we found the 13th predicted 9-mer PRAME peptide (PRA^{44-52}) actually to lack binding capacity for HLA-A0201, whereas

Table 2. Accuracy of binding prediction.

Experimental[a]		Prediction[b]	Peptide[c]	
rank	IC_{50}	rank	sequence	aa.
1	2.5	16	NLTHVLYPV	435
2	2.9	1	QLLALLPSL	394
3	3.7	2	SLLQHLIGL[g]	425
4	4.6	15	TLAKFSPYL	248
5	5.1	5	ALAIAALEL	39
6	5.2	8	VLDGLDVLL[g]	100
7	5.4	4	YLHARLREL	462
8	**5.7**	**34**[d]	**SISALQSLL**	**419**
9	6.8	12	GLSNLTHVL	432
10	9.2	21	ITDDQLLAL	390
11	**9.3**	**245**[d]	**CTWKLPTLA**	**242**
12	10.2	22	LLKDEALAI	34
13	**11.0**	**33**[d]	**TLSFYGNSI**	**410**
14	**11.1**	**53**[d]	**HLHLETFKA**	**91**
15	**13.2**	**41**[d]	**TLSITNCRL**	**326**
16	13.4	9	AVLDGLDVL	99
17	14.0	3	RLRELLCEL	466
18	14.2	7	ALQSLLQHL	422
19	15.7	10	RLDQLLRHV	312
20	**15.8**	**215**[d]	**RTFYDPEPI**	**493**
Predicted binders (in best 20) not (efficiently) binding[e]				
35	**35.0**	**11**[e]	**LLERASATL**	**372**
43	**71.3**	**13**[e]	**ALELLPREL**	**44**
45	**79.4**	**18**[e]	**SLFFLRGRL**	**305**
59	**>100**	**14**[e]	**KMILKMVQL**	**224**

[a] As measured in HLA-A2 binding assay.
[b] Prediction by SYFPEITHI algorithm of 9-mer peptides derived from PRAME binding in HLA-A*0201.
[c] Start aa. position in PRAME and sequence.
[d] False negative prediction of binding.
[e] False positive prediction of binding.
[g] Proven CTL epitope.

the 16th predicted binder ($PRA^{435-443}$) bound with highest affinity (Table 2).
As discussed in chapters 1 and 2, in silico prediction of proteasomal excision is inaccurate as well. This was also revealed when we tested the performance of the five algorithms that integrate prediction of class I binding, proteasomal excision and TAP translocation, being MHC-Pathway [5], WAPP [6], NetCTL [7], IEDB [8], and EpiJen [9]). As test-set the 64 nonameric peptides from PRAME that were in vitro tested for HLA-A2-binding capacity and proteasomal liberation to identify two CTL

epitopes (chapter 2 [10]) were used (Table 3). The two proven nonameric CTL epitopes were predicted in the 10 best ranked predicted epitopes by four of the five algorithms. MHC-Pathway, which did not rank the $PRA^{100-108}$ epitope in the top-10, but instead correctly predicted $PRA^{301-309}$ (which is a natural epitope; our unpublished results). The combined algorithm from the 'immune epitope database and analysis resource' (IEDB [8]) predicted all three epitopes. However, the five predictions contained a considerable number (> 50%) of falsely predicted epitopes, mainly because the C-terminus was (in vitro) not generated by the proteasome (Table 3). This implies that experimental verification of proteasomal cleavages is needed to improve the selection of putative epitopes. Another practical weakness of the integrative algorithms is that they mostly allow the prediction of only nonamers (with exceptions for certain alleles), and their coverage of prevalent HLA class I alleles is incomplete. Generally for CTL epitope prediction, the challenge is to find a balance between the reduction of work in the prediction phase when predictions are accomplished solely in silico (without experimental verification) on the one hand and on the other hand the loss in quality of the final prediction, which will lead to more laborious work and reduced success rates (more and failed T cell inductions) in the validation phase. This balance may depend on the precise research question, including the length of the source protein under study: the longer the protein, the better the chance that top-scoring predicted epitopes will be genuine epitopes.

For an overall judgement of the efficiency of the reverse immunology approach it is important to note that the prediction of class I ligands aims to select only those peptides that are enzymatically generated, survive further

Table 3. Prediction by combined algorithms.

Start aa.	Nonamer	Predict. Rank[a]	Experimental Bind.[b]	Experimental C-term.[c]
MHC-Pathway				
248	TLAKFSPYL	1	4.6	-
425	**SLLQHLIGL**[e]	2	3.7	++
435	NLTHVLYPV	3	2.5	-
394	QLLALLPSL	4	2.9	-
432	GLSNLTHVL	5	6.8	-
284	YIAQFTSQF	6	n.t.[d]	n.t.[d]
301	**LYVDSLFFL**[e]	7	6.3	++
410	TLSFYGNSI	8	11.0	-
340	MHLSQSPSV	9	n.t.	n.t.
353	VLSLSGVML	10	17.4	n.t.
WAPP				
425	**SLLQHLIGL**[e]	1	3.7	++
432	GLSNLTHVL	2	6.8	-
308	FLRGRLDQL	3	16.1	n.t.
435	NLTHVLYPV	4	2.5	-
100	**VLDGLDVLL**[e]	5	5.2	++
305	SLFFLRGRL	6	**79.4**	-
312	RLDQLLRHV	7	15.7	n.t.
394	QLLALLPSL	8	2.9	-
39	ALAIAALEL	9	5.1	-
177	VLVDLFLKE	10	n.t.	-
NetCTL				
394	QLLALLPSL	1	2.9	-
248	TLAKFSPYL	2	4.6	-
425	**SLLQHLIGL**[e]	3	3.7	++
435	NLTHVLYPV	4	2.5	-
100	**VLDGLDVLL**[e]	5	5.2	++
462	YLHARLREL	6	5.4	+
432	GLSNLTHVL	7	6.8	-
39	ALAIAALEL	8	5.1	-
224	KMILKMVQL	9	**>100**	n.t.
422	ALQSLLQHL	10	14.2	-
IEDB[g]				
394	QLLALLPSL	1	2.9	-
425	**SLLQHLIGL**[e]	2	3.7	++
301	**LYVDSLFFL**	3	6.3	++
248	TLAKFSPYL	4	4.6	-
294	SLQCLQALY	5	n.t.	-
100	**VLDGLDVLL**[e]	6	5.2	++
462	YLHARLREL	7	5.4	+
435	NLTHVLYPV	8	2.5	-
422	ALQSLLQHL	9	14.2	-
224	KMILKMVQL	10	**>100**	n.t.
EpiJen				
394	QLLALLPSL	1	2.9	-
425	**SLLQHLIGL**[e]	2	3.7	++
435	NLTHVLYPV	3	2.5	-
100	**VLDGLDVLL**[e]	4	5.2	++
224	KMILKMVQL	5	**>100**	n.t
248	TLAKFSPYL	6	4.6	-
432	GLSNLTHVL	7	6.8	-
51	ELFPPLFMA	8	**>100**	-
312	RLDQLLRHV	9	15.7	n.t
308	FLRGRLDQL	10	16.1	n.t

[a] Ten best scoring nonamers from PRAME.
[b] Binding score in IC_{50} (lower score, is higher affinity).
[c] Cleavages with immunoproteasomes after 1 h digestion. Index: (-) no cleavage behind C-term. of peptide, (+) low abundant cleavage, (++) abundant cleavage.
[d] n.t.; not tested.
[e] published (two) and unpublished (see text) epitopes.
[g] Prediction using the ARB method (see chapter 1, table 2).

cytosolic degradation by efficient translocation into the ER and bind with high affinity to an available class I molecule. Especially the constraints of proteolysis and class I binding render only a small fraction of all 9-, 10- and 11-mer peptides in a protein available for cell surface presentation. Furthermore, on the basis of kinetic data, it has been calculated that more than 99% of intracellular peptides are destroyed before encountering TAP [11]. Consequently, the production of class I ligands is an inefficient process. A low number of predicted epitopes, therefore, is a sign of strength of the prediction phase, provided that these peptides are genuine epitopes.

Our experience (chapters 2 and 5 [10,12]) and that of others [13], is that the extended prediction procedure including proteasomal digestion analysis, which selects peptides with high class I binding affinity that are C-terminally liberated by an abundant proteasomal cleavage site, very accurately predicts CTL epitopes. Obviously, selection of peptides according to less stringent selection criteria may result in the prediction of non-existing class I ligands. Factors that may severely hamper the validation of putative epitopes are the lack of peptide-specific precursor T cells in the repertoire of the chosen blood donor, which may be caused by tolerance in the case of tumor differentiation antigens, the possibly low sensitivity of the induced CTL, and unfavourable growth characteristics of CTL clones. To improve success rates, several adaptations have been made in the T cell induction protocols [14] and procedures were developed to accelerate expansion of specific CTL by selecting cytokine-secreting or tetramer-positive T cell populations [15].

An issue related to efficiency is the question how often peptides are predicted falsely negative, in other words: how many epitopes are missed? Apart from selection criteria that may have been chosen too stringent, falsely negative predictions can result from intrinsic weaknesses. First, peptides with high binding affinity may be missed because the algorithms are not completely covering all possible positive and negative effects on binding and some peptides lacking the canonical binding motif [16], which will score low in binding prediction and will not be selected (false negative prediction), may have high actual binding capacity (an example is shown in Table 2: peptide CTWKLPTLA lacks the canonical HLA-A2 anchors at position two and the C-terminus). Another important point is that to date the majority of binding prediction algorithms allow only predictions of nonameric and decameric peptides for most HLA class I molecules, despite the fact that a substantial number of class I ligands are 11-mers (e.g. 25 out of 298 HLA-A2 ligands in the SYFPEITHI database [17]) and still longer CTL epitopes have been reported [18-20]. Secondly, our still incomplete understanding of antigen processing will also contribute. The incorporation of only proteasomal C-terminal excision in the procedure results in falsely negative prediction of the unknown fraction of CTL epitopes that are C-terminally liberated by a proteasome-independent mechanism [21,22]. It has been calculated from experimental digestion results, for instance, that the likelihood of a proteasomal cleavage after a lysine is very low, although a high number of (mainly HLA-A3 presented) epitopes own a C-terminal lysine [5]. This is suggestive for the involvement of supplementary enzymes. Thus, verification of in vitro proteasomal C-terminal generation predicts the intracellular generation of most class I ligands, but will miss those that are C-terminally liberated by cytosolic endopeptidases like nardilysin and TOP (chapter 6, discussed below). Incorpora-

tion of nardilysin-dependent processing in epitope-prediction may result in the identification of novel epitopes (see also below). An additional category of class I ligands being missed by reverse immunology are peptides that are posttranslationally modified [23,24] or are produced intracellularly by uncommon mechanisms like peptide splicing [25,26].

In summary, when applying experimental verifications with stringent selection criteria in the prediction phase, reverse immunology is extremely well suited to successfully predict HLA class I presented ligands of which the immunogenicity is sometimes hard to confirm (or absent) due to absence of specific T cells. On the other hand reverse immunology will miss an unknown percentage of ligands/epitopes of which the restriction may be biased to certain alleles (e.g. HLA-A3). Finally, to successfully identify ligands for a certain HLA class I molecule, the length of the source antigen is obviously a relevant factor. For instance, in chapter 2, only four HLA-A2-restricted epitopes were found in the PRAME protein with a length of 509 aa [10]. Although being not necessarily the full picture, this would very roughly mean one nonameric or decameric epitope per 100 aa. protein length per class I allele (which means approximately 0.5% of the approximately 200 possible nonamers and decamers).

The future of CTL epitope identification

Although the reverse immunology strategy has been proven invaluable for the identification of HLA class I presented peptides, this approach may be superseded by more efficient methods in the near future. The advent of genomics and proteomics in the recent past has enabled the introduction of large-scale high-throughput screening methods, both for tumor antigen discovery and T cell epitope identification. It is expected that the rapidly increasing power of mass spectrometric techniques will have a tremendous impact on the unraveling of the cancer-specific and pathogen-specific HLA class I-bound 'ligandomes'. Thus, the identification of HLA class I ligands by reverse immunology-based predictions may eventually be bypassed by direct identification of cell surface presented peptides with mass spectrometry (see chapter 1, §8.4). Analysis of T cell responses against such identified (proven) HLA class I ligands is then needed only to test the immunogenicity of the epitope, and no longer to validate its cell surface expression. An advantage of direct sequencing by tandem mass spectrometry of peptides eluted from the cell surface is that it will probably identify, next to numerous novel 'conventional' ligands, also HLA class I ligands with non-canonical binding motifs, extraordinary length or post-translational modifications. Moreover these ligands may have been produced by non-conventional enzymatic mechanisms, possibly even in a proteasome-independent manner. These categories of class I presented peptides will not be identified by reverse immunology predictions.

Post proteasomal and proteasome-independent class I antigen processing

As summarized in chapter 1 (§4.4.6.), several important issues are still unresolved in class I antigen processing. A major question is the involvement of cytosolic endopeptidases in the generation of class I presented peptides. An accumulating body of published evidence points to a significant contribution of proteolysis independent of the proteasome [21,22,27-30]. However, as these studies almost without exception use proteasome-inhibitors as primary tool, and because it is known

that these inhibitors still allow significant residual proteasome activity, the real contribution, both qualitatively and quantitatively, of non-proteasomal proteolysis remains to be determined. Therefore, in chapter 6 the primary goal was to identify a CTL epitope that is indisputably made at its C-terminus by a proteasome-independent mechanism. Using a reverse immunology approach and starting with a high affinity HLA-A3 binding peptide from PRAME (aa 190-198) that was not excised at its C-terminus by the proteasome, nardilysin and thimet oligopeptidase (TOP) were demonstrated to jointly liberate the C-terminus of the PRAME$^{190-198}$ epitope. Nardilysin has the preference to cleave before or in the middle of dibasic motifs in peptides up to ~30 aa in length [31]. This cytosolic endopeptidase is implicated in the N-terminal excision of the high fraction of HLA-B27-presented peptides with a dibasic N-terminal motif (Chapter 6, Fig. 4B,C,D). Remarkably, HLA-B27 was previously found to present a high proportion of proteasome-inhibitor insensitive peptides [30]. However, when four of the peptides from this study were tested (Ch. 6, Fig. 4B, 1st, 2nd, 4th and 7th peptide), only the N-terminus was liberated by nardilysin. For one of these peptides, the epitope is located at the C-terminus of the protein (7th peptide), explaining proteasome-independence, but the other three peptides apparently need either another endopeptidase for the liberation of their C-terminus (a role of TPPII was excluded [32]) or residual proteasome-activity is capable to do so.

Having identified nardilysin and TOP in class I antigen processing, the question whether, and if so, how often, completely proteasome-independent CTL epitopes really exist is not yet answered. It is important to appreciate that a difference exists between proteasome-independent generation of the epitope's N-terminus (which is a frequent event) or its C-terminus (occurring in an unknown proportion of epitopes) as a result of post-proteasomal processing, on the one hand, and completely proteasome-independent epitope-generation on the other hand. For the time being, the existence of the latter category of epitopes will remain an open issue, because complete inhibition or silencing of the proteasome is impossible, although novel inhibitors specifically and completely inhibiting one catalytic activity of the proteasome are coming available (Ch. 6, suppl. Fig. 7 and ref. 33). This research question is also strongly related to one of the other unknowns of class I antigen processing, being the precise source and nature of the substrates that enter the processing pathway. Relatively short DRiPs may be hydrolyzed without any involvement of the proteasome, because such substrates would not need to be unfolded and are possibly within the length restrictions of some endopeptidases.

Quantitative assessment of the fractions of class I presented peptides that are C-terminally liberated in a proteasome-independent fashion is within reach of current technologies. Identification by tandem mass spectrometry of all peptides from one source protein that are presented on the cell surface of single-class I allele expressing cells and subsequently analyzing their C-terminal liberation by in vitro proteasome-mediated digestions will provide quantitative insights. This is an important question to answer, not only from a basal perspective because it possibly challenges the dogma of the proteasome being required for most C-terminal epitope excisions, but also for purposes of epitope-identification and vaccine development. At this time, we can only speculate whether the PRA$^{190-198}$ epitope (chapter 6) and the TPPII-dependent HIV Nef epitope [22] are merely exceptions or examples

of a substantial category. TPPII was proposed [34] to liberate more often peptides presented in HLA-A3 with a C-terminal lysine like the HIV Nef epitope [22]. This would make sense because of the relatively inefficient capacity of the proteasome to cleave behind a lysine [5]. However, despite considerable efforts [32,35-38] a second CTL epitope that unequivocally relies upon the endoproteolytic activity of TPPII has not been discovered.
Reverse immunology will also help to shed light on this issue. If several putative epitopes predicted to be C-terminally released by nardilysin (see e.g. Ch. 6, Fig. 4A) are indeed confirmed to be naturally presented in a nardilysin-dependent fashion, this would indicate that nardilysin is often involved in efficient antigen processing. Without doubt more epitopes will be found to be C-terminally liberated by alternative endopeptidases. As suggested by the literature [21,27,30], there may be a correlation between the chemical nature of the C-terminal anchor and frequencies of proteasome-independence. Especially HLA-A3 [21,27] and HLA-B27 [21,30], which both preferentially harbour peptides with a basic C-terminus, were found to be proteasome-inhibitor insensitive. This may possibly reflect co-evolution of endopeptidases and MHC class I peptide-binding structures. However, as thimet oligopeptidase (TOP), which has only little sequence specificity [39,40], was found to function as C-terminal trimming endopeptidase making the final C-terminal cut by releasing 3–5 residues from epitope precursors (chapter 6), proteasome-independence may not be skewed too much towards a specific C-terminal binding motif. Therefore, it will be especially interesting to investigate whether a class I molecule like HLA-A2, which has not been found to be especially proteasome-inhibitor insensitive, presents peptides that are C-terminally excised by non-proteasomal hydrolysis.
Until now the role of TOP in class I antigen processing was considered to be destructive, however this notion is heavily based on results from over-expression experiments that skew the system to antigen destruction [41-43]. The discovery of TOP's excision of the ELFSYLIEK-epitope (chapter 6) reveals a dual role for TOP in class I antigen processing: on the one hand it limits the presentation – by partial destruction – of epitopes whose correct C-terminus has already been made by (e.g.) the proteasome (like SIINFEKL [43,109]), and, on the other hand, it produces class I binding peptides by trimming 12–14 meric C-terminally extended epitope precursors that lack a C-terminal class I binding anchor. This model is in perfect accordance with a recent biochemical study demonstrating that TOP both destroys and generates peptides of the length of class I ligands [110].
A further question concerning protein degradation in the cytosol is whether next to TPPII [22,44-46], nardilysin and TOP still other cytosol-resident endopeptidases are involved in class I ligand production. The correct answer here is that there is no reason why any cytosolic endopeptidase would not be involved in protein degradation, and, thus, possibly also in epitope-generation. Examples of such enzymes are neurolysin [47] and insulin degrading enzyme [48], the latter enzyme has been suggested to be associated with the proteasome [49] and involved in the regulation of proteasomal processing [50,51].

Practical and theoretical implications of nardilysin- and TOP-dependent antigen processing

The knowledge that nardilysin is capable to excise epitopes can be used for practical purposes. Nardilysin-dependent processing can

be incorporated as selection screen in the prediction procedure of the reverse immunology approach for CTL epitope identification. Especially C-terminal excision is interesting, because of redundancy in N-terminal processing. Although nardilysin-mediated cleavages are much more predictable than proteasomal cleavages, the actual cleavage can still occur either before or in the middle of a dibasic motif. Therefore, like proteasomal cleavages, nardilysin-dependent excision of a predicted epitope should be tested experimentally by in vitro digestion of long epitope-encompassing peptides. The question must be addressed whether nardilysin-dependent processing is expected to significantly raise the number of identified CTL epitopes. This was analysed for two tumor antigens, PRAME (509 aa) and P53 (393 aa), containing eight and seven dibasic or tribasic motifs respectively. P53 was found to contain 14 class I binding peptides epitopes with a di- or tribasic motif at the C-terminus, and PRAME four predicted nardilysin-dependent class I ligands (data not shown). As expected, many of these predicted epitopes are high affinity binders for class I molecules that harbour basic C-termini. Like the $PRA^{190-198}$ CTL epitope (chapter 6), other nardilysin-dependent CTL epitopes may also be over-expressed in cells that are treated with proteasome inhibitors. Proteasome-inhibitor insensitive targeting of CTL epitopes could be used as adjuvant immunotherapeutic strategy in relapsed/refractory multiple myeloma, which is currently treated with the proteasome inhibitor Bortezomib [52].

Another promising practical relevance of nardilysin-dependent processing is envisaged to be the insertion of dibasic motifs between epitopes in multi-epitope 'string-of-bead' vaccine sequences [53,54] (see above). This would promote the efficient processing and release of the vaccine-epitopes, thereby promoting immunogenicity and vaccine efficacy. Interestingly, proof of concept of this approach can be found in the literature [55-57]. In these studies, the insertion of a dibasic motif was shown to strongly enhance presentation of both $CD4^+$ and $CD8^+$ T cell epitopes. Insertion of the dibasic motif reversed a cryptic $CD4^+$ T cell epitope from mouse lysozyme M into an immunodominant epitope in mice experiments [56]. In this study, the motif was inserted N-terminally and not directly adjacent of the epitope. Hence, improved processing of the cryptic antigenic determinant may also be attributable to dibasic-motif-induced pre-processing – by nardilysin possibly, but involvement of so-called proprotein convertases in the secretory pathway [58] can not be excluded. Such pre-processing may facilitate the accessibility to pre-existing 'silent' proteolytic cleavage sites. A similarly enhanced presentation of a $CD4^+$ T cell epitope that was induced after C-terminal insertion of a dibasic motif has been observed for exogenously loaded hen egg lysozyme [55]. Importantly, insertion of the dibasic motif directly C-terminally of a model CTL epitope from HIV-1 Gag strongly enhanced epitope production and presentation in living cells [57]. Precisely the same motif (KKYK) induced efficient nardilysin-mediated epitope-excision in vitro (see Ch. 6, Fig. 4A, 1st peptide).

As TOP-mediated C-terminal epitope excision has strong length preferences (removing 3–5 aa from the C-terminus [59,60]), but only very moderate sequence-specificity [39,40], a specific TOP-cleavage motif enabling CTL epitope-discovery (by prediction) or improved 'guided'-processing, is not available. This also hampers the discovery of additional TOP-dependent epitopes.

From a theoretical perspective, the involvement of nardilysin and TOP in epitope-pro-

duction adds again another level of diversity and complexity to class I antigen processing. Strong diversity in antigen processing, directly linked to the equally diverse binding-preferences of the highly polymorphic class I molecules, confers a significant evolutionary advantage in immunity to infectious diseases. In the case of HIV, which is subject to a very high mutation rate, antigen processing escape variants frequently occur within hosts. But, importantly, this does not result in accumulated adaptations in the HIV proteome [61]. The total number of predicted CTL epitopes in HIV-1 (clade B) has remained relatively constant over the last 30 years. This is caused by the overall reduction of viral fitness caused by mutations [62], but, importantly, also – as estimated [61] – by the fact that upon transmission of a virus to a new host up to 66% of the mutations that caused epitope (precursor) escape are released from immune pressure due to the highly diverse and polymorphic processing and presentation mechanisms.

Antigen processing, CTL epitopes and T cell-based immunotherapy

Processing of viral, bacterial, parasitic or tumor-associated proteins results in HLA class I presented peptides that can be used as target epitopes of therapeutically induced CTL. Insight in antigen processing is needed to identify and choose appropriate target epitopes and help to explain the nature (e.g. breadth, magnitude, immunodominance and immunohierarchy) of the T cell response and escape from this response by the tumor or pathogen. The procedure to identify CTL epitopes from PRAME and BCR-ABL, as described in chapters 2 and 5, can be used in a completely similar fashion for the discovery of epitopes from intracellular pathogens. Obviously, T cell-based immunotherapy for cancer relies on the same basic rules as T cell therapy for intracellular pathogens, however important differences exist as well.

Exploiting the full T cell potential in immunotherapy of cancer

Taking advantage of the full potential of the dormant anti-tumor T cell immunity of the patient will in principle greatly enhance the clinical efficacy of immunotherapy. Numerous studies indicate that the natural anti-tumor T cell repertoire is directed towards multiple CTL epitopes derived from different antigens [63-69]. Therapeutic exploitation of the complete potential of anti-tumor T cells can be achieved by adoptive transfer of ex vivo expanded tumor infiltrating lymphocytes (TILs) or vaccination with autologous tumor cells (or lysates, HSPs, mRNA derived from the tumor). However, the cumbersome procedure to generate high numbers of autologous TILs, and often the failure to obtain tumor samples, severely hampers the application of these forms of non-defined personalized immunotherapy. Therefore, worldwide efforts are mostly pursuing a TAA-defined and epitope-defined form of immunotherapy – either by adoptive transfer of PBL transduced to express a specific TCR or by vaccination with e.g. peptides encompassing CTL epitopes. This allows immunotherapy to be standardized, less laborious and possible for more patients. Targeting of defined epitopes requires the careful choice, by several criteria, of the TAA and the T cell epitopes in the TAA.

As example one may consider a specific T cell-based therapy for chronic myeloid leukemia (CML). The Philadelphia translocation-induced BCR-ABL fusion protein has a direct role in leukemogenesis. It was for that reason believed to be an advantageous target antigen because antigen escape variants that may

arise under immune pressure would not be cancerous. However, as revealed in chapter 5, only few of the neo-antigenic breakpoint-encompassing peptides in the variant BCR-ABL fusion regions are both binding to a class I molecule and C-terminally generated by the proteasome. The vaccination trials that have been conducted with non-processed class I-binding BCR-ABL fusion-peptides seem to be a piteous waste of energy [70-72]. Given our results, it is no surprise that natural T cell immunity against CML, which interestingly appeared to be multi-epitopic [73,74] (see also below), was either not [73] or hardly [74] found to be directed towards BCR-ABL breakpoint peptides. A more favourable antigen to target in CML, and other leukemias, will likely be TAA PRAME. Although PRAME is not uniformly expressed in CML [75], nor in other leukemias, this protein contains a multitude of CTL epitopes (chapter 2, and our unpublished results). Furthermore, although contradictory findings have been reported [76,77], it is strongly suggested that loss of PRAME expression may decrease the rate of distant metastases and increase patient survival [78-82]. Mechanistically, PRAME was reported to be instrumental in cancer progression through its binding to retinoic-acid receptor thereby inhibiting retinoic-acid-induced differentiation, growth arrest, and apoptosis [79]. For efficacious immunotherapy, apart from being presented on the cell surface, CTL epitopes should be sufficiently immunogenic. Therefore, we tested ex vivo T cell responses towards the four identified HLA-A2-restricted epitopes from PRAME (chapter 2) in blood from healthy donors and cancer patients (chapter 3). Especially $PRAME^{100-108}$ was demonstrated to be immunogenic, whereas $CD8^+$ T cells reactive to the three other epitopes were only found in lower frequencies. These result are not in complete accordance with similar studies conducted by others [83-86] demonstrating the anti-PRAME response to be skewed towards either $PRAME^{100-108}$ and $PRAME^{300-309}$ [85] or $PRAME^{300-309}$ and $PRAME^{142-151}$ [86]. Technical aspects and/or specific donor/patient T cell reactivity profiles may account for these differences. Apart from the breadth and the magnitude of the CTL response, relevant factors to assess are T cell avidity and effector functions before immunotherapy is being pursued. Keeping to CML as example, the targeting of only PRAME will not be enough to eradicate minimal residual disease (what is believed a realistic goal for specific T cell immunotherapy). Thus the targeting of multiple TAA, in the case of CML for instance PRAME, WT1 and proteinase 3 [73], is needed. This would exploit the full potential of the multi-epitopic tumor-specific $CD8^+$ T cell responses that occur naturally in most CML patients [73,74].

Only a few cancer types express TAA that are both directly linked to carcinogenesis and sufficiently immunogenic allowing the successful immunotherapeutic-targeting of only a single TAA. Viral oncogenic proteins like HPV E6 and E7 may constitute examples of such antigens, as is supported by clinical regressions of lesions from vulvar intraepithelial neoplasia (VIN) induced by therapeutic peptide vaccinations targeting these proteins [87].

Therapeutic vaccines for infectious diseases and some differences with cancer vaccines

Major health problems worldwide are the pandemic infectious diseases originating from human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), mycobacterium tuberculosis (*M. tuberculosis*), and the plasmodium parasites causing malaria. For instance, HIV/AIDS and tuberculosis (TB)

annually cause approximately three million and two million deaths, respectively, and in 2003 two billion individuals were infected with *M. tuberculosis* [88] and nowadays 200 million carriers are chronically infected with HBV [3]. Huge efforts are currently directed towards the development of not only prophylactic but also therapeutic vaccines for these life-threatening infections [2,3]. Vaccines have the advantage that they can be affordable and easy to administer. By vaccination, these diseases – for which antimicrobial agents are (1) often not eradicating the pathogen, thereby selecting for resistance, (2) toxic and (3) not affordable for most patients – could become containable and hopefully curable, preventing disease progression. T cell immunity, and especially $CD8^+$ T cell responses, have been shown to be essential in natural immunity partly controlling and limiting HIV/AIDS [89,90], HBV [91] and HCV [92] infections [91], TB [93], and malaria [94]. Thus, unlike the protective vaccines that mostly rely completely upon sufficiently induced humoral immunity, therapeutic vaccination for infectious diseases, requires the induction of effective CTL responses to kill the cells that are infected.

Fundamental differences between cancer vaccines and vaccines against pathogens lie in the characteristics of the antigens that need to be targeted. Tumors express on the one hand a relatively limited number of shared tumor assocated antigens (TAA) that are relatively stable, but not always really tumor-specific and they may be lost upon immune pressure and, on the other hand, unique antigens that are expressed in the tumor of only one patient (see chapter 1, §7). The often huge proteomes of pathogens are the source of a wealth of potential antigens, all harbouring numerous B- and T cell epitopes. For instance, 4000 proteins were predicted to be encoded by the *M. tuberculosis* genome [95]. Importantly, vaccines for viral, bacterial and parasitic infections need to deal with the sometimes high antigen variability. Differences in viral strains, each with their specific regional prevalence, and antigenic drift both contribute to variability in antigens [96]. HIV, for instance, by lacking proof-editing of the genetic code, can generate antigenically different forms each day [97]. The advent of genomics allows, in a strategy that is called 'reverse vaccinology', the search for conserved antigens with limited antigenic variability in the full genomes of different strains, allowing universal vaccines [98]. Obviously, these conserved antigens, and specifically the subdominant CTL epitopes that do not lead to escape under pressure of natural immunity, are the favourable targets to include in a therapeutic HIV vaccine.

Conclusion and prospects for T cell immmunotherapy

The accurate and broad identification of CTL epitopes has the advantage that it allows precise assessment of pre-existent immunity and accurate monitoring of therapy-induced immunity. Furthermore, it will help to efficiently guide the CTL responses towards multiple antigenic determinants, including immunodominant and subdominant epitopes. This will result in a broader exploitation of the full T cell potential, thereby hampering immune-evasion of the tumor or pathogen through e.g. loss of antigens, mutations or class I down regulation.

T cell inducing vaccines for both cancer and infectious pathogens have to meet the same tremendous challenges of overcoming immune evasion strategies and mounting T cell responses that are superior to the – apparently insufficient – natural immunity [99]. The correct application of emerging principles, like (1)

avoidance of tolerance induction through non-professional antigen presentation, (2) provision of sufficient help (by $CD4^+$ Th cells) and costimulation, (3) avoidance and down regulation of negative regulatory T cell responses, and, as elaborated in this thesis, (4) an appropriate multipotent target choice, will help to improve vaccine efficacy. Next to vaccines, adoptive T cell transfer is an important immunotherapeutic option (for both cancer and some viral infections) that will also strongly benefit from precise CTL epitope definition.
Important developments in cancer treatment can be expected and are needed in the application of combined conventional therapy and immunotherapy, for instance by exploiting the beneficial effects for anti-tumor immunity of radiotherapy and some chemotherapies that induce apoptosis and/or autophagy. Recent evidence indicates that the host immune system also contributes to therapeutic outcome of conventional chemo- and radiotherapy based cancer treatments. For instance, anthracyclines, which have been used to treat a broad range of cancers, can boost the host's immune system to improve the efficacy of chemotherapy [100]. Both innate and adaptive immune responses can be induced by dying tumor cells [101,102]. These anti-cancer immune responses then help to eliminate residual cancer cells and can potentially maintain micrometastases in a stage of dormancy [103]. Currently, the molecular and cellular bases of the immunogenicity of cell death that is induced by cytotoxic agents are being progressively unravelled. We begin to learn under which circumstances cellular demise induces an immune response. The immunogenicity of dying tumor cells is a function of the cell death modality: apoptotic, autophagic or necrotic cells may be either immunogenic, immunologically silent or tolerogenic for the immune system [104]. Induction of autophagy, for instance, has been demonstrated to forcefully enhance class II-restricted immunity [105] and cross-presentation [102]. Hence, induction of autophagy in cancer cells may be exploited for the induction of anti-cancer $CD4^+$ Th cells [106]. Recent studies have already demonstrated that autophagy-induced MHC class II presentation mediates resistance to pathogens and is targeted for immune evasion by viruses and bacteria [107,108]. In one system, autophagy in tumor cells has also been shown to be indispensable for the cross presentation of tumor-derived antigens by DC [102].
Thus, an optimal treatment of cancer has to implement results from different research areas.

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CHAPTER 8

CHAPTER 8

Samenvatting voor de niet-ingewijde

De titel van dit proefschrift luidt: 'De generatie van cytotoxische T cel epitopen en hun identificatie voor immunotherapie van kanker'. Er komen een paar vragen op:
– *Wat zijn cytotoxische T cellen?*
– *Wat zijn die epitopen?*
– *Waarom die epitopen identificeren voor een immunotherapie tegen kanker?*
Hier wil ik deze vragen in woord en beeld (zie figuur) beantwoorden voor de niet-immunoloog. Om te beginnen is het goed om je te realiseren dat het onderzoek veel meer met immunologie te maken heeft dan met kanker.
Het uiteindelijke doel is om de strategie die het lichaam gebruikt in de afweer tegen virussen en bacteriën therapeutisch toe te passen voor het dood maken van kankercellen.

Het immuunsysteem (afweersysteem) bestaat hoofdzakelijk uit de witte bloedcellen (ook leukocyten genoemd) en diverse immunologische herkenningsmoleculen en stimulatiemoleculen die door de afweercellen worden uitgescheiden. Afweercellen bevinden zich in de lymfeklieren, de lymfe en het bloed en kunnen zodoende in alle organen doordringen. De afweercellen gaan ingenieuze interacties aan met elkaar en vooral ook met (componenten van) pathogene indringers waardoor de afweer tegen de ziekteverwekkers wordt geïnitieerd. Het immuunsysteem beschermt het menselijk lichaam tegen besmettelijke ziekten die door bacteriën, virussen en parasieten worden overgebracht en veroorzaakt. Deze pathogene indringers komen het lichaam meestal binnen via de gastrointestinale, urogenitale of nasofaryngale routes, en na verwonding ook via de huid. Ziekteverwekkers moeten eerst worden herkend door (componenten van) het immuunsysteem voordat ze geëlimineerd kunnen worden. **Herkenning** van pathogene structuren ligt daarom ten grondslag aan het functioneren van het immuunsysteem. Naast herkenning, bepaald **de mate van stimulering** van het immuunsysteem of de afweer voldoende, te weinig of te veel zal zijn. Afhankelijk van de aard van het pathogeen en de plaats van de infectie gebruikt het immuunsysteem een andere afweerstrategie. Een belangrijk verschil is er tussen de afweer tegen pathogenen die buiten de cel blijven en afweer tegen pathogenen die zich in cellen nestelen. Alle virussen (o.a. gewone verkoudheidsvirussen), sommige bacteriën (bv. degenen die TBC of tyfus veroorzaken) en sommige parasieten (bv. de malaria parasiet) infecteren lichaamscellen en vermenigvuldigen zich in de cel. Voor de eliminatie van deze pathogenen moeten de geïnfecteerde cellen worden opgeruimd door afweercellen.

Herkenning: hoe herkennen afweercellen dat een lichaamscel geïnfecteerd is?
Een subpopulatie van de witte bloedcellen, **de cytotoxische T-lymfocyten (ofwel T-cel, afgekort CTL)**, is daartoe uitgerust met een receptor **(T-cel receptor, ofwel TCR)** op zijn membraan waarmee hij continue alle lichaamscellen 'afsnuffelt' op kleine stukjes eiwit, **peptiden** genaamd, die op hun oppervlakte tentoongespreid worden. Als een CTL een peptide op de oppervlakte van de lichaamscel herkent komt hij in actie: hij bindt aan de cel en maakt de cel dood. Dus een CTL is cytotoxisch. Elke CTL heeft duizenden (circa 50.000) identieke copies van

dezelfde TCR op zijn oppervlakte, en die TCR kan slechts één peptide goed herkennen, daarom heeft een CTL slechts specificiteit (bindingskracht) voor één peptide. De **herkenning van geïnfecteerde lichaamscellen** door de CTL vindt plaats middels de herkenning van **peptiden die afkomstig zijn van bacteriële of virale eiwitten** (bacteriën en virussen bestaan uit eiwitten) op de oppervlakte van die cellen.

Herkenning: hoe worden de herkende peptiden gemaakt?

Cellen bestaan voor een groot deel uit eiwitten (proteïnen) welke van cruciaal belang zijn voor de structuur en functie van de cel. Eiwitten zijn opgebouwd uit **aminozuren (afgekort aa. – van amino acids)**, waarvan er 20 verschillende zijn, die als een kralensnoer (ongeveer 100 tot 500 aa. per eiwit) in een voor ieder verschillend eiwit unieke volgorde aan elkaar gekoppeld zijn. In elke cel bevinden zich duizenden verschillende eiwitten, en van elk eiwit weer vele kopieën. De eiwitten hebben afhankelijk van hun rol in de cel een verschillende levensduur, variërend van enkele uren tot een week of langer. Eiwitmoleculen die aan het einde van hun levensduur zijn, en degene die met fouten gemaakt zijn, worden afgebroken tot aminozuren welke dan weer gebruikt worden voor de synthese van nieuwe eiwitten.

De **afbraak van eiwitten** in de cel is een stapsgewijs proces waarbij de koppelingen tussen de aminozuren worden 'losgeknipt'. Dit proces wordt gecontroleerd door enzymen (katalysatoren van chemische reacties). Een enzym dat een eiwit of een deel van een eiwit doorknipt wordt respectievelijk een **proteinase of peptidase** genoemd. Het belangrijkste proteinase in de cel is het zogenaamde **proteasome** dat de eerste stap van de eiwitafbraak uitvoert waarbij **eiwitbrokstukken (peptiden)** van ca 10 á 20 aa. lang overblijven. Deze peptiden worden vervolgens door peptidasen in de cel in weer kleinere stukken geknipt. Het proteasome en de peptidases hebben een bepaalde specificiteit, d.w.z. dat ze een bepaalde aminozuur volgorde (sequentie) al dan niet graag kapot knippen. De specificiteit van het proteasome en peptidasen bepaald dus waar (tussen welke aa.) in een eiwit geknipt wordt en daardoor welke peptiden uit een eiwit molecuul zullen ontstaan. De afbraak van eiwitten gaat **in principe** door tot er van elk peptide slechts losse aminozuren overblijven.

Echter, gedurende de evolutie is er een mechanisme ontstaan waarbij het immuunsysteem gebruik maakt van de peptiden die bij eiwitafbraak ontstaan! Een klein percentage van deze peptiden zal namelijk ontsnappen naar een door membranen afgeschermd organel in de cel, het zogenaamde **endoplasmatisch reticulum (ER) (zie figuur 1, blz. 18)**. Daar zijn de peptiden beschermd tegen verdere afbraak omdat er zich minder peptidasen bevinden. Bovendien zullen de peptiden als ze in het ER zijn – en de goede lengte en aminozuurvolgorde hebben – binden in de groeve van daar aanwezige **HLA moleculen (HLA staat voor 'Human Leucocyte Antigen')**. Er ontstaat zo een **HLA-peptide complex**. Deze complexen worden vervolgens getransporteerd naar het celoppervlakte waar ze zich verankeren in de membraan. Het peptide wijst naar buiten en kan worden herkend door een CTL (die in de buurt is) indien de CTL een TCR heeft met de juiste specificiteit (bindingskracht) voor het peptide (gebonden in het HLA molecuul).

Van elk eiwit dat wordt afgebroken in de cel worden op deze manier peptiden gemaakt die op de celoppervlakte **gepresenteerd** (tentoongespreid) worden aan CTL. Dit mechanisme vindt net zo goed plaats in gezonde cellen als in cellen die geïnfecteerd zijn door virussen of bacteriën. In het

laatste geval zullen dus ook virale of bacteriële eiwitten die zich in de cel bevinden worden afgebroken tot peptiden die dan aan CTL gepresenteerd worden. Door de unieke aminozuurvolgorde van elk eiwit zijn de honderden tot duizenden verschillende peptiden op de oppervlakte van een cel een representatie van alle eiwitten in de cel, inclusief bacteriële of virale eiwitten als de cel geïnfecteerd is. Anders gezegd: elk peptide gepresenteerd op de oppervlakte van lichaamscellen is een **uniek 'visitekaartje'** van het eiwit waar het van afkomstig is en verraad de aanwezigheid van dat eiwit in de cel. Van dit feit maakt het immuunsysteem gebruik om te bepalen of een cel al dan niet geïnfecteerd is door een virus of bacterie.

Herkenning: waarom worden gezonde cellen niet herkend en aangevallen door CTL?
Als een gepresenteerd peptide (HLA-peptide complex) herkend wordt door een TCR op de CTL, d.w.z. er aan bind, dan wordt het peptide een **CTL-epitoop** genoemd. De herkenning door de CTL van een CTL-epitoop activeert de CTL zodat hij cytotoxische moleculen uitscheid in zijn naaste omgeving die de lichaamscel waar hij aan gebonden is dood maakt.
Zoals gezegd heeft elke CTL, middels zijn TCR, een unieke specificiteit (bindingskracht) voor slechts één CTL-epitoop. **Uiteraard is het niet de bedoeling dat een CTL gezonde lichaamscellen herkend en aanvalt.** Ieder mens heeft in zijn lichaam een repertoire van miljoenen verschillende CTL (met elk veel kopieën van één TCR) zodat er evenveel verschillende peptiden herkend kunnen worden. Dus zou er gevaar zijn voor de herkenning van CTL epitopen afkomstig van gezonde eiwitten (lichaamseigen of 'zelf' eiwitten), wat auto-immuniteit (afweer tegen gezonde cellen) zou veroorzaken.
Echter, tijdens de ontwikkeling van de T cellen (in de thymus) zijn alle CTL die een CTL-epitoop herkennen dat afkomstig is van een gezond (lichaamseigen) eiwit er uitgeselecteerd! Deze zogenaamde **negatieve selectie** zorgt ervoor dat de overblijvende CTL in het repertoire alleen epitopen zullen herkennen die afkomstig zijn van pathogene (lichaamsvreemde) eiwitten, en dus op geïnfecteerde cellen gepresenteerd worden. Gezonde lichaamscellen worden niet aangevallen omdat er geen CTL zijn die peptiden van gezonde eiwitten herkennen.

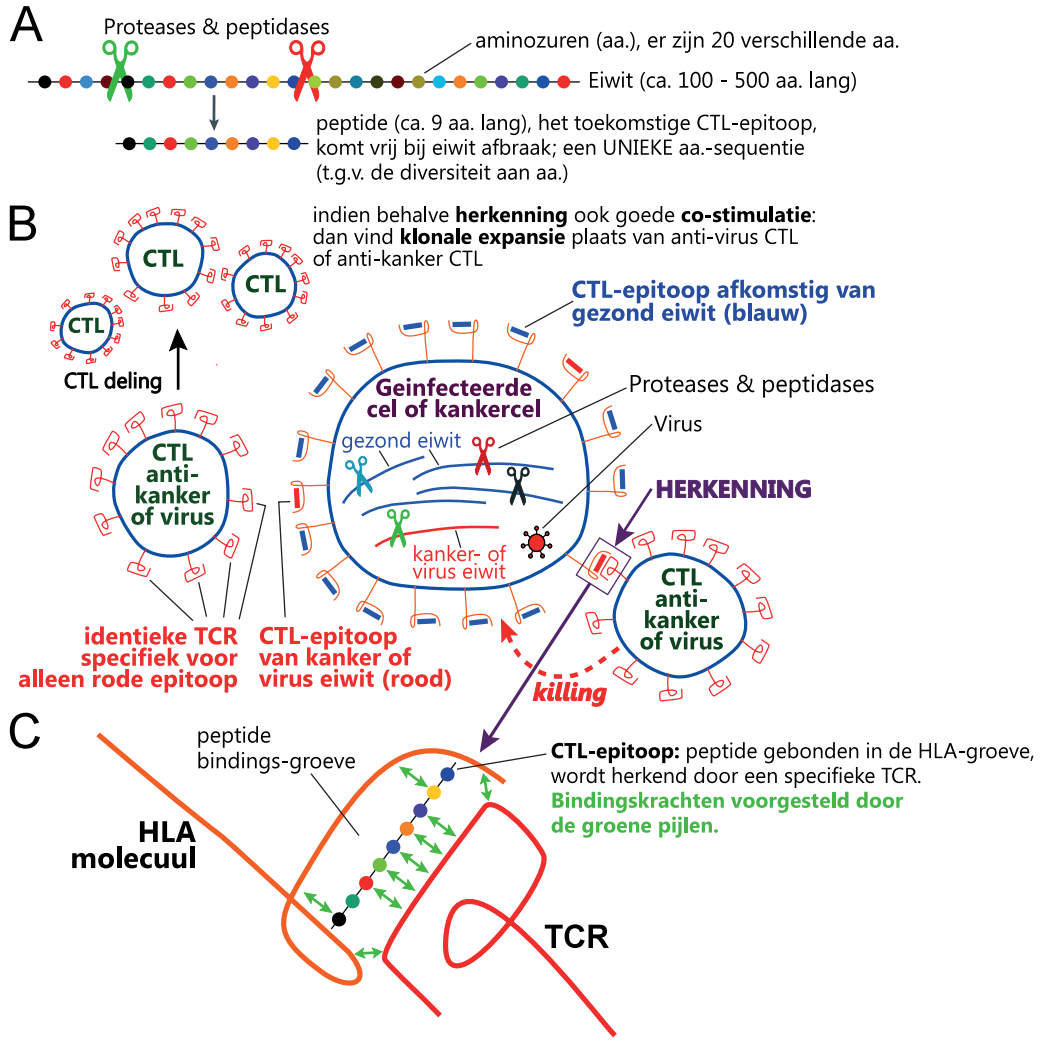
De afweer tegen kanker: hoe zit het daarmee?
In geval van kanker is er wel een sluimerende CTL-afweer tegen de tumorcellen, maar die is niet afdoende. In een kankercel worden eiwitten gemaakt die een gezonde cel niet produceert, waardoor de kankercel zich blijft delen. Omdat deze eiwitten nieuw zijn – ofwel lichaamsvreemd – voor het immuunsysteem, heeft er geen negatieve selectie plaatsgevonden van CTL die epitopen herkennen welke afkomstig zijn van de afbraak van de kankereiwitten. **Er zijn dus wel degelijk CTL beschikbaar die tumorspecifieke CTL-epitopen herkennen en zo de kankercel kunnen aanvallen.** Echter, in tegenstelling tot de afweer tegen viraal- of bacterieel geïnfecteerde cellen, blijft de afweer tegen kankercellen meestal slechts sluimeren of is geheel afwezig.
Waar komt dat door? Elke CTL die een epitoop herkend wordt in voldoende mate geactiveerd om de herkende cel aan te vallen. In geval van een virale of bacteriële infectie wordt de CTL bovendien nog **extra geactiveerd** door een tweede signaal. Deze zogenaamde co-stimulatie leidt er toe dat de CTL zich 'klonaal' gaat delen. Zo komen er duizenden tot miljoenen identieke CTL beschikbaar die hetzelfde CTL-epitoop herkennen en daarmee alle geïnfecteerde cellen kunnen

opruimen die dat epitoop op hun membraan presenteren. **Het probleem bij kanker is dat het co-stimulatie signaal niet door kankercellen wordt gegeven.** Daarom is er **geen klonale expansie** van CTL die tegen de tumorcellen gericht zijn en zijn er niet genoeg CTL om de kankercellen op te ruimen.

De afweer tegen kanker: kan die therapeutisch versterkt worden?
Ook al laat de natuurlijke afweer tegen kanker het afweten, deze valt wel op te wekken met immunotherapie. Als we de identiteit van de tumorspecifieke CTL-epitopen weten, kunnen we die peptiden artificieel synthetiseren en inspuiten in de patiënt (vaccinatie tegen kanker). Als dan ook nog het co-stimulatie signaal in de patiënt wordt bevorderd dan zal het synthetische peptide (na binding aan een HLA molecuul op lichaamscellen) de CTL die het peptide herkend activeren en doen laten expanderen, zodat er genoeg CTL zijn om zoveel mogelijk tumorcellen te herkennen en aan te vallen. In veel dierexperimenten en ook in klinische trials in patiënten is inmiddels aangetoond dat aldus opgewekte CTL de tumor geheel kan doen laten verdwijnen.

Tenslotte: wat is er onderzocht?
Als het bekend is welke proteasen en peptidasen waar knippen in een eiwit, dan valt het te voorspellen en te testen welke peptiden bij de afbraak vrijkomen. Dit is een universeel mechanisme, de eiwitafbraak verloopt in elk individu hetzelfde: er komt een zelfde set peptiden vrij uit een zelfde (kankerspecifiek) eiwitmolecuul in ieder individu. In **hoofdstuk 2** is gekeken naar de cruciale rol van het proteasome bij eiwitafbraak en in **hoofdstuk 6** is gezocht naar nog onbekende peptidasen die bijdragen aan eiwitafbraak en daarbij CTL-epitopen maken.
Eiwitafbraak is hetzelfde in elke individu, maar een complicerende factor is dat de HLA moleculen wel verschillen per individu. Er bestaan veel verschillende HLA moleculen die elk iets anders zijn en een eigen peptide bindingsspecificiteit hebben. Aangezien elk individu een bepaalde set van HLA moleculen op zijn lichaamscellen heeft zullen mensen ook verschillende peptiden 'kiezen' uit alle beschikbare afbraakpeptiden om als CTL-epitoop te presenteren. Daarom moet getest worden welke afbraakpeptiden kunnen binden in de groeve van de HLA moleculen die op de lichaamscellen van de patiënt aanwezig zijn. In **hoofdstuk 4** zijn er testen ontwikkeld waarmee die binding bepaald kan worden. Weten we welke peptiden kunnen binden, dan zijn de gepresenteerde CTL-epitopen bekend, zodat die in de patiënt therapeutisch kunnen worden ingespoten (vaccinatie). In **hoofdstuk 2** zijn er vier CTL-epitopen geïdentificeerd die afkomstig zijn van het kankerspecifieke eiwit 'PRAME'. En in **hoofdstuk 3** is hetzelfde gedaan: er is geïnventariseerd welke CTL-epitopen worden gemaakt uit het eiwit 'BCR-ABL' dat alleen in leukemiecellen voorkomt. In **hoofdstuk 5** is er tenslotte gekeken of er in het bloed van gezonde mensen en van kankerpatiënten CTL aanwezig zijn die de vier CTL-epitopen die geïdentificeerd zijn in hoofdstuk 2 kunnen herkennen.

Kortom, de bestudering van de manier waarop CTL-epitopen gemaakt worden en identificatie van deze epitopen staan in dit proefschrift centraal ten behoeve van het ontwikkelen van immunotherapie voor kanker.



Figuur 1. Herkenning van geïnfecteerde cellen of kanker cellen door CTL in a nutshell.
A. Detail eiwitafbraak: eiwitten hebben een unieke aa. volgorde, dus de peptiden die ontstaan bij afbraak zijn ook uniek: ze 'veraden' de aanwezigheid van het eiwit binnen in de cel.
B. Overzicht CTL herkenning: geïnfecteerde cellen of kanker cellen hebben eiwitten (rood) die gezonde cellen niet hebben. Bij afbraak komen daar peptiden (rood) uit vrij die op de celmembraan gepresenteerd worden. In het lichaam zijn er CTL die deze rode epitopen zullen herkennen. Herkenning van het epitoop activeert de CTL die daarop aanvalt en de cel dood maakt. Indien er tevens een extra co-stimulatie signaal aanwezig is, zal de CTL ook gaan delen (klonale expansie).
C. Detail CTL herkenning: het CTL-epitoop is a.h.w. een sleutel die op twee sloten past en daarmee de herkenning van de geïnfecteerde lichaamscel of kanker cel door de CTL mogelijk maakt. Twee bindingsfenomenen spelen daarbij een rol (groene pijlen): **(1)** binding v.d. buitenste aa. van het epitoop in de groeve van het HLA-molecuul (= epitoop-presentatie). Terzijde: zolang de buitenste aa. van een peptide de goede eigenschappen hebben zal het peptide kunnen binden, en **(2)** binding v.d. TCR aan de binnenste aa. van het epitoop en tevens aan delen van het HLA-molecuul (= epitoop herkenning). Terzijde: de bindingcapaciteit van een TCR beperkt zich tot slechts één of ten hoogste een paar epitopen (dwz een peptide gebonden in een bepaald HLA molecuul).

CV & PUBLICATIONS

CV & PUBLICATIONS

Curriculum vitae

Jan Kessler werd geboren op 7 oktober 1959 in Amsterdam. Na het behalen van het VWO diploma aan het Willem de Zwijger College in Bussum heeft hij verschillende opleidingen gevolgd: propedeuse psychologie aan de Vrije Universiteit, afgeronde opleiding tot meubelmaker aan de Vakschool voor meubelmakers, behangers en stoffeerders in de Jordaan en doctoraal geneeskunde (Universiteit van Amsterdam, UvA). Begin jaren '90 werd de steven gewend richting 'de wetenschap' met het doctoraal medische biologie (UvA), waaronder een 14-maandse stage in de groep van Arthur Verhoeven binnen de afdeling Bloedcelchemie aan het Centraal Laboratorium voor de Bloedtransfusie (CLB) in Amsterdam waar aan de celbiologie van de neutrofiele granulocyt werd gewerkt. Sinds september 1994 werkt hij binnen de tumorimmunologie groep van de afdeling Immunohematologie en Bloedtransfusie aan het LUMC te Leiden wat heeft geresulteerd in het hier gepresenteerde werk. In 2004 heeft hij samen met Jan Paul Medema onderzoek geïnitieerd naar betulinezuur als potentieel anti-kanker therapeuticum, waar hij nog mede vorm aan geeft.

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NAWOORD

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Nawoord

Vanaf 1994, mijn start in de tumorimmunologiegroep, is er veel veranderd binnen de groep en binnen de afdeling IHB. Zelfs het meubilair is tenminste twee keer vernieuwd. Desalniettemin zijn er een aantal vaste waarden geweest die het werk effectief hebben doen laten verlopen, er onontbeerlijk voor waren, en het sociaal gesproken leuk hebben gemaakt om te werken. Daar wil ik hier graag even bij stilstaan en dank voor zeggen. Jan Wouter, jouw altijd makkelijk toegankelijke input in praktische en theoretische zin, o.a. bij de bindingsstudies en bij substraatontwerp, waren cruciaal en steeds bemoedigend. Uiteraard dank voor de vele uren werk van alle peptidologen: Willemien, Peter, Antoinette, 2e generatie Drijfhout en de nieuwe leden – dropjes kunnen daar nooit tegenop. Ferry, de implementatie van de door jou opgezette proteasome-digestie analyse in mijn project heeft het epitope-identificatie werk een backbone gegeven en het overleg over het werk was nuttig en prettig. Peter en Arnoud, massspec analyse stond centraal in de meeste studies. Jullie giga vele metingen en jouw theoretische bijstand waren vaak de drijfveer van het werk. Bovendien altijd gezellig even op het MS-lab of andere hangplek te vertoeven. Selina, jouw bijdrage heeft een belangrijke hobbel helpen nemen. Marieke G, het was goed een tijdelijke PRAME-genote te hebben. De stagiaires Ivo Huijbers, Pauline Verdijk, Debby Vissers en Nadine hebben met grote inzet, ieder voor 9 maanden, het werk een push gegeven. Bovendien, Nadine, hoor je met Marieke, Martijn, Reza en Sytse bij de recente AIO's met wie het figuurlijk én letterlijk goed fietsen is over werk en al dan niet verwante zaken. Sytse bedankt voor je spoeduitleg InDesign. Heel veel labgenoten door de jaren heen waren prettige collega's. Uit deze categorie – subcategorie flowgenoten – wil ik Anke noemen: behalve je fleurige kledingadviezen waardeer ik ook de leuke gesprekjes in de flow. IHB-ers: met name de bijdragen van Ilias en Yvonne Z, Els G, Arend en Kees F waren belangrijk. Kamergenoten, velen, steeds belangrijk voor alle intermezzo's en de gezelligheid. In de categorie oude garde en educatie, Antoinette: het is lang geleden dat die T175 op z'n kop de hele stoof doorlekte; dankzij jouw adviezen toen, en inzet gedurende een paar maanden daarna, "mag je dit nog meemaken". Afdeling oude garde en weddenschappen: Rene, altijd een goede oneliner, leuk. Hoe ging die weddenschap ook al weer, Maaike? Sandra L, jouw adviezen bij de peptide-eluties waren echt nodig, en let's continue met die ijsjes & lunches. Sandra B, je was cruciaal voor molbiol-educatie en niet te vergeten productie. Maar vooral ben je een vaste waarde om 't werk een beetje gezellig te houden en te relativeren. Voor de broodnodige afleiding en inspiratie buiten de immunologie: Jan Paul en Franziska, die interessante en gezellige wandelingen in het grote dichte berkenbos waren belangrijk voor me. Tenslotte, Kees, laat ik het zoals hier vereist is sec stellen: we waren goede 'brothers in crime' – de inhoud gaat boven alles, zoals het hoort.

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