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

Kessler, J.

CHAPTER 7

CHAPTER 7

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, 1^{st} 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.

References

1. Goldman B, DeFrancesco L: The cancer vaccine roller coaster. Nat Biotechnol 27:129-139, 2009.
2. Sela M, Hilleman MR: Therapeutic vaccines: realities of today and hopes for tomorrow. Proc Natl Acad Sci U S A 101 Suppl 2:14559, 2004.
3. Autran B, Carcelain G, Combadiere B, Debre P: Therapeutic vaccines for chronic infections. Science 305:205-208, 2004.
4. Yewdell JW, Bennink JR: Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu Rev Immunol 17:51-88, 1999.
5. Tenzer S, Peters B, Bulik S, Schoor O, Lemmel C, Schatz MM, Kloetzel PM, Rammensee HG, Schild H, Holzhutter HG: Modeling the MHC class I pathway by combining predictions of proteasomal cleavage, TAP transport and MHC class I binding. Cell Mol Life Sci 62:1025-1037, 2005.
6. Donnes P, Kohlbacher O: Integrated modeling of the major events in the MHC class I antigen processing pathway. Protein Sci 14:2132-2140, 2005.
7. Larsen MV, Lundegaard C, Lamberth K, Buus S, Brunak S, Lund O, Nielsen M: An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions. Eur J Immunol 35:2295-2303, 2005.
8. Zhang Q, Wang P, Kim Y, Haste-Andersen P, Beaver J, Bourne PE, Bui HH, Buus S, Frankild S, Greenbaum J, Lund O, Lundegaard C, Nielsen M, Ponomarenko J, Sette A, Zhu Z, Peters B: Immune epitope database analysis resource (IEDB-AR). Nucleic Acids Res 36:W513-W518, 2008.
9. Doytchinova IA, Guan P, Flower DR: EpiJen: a server for multistep T cell epitope prediction. BMC Bioinformatics 7:131-141, 2006.
10. Kessler JH, Beekman NJ, Bres-Vloemans SA, Verdijk P, van Veelen PA, Kloosterman-Joosten AM, Vissers DC, ten Bosch GJ, Kester MG, Sijts A, Drijfhout JW, Ossendorp F, Offringa R, Melief CJM: Efficient identification of novel HLA-A(*)0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis. J Exp Med 193:73-88, 2001.
11. Yewdell JW, Reits E, Neefjes J: Making sense of mass destruction: quantitating MHC class I antigen presentation. Nat Rev Immunol 3:952-961, 2003.
12. Kessler JH, Bres-Vloemans SA, van Veelen PA, de Ru A, Huijbers IJ, Camps M, Mulder A, Offringa R, Drijfhout JW, Leeksma OC, Ossendorp F, Melief CJ: BCR-ABL fusion regions as a source of multiple leukemia-specific CD8(+) T-cell epitopes. Leukemia 20:1738-1750, 2006.
13. Hassainya Y, Garcia-Pons F, Kratzer R, Lindo V, Greer F, Lemonnier FA, Niedermann G, van Endert PM: Identification of naturally processed HLA-A2--restricted proinsulin epitopes by reverse immunology. Diabetes 54:2053-2059, 2005.
14. Schultze JL, Michalak S, Seamon MJ, Dranoff G, Jung K, Daley J, Delgado JC, Gribben JG, Nadler LM: CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. J Clin Invest 100:2757-2765, 1997.
15. Ayyoub M, Stevanovic S, Sahin U, Guillaume P, Servis C, Rimoldi D, Valmori D, Romero P, Cerottini JC, Rammensee HG, Pfreundschuh M, Speiser D, Levy F: Proteasome-assisted identification of a SSX-2-derived epitope recognized by tumor-reactive CTL infiltrating metastatic melanoma. J Immunol 168:1717-1722, 2002.
16. Bredenbeck A, Losch FO, Sharav T, Eichler-Mertens M, Filter M, Givehchi A, Sterry W, Wrede P, Walden P: Identification of noncanonical melanoma-associated T cell epitopes for cancer immunotherapy. J Immunol 174:6716-6724, 2005.
17. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S: SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 50:213-219, 1999.
18. Probst-Kepper M, Stroobant V, Kridel R, Gaugler B, Landry C, Brasseur F, Cosyns JP, Weynand B, Boon T, Van den Eynde BJ: An alternative open reading frame of the human macrophage colony-stimulating factor gene is independently translated and codes for an antigenic peptide of 14 amino acids recognized by tumor-infiltrating CD8 T lymphocytes. J Exp Med 193:1189-1198, 2001.
19. Tynan FE, Burrows SR, Buckle AM, Clements CS, Borg NA, Miles JJ, Beddoe T, Whisstock JC, Wilce MC, Silins SL, Burrows JM, Kjer-Nielsen L, Kostenko L, Purcell AW, McCluskey J, Rossjohn J: T cell receptor recognition of a 'super-bulged' major histocompatibility complex class I-bound peptide. Nat Immunol 6:1114-1122, 2005.
20. Samino Y, Lopez D, Guil S, Saveanu L, van Endert PM, Del Val M: A long N-terminally extended nested set of abundant and antigenic MHC class I natural ligands from HIV envelope protein. J Biol Chem 281:6358-6365, 2006.
21. Luckey CJ, Marto JA, Partridge M, Hall E, White FM, Lippolis JD, Shabanowitz J, Hunt DF, Engelhard VH: Differences in the expression of human class I MHC alleles and their associated peptides in the presence of proteasome inhibitors. J Immunol 167:1212-1221, 2001.

22. Seifert U, Maranon C, Shmueli A, Desoutter JF, Wesoloski L, Janek K, Henklein P, Diescher S, Andrieu M, de la SH, Weinschenk T, Schild H, Laderach D, Galy A, Haas G, Kloetzel PM, Reiss Y, Hosmalin A: An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. Nat Immunol 4:375-379, 2003.
23. Skipper JC, Hendrickson RC, Gulden PH, Brichard V, Van Pel A, Chen Y, Shabanowitz J, Wolfel T, Slingluff CL, Jr., Boon T, Hunt DF, Engelhard VH: An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. J Exp Med 183:527-534, 1996.
24. Engelhard VH, Altrich-Vanlith M, Ostankovitch M, Zarling AL: Post-translational modifications of naturally processed MHC-binding epitopes. Curr Opin Immunol 18:92-97, 2006.
25. Vigneron N, Stroobant V, Chapiro J, Ooms A, Degiovanni G, Morel S, van der BP, Boon T, Van den Eynde BJ: An antigenic peptide produced by peptide splicing in the proteasome. Science 304:587-590, 2004.
26. Hanada K, Yewdell JW, Yang JC: Immune recognition of a human renal cancer antigen through post-translational protein splicing. Nature 427:252-256, 2004.
27. Benham AM, Gromme M, Neefjes J: Allelic differences in the relationship between proteasome activity and MHC class I peptide loading. J Immunol 161:83-89, 1998.
28. Luckey CJ, King GM, Marto JA, Venketeswaran S, Maier BF, Crotzer VL, Colella TA, Shabanowitz J, Hunt DF, Engelhard VH: Proteasomes can either generate or destroy MHC class I epitopes: evidence for nonproteasomal epitope generation in the cytosol. J Immunol 161:112-121, 1998.
29. Schwarz K, de Giuli R, Schmidtke G, Kostka S, van Den BM, Kim KB, Crews CM, Kraft R, Groettrup M: The selective proteasome inhibitors lactacystin and epoxomicin can be used to either up- or down-regulate antigen presentation at nontoxic doses. J Immunol 164:6147-6157, 2000.
30. Marcilla M, Cragnolini JJ, Lopez de Castro JA: Proteasome-independent HLA-B27 ligands arise mainly from small basic proteins. Mol Cell Proteomics 6:923, 2007.
31. Chow KM, Csuhai E, Juliano MA, St Pyrek J, Juliano L, Hersh LB: Studies on the subsite specificity of rat nardilysin (N-arginine dibasic convertase). J Biol Chem 275:19545-19551, 2000.
32. Marcilla M, Villasevil EM, de Castro JA: Tripeptidyl peptidase II is dispensable for the generation of both proteasome-dependent and proteasome-independent ligands of HLA-B27 and other class I molecules. Eur J Immunol 38:631-639, 2008.
33. van Swieten PF, Samuel E, Hernandez RO, van den Nieuwendijk AM, Leeuwenburgh MA, van der Marel GA, Kessler BM, Overkleeft HS, Kisselev AF: A cell-permeable inhibitor and activity-based probe for the caspase-like activity of the proteasome. Bioorg Med Chem Lett 17:3402-3405, 2007.
34. Geier E, Pfeifer G, Wilm M, Lucchiari-Hartz M, Baumeister W, Eichmann K, Niedermann G: A giant protease with potential to substitute for some functions of the proteasome. Science 283:978-981, 1999.
35. Guil S, Rodriguez-Castro M, Aguilar F, Villasevil EM, Anton LC, Del Val M: Need for Tripeptidyl-peptidase II in Major Histocompatibility Complex Class I Viral Antigen Processing when Proteasomes are Detrimental. J Biol Chem 281:39925-39934, 2006.
36. Wherry EJ, Golovina TN, Morrison SE, Sinnathamby G, McElhaugh MJ, Shockey DC, Eisenlohr LC: Re-evaluating the generation of a "proteasome-independent" MHC class I-restricted CD8 T cell epitope. J Immunol 176:2249-2261, 2006.
37. York IA, Bhutani N, Zendzian S, Goldberg AL, Rock KL: Tripeptidyl Peptidase II Is the Major Peptidase Needed to Trim Long Antigenic Precursors, but Is Not Required for Most MHC Class I Antigen Presentation. J Immunol 177:1434-1443, 2006.
38. Basler M, Groettrup M: No essential role for tripeptidyl peptidase II for the processing of LCMV-derived T cell epitopes. Eur J Immunol 37:896-904, 2007.
39. Oliveira V, Campos M, Melo RL, Ferro ES, Camargo AC, Juliano MA, Juliano L: Substrate specificity characterization of recombinant metallo oligopeptidases thimet oligopeptidase and neurolysin. Biochemistry 40:4417-4425, 2001.
40. Sigman JA, Patwa TH, Tablante AV, Joseph CD, Glucksman MJ, Wolfson AJ: Flexibility in substrate recognition by thimet oligopeptidase as revealed by denaturation studies. Biochem J 388:255-261, 2005.
41. Saric T, Beninga J, Graef CI, Akopian TN, Rock KL, Goldberg AL: Major histocompatibility complex class I-presented antigenic peptides are degraded in cytosolic extracts primarily by thimet oligopeptidase. J Biol Chem 276:36474-36481, 2001.
42. Kim SI, Pabon A, Swanson TA, Glucksman MJ: Regulation of cell-surface major histocompatibility complex class I expression by the endopeptidase EC3.4.24.15 (thimet oligopeptidase). Biochem J 375:111-120, 2003.
43. York IA, Mo AX, Lemerise K, Zeng W, Shen Y, Abraham CR, Saric T, Goldberg AL, Rock KL: The cytosolic endopeptidase, thimet oligopeptidase,

destroys antigenic peptides and limits the extent of MHC class I antigen presentation. Immunity 18:429-440, 2003.
44. Reits E, Neijssen J, Herberts C, Benckhuijsen W, Janssen L, Drijfhout JW, Neefjes J: A major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation. Immunity 20:495-506, 2004.
45. Tomkinson B, Lindas AC: Tripeptidyl-peptidase II: a multi-purpose peptidase. Int J Biochem Cell Biol 37:1933-1937, 2005.
46. van Endert P: Role of tripeptidyl peptidase II in MHC class I antigen processing – the end of controversies? Eur J Immunol 38:609-613, 2008.
47. Brown CK, Madauss K, Lian W, Beck MR, Tolbert WD, Rodgers DW: Structure of neurolysin reveals a deep channel that limits substrate access. Proc Natl Acad Sci U S A 98:3127-3132, 2001.
48. Shen Y, Joachimiak A, Rosner MR, Tang WJ: Structures of human insulin-degrading enzyme reveal a new substrate recognition mechanism. Nature 443:870-874, 2006.
49. Bennett RG, Hamel FG, Duckworth WC: Identification and isolation of a cytosolic proteolytic complex containing insulin degrading enzyme and the multicatalytic proteinase. Biochem Biophys Res Commun 202:1047-1053, 1994.
50. Duckworth WC, Bennett RG, Hamel FG: A direct inhibitory effect of insulin on a cytosolic proteolytic complex containing insulin-degrading enzyme and multicatalytic proteinase. J Biol Chem 269:24575-24580, 1994.
51. Hamel FG, Bennett RG, Duckworth WC: Regulation of multicatalytic enzyme activity by insulin and the insulin-degrading enzyme. Endocrinology 139:4061-4066, 1998.
52. Orlowski RZ, Kuhn DJ: Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 14:1649-1657, 2008.
53. Toes RE, Hoeben RC, van der Voort EI, Ressing ME, van der Eb AJ, Melief CJ, Offringa R: Protective anti-tumor immunity induced by vaccination with recombinant adenoviruses encoding multiple tumor-associated cytotoxic T lymphocyte epitopes in a string-of-beads fashion. Proc Natl Acad Sci U S A 94:14660-14665, 1997.
54. Livingston BD, Newman M, Crimi C, McKinney D, Chesnut R, Sette A: Optimization of epitope processing enhances immunogenicity of multiepitope DNA vaccines. Vaccine 19:4652-4660, 2001.
55. Schneider SC, Ohmen J, Fosdick L, Gladstone B, Guo J, Ametani A, Sercarz EE, Deng H: Cutting edge: introduction of an endopeptidase cleavage motif into a determinant flanking region of hen egg lysozyme results in enhanced T cell determinant display. J Immunol 165:20-23, 2000.
56. Zhu H, Liu K, Cerny J, Imoto T, Moudgil KD: Insertion of the dibasic motif in the flanking region of a cryptic self-determinant leads to activation of the epitope-specific T cells. J Immunol 175:2252-2260, 2005.
57. Le Gall S, Stamegna P, Walker BD: Portable flanking sequences modulate CTL epitope processing. J Clin Invest 117:3563-3575, 2007.
58. Steiner DF: The proprotein convertases. Curr Opin Chem Biol 2:31-39, 1998.
59. Knight CG, Dando PM, Barrett AJ: Thimet oligopeptidase specificity: evidence of preferential cleavage near the C-terminus and product inhibition from kinetic analysis of peptide hydrolysis. Biochem J 308 (Pt 1):145-150, 1995.
60. Oliveira V, Gatti R, Rioli V, Ferro ES, Spisni A, Camargo AC, Juliano MA, Juliano L: Temperature and salts effects on the peptidase activities of the recombinant metallooligopeptidases neurolysin and thimet oligopeptidase. Eur J Biochem 269:4326-4334, 2002.
61. Schmid BV, Kesmir C, de Boer RJ: The specificity and polymorphism of the MHC class I prevents the global adaptation of HIV-1 to the monomorphic proteasome and TAP. PLoS ONE 3:e3525, 2008.
62. Smith SM: HIV CTL escape: at what cost? Retrovirology 1:8, 2004.
63. Yamshchikov GV, Mullins DW, Chang CC, Ogino T, Thompson L, Presley J, Galavotti H, Aquila W, Deacon D, Ross W, Patterson JW, Engelhard VH, Ferrone S, Slingluff CL, Jr.: Sequential immune escape and shifting of T cell responses in a long-term survivor of melanoma. J Immunol 174:6863-6871, 2005.
64. Zhou X, Jun DY, Thomas AM, Huang X, Huang LQ, Mautner J, Mo W, Robbins PF, Pardoll DM, Jaffee EM: Diverse CD8+ T-cell responses to renal cell carcinoma antigens in patients treated with an autologous granulocyte-macrophage colony-stimulating factor gene-transduced renal tumor cell vaccine. Cancer Res 65:1079-1088, 2005.
65. Khong HT, Wang QJ, Rosenberg SA: Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. J Immunother 27:184-190, 2004.
66. Lennerz V, Fatho M, Gentilini C, Frye RA, Lifke A, Ferel D, Wolfel C, Huber C, Wolfel T: The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc Natl Acad Sci U S A 102:16013-16018, 2005.
67. Parmiani G, De Filippo A, Novellino L, Castelli C: Unique human tumor antigens: immunobiology

and use in clinical trials. J Immunol 178:1975-1979, 2007.
68. Slingluff CL, Jr., Colella TA, Thompson L, Graham DD, Skipper JC, Caldwell J, Brinckerhoff L, Kittlesen DJ, Deacon DH, Oei C, Harthun NL, Huczko EL, Hunt DF, Darrow TL, Engelhard VH: Melanomas with concordant loss of multiple melanocytic differentiation proteins: immune escape that may be overcome by targeting unique or undefined antigens. Cancer Immunol Immunother 48:661-672, 2000.
69. Khong HT, Rosenberg SA: Pre-existing immunity to tyrosinase-related protein (TRP)-2, a new TRP-2 isoform, and the NY-ESO-1 melanoma antigen in a patient with a dramatic response to immunotherapy. J Immunol 168:951-956, 2002.
70. Pinilla-Ibarz J, Cathcart K, Korontsvit T, Soignet S, Bocchia M, Caggiano J, Lai L, Jimenez J, Kolitz J, Scheinberg DA: Vaccination of patients with chronic myelogenous leukemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. Blood 95:1781-1787, 2000.
71. Cathcart K, Pinilla-Ibarz J, Korontsvit T, Schwartz J, Zakhaleva V, Papadopoulus EB, Scheinberg DA: A multivalent bcr-abl fusion peptide vaccination trial in patients with chronic myeloid leukemia. Blood 103:1037, 2003.
72. Bocchia M, Gentili S, Abruzzese E, Fanelli A, Iuliano F, Tabilio A, Amabile M, Forconi F, Gozzetti A, Raspadori D, Amadori S, Lauria F: Effect of a p210 multipeptide vaccine associated with imatinib or interferon in patients with chronic myeloid leukaemia and persistent residual disease: a multicentre observational trial. Lancet 365:657-662, 2005.
73. Gannage M, Abel M, Michallet AS, Delluc S, Lambert M, Giraudier S, Kratzer R, Niedermann G, Saveanu L, Guilhot F, Camoin L, Varet B, Buzyn A, Caillat-Zucman S: Ex vivo characterization of multiepitopic tumor-specific CD8 T cells in patients with chronic myeloid leukemia: implications for vaccine development and adoptive cellular immunotherapy. J Immunol 174:8210-8218, 2005.
74. Grunebach F, Mirakaj V, Mirakaj V, Muller MR, Brummendorf T, Brossart P: BCR-ABL is not an immunodominant antigen in chronic myelogenous leukemia. Cancer Res 66:5892-5900, 2006.
75. Paydas S, Tanriverdi K, Yavuz S, Seydaoglu G: PRAME mRNA levels in cases with chronic leukemia: Clinical importance and review of the literature. Leuk Res 31:365-369, 2007.
76. Tajeddine N, Gala JL, Louis M, Van Schoor M, Tombal B, Gailly P: Tumor-Associated Antigen Preferentially Expressed Antigen of Melanoma (PRAME) Induces Caspase-Independent Cell Death In vitro and Reduces Tumorigenicity In vivo. Cancer Research 65:7348-7355, 2005.
77. Steinbach D, Pfaffendorf N, Wittig S, Gruhn B: PRAME expression is not associated with downregulation of retinoic acid signaling in primary acute myeloid leukemia. Cancer Genet Cytogenet 177:51-54, 2007.
78. 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der KK, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH: Gene expression profiling predicts clinical outcome of breast cancer. Nature 415:530-536, 2002.
79. Epping MT, Wang L, Edel MJ, Carlee L, Hernandez M, Bernards R: The human tumor antigen PRAME is a dominant repressor of retinoic acid receptor signaling. Cell 122:835-847, 2005.
80. Epping MT, Bernards R: A causal role for the human tumor antigen preferentially expressed antigen of melanoma in cancer. Cancer Res 66:10639-10642, 2006.
81. Epping MT, Hart AA, Glas AM, Krijgsman O, Bernards R: PRAME expression and clinical outcome of breast cancer. Br J Cancer 99:398-403, 2008.
82. Paydas S: Is everything known in all faces of iceberg in PRAME? Leuk Res 32:1356-1357, 2008.
83. Greiner J, Schmitt M, Li L, Giannopoulos K, Bosch K, Schmitt A, Dohner K, Schlenk RF, Pollack JR, Dohner H, Bullinger L: Expression of tumor-associated antigens in acute myeloid leukemia: implications for specific immunotherapeutic approaches. Blood 108:4109-4117, 2006.
84. Li L, Giannopoulos K, Reinhardt P, Tabarkiewicz J, Schmitt A, Greiner J, Rolinski J, Hus I, Dmoszynska A, Wiesneth M, Schmitt M: Immunotherapy for patients with acute myeloid leukemia using autologous dendritic cells generated from leukemic blasts. Int J Oncol 28:855-861, 2006.
85. Quintarelli C, Dotti G, De Angelis B, Hoyos V, Mims M, Luciano L, Heslop HE, Rooney CM, Pane F, Savoldo B: Cytotoxic T lymphocytes directed to the preferentially expressed antigen of melanoma (PRAME) target chronic myeloid leukemia. Blood 112:1876-1885, 2008.
86. Rezvani K, Yong AS, Tawab A, Jafarpour B, Eniafe R, Mielke S, Savani BN, Keyvanfar K, Li Y, Kurlander R, Barrett AJ: Ex-vivo characterization of polyclonal memory CD8+ T-cell responses to PRAME-specific peptides in patients with acute lymphoblastic leukemia and acute and chronic myeloid leukemia. Blood 113:2245-2255, 2008.
87. Kenter GG, Welters MJP, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, Essahsah F, Fathers LM, Offringa R, Drijfhout JW, Wafelman AR, Oostendorp J, Fleuren GJ, van der Burg SH,

Melief CJM: Vaccination against Human Papillomavirus 16 for Vulvar Intraepithelial Neoplasia. N Engl J Med, In Press: 2009.
88. Kaufmann SH, Mcmichael AJ: Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. Nat Med 11:S33-S44, 2005.
89. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD: Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 68:4650-4655, 1994.
90. Benito JM, Lopez M, Soriano V: The role of CD8+ T-cell response in HIV infection. AIDS Rev 6:79-88, 2004.
91. Webster GJ, Reignat S, Brown D, Ogg GS, Jones L, Seneviratne SL, Williams R, Dusheiko G, Bertoletti A: Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol 78:5707-5719, 2004.
92. Klenerman P, Semmo N: Cellular immune responses against persistent hepatitis C virus: gone but not forgotten. Gut 55:914-916, 2006.
93. Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Andersen P, Dockrell H, Pasvol G, Hill AV: Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 95:270-275, 1998.
94. Morrot A, Zavala F: Effector and memory CD8+ T cells as seen in immunity to malaria. Immunol Rev 201:291-303, 2004.
95. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE, III, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG: Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393:537-544, 1998.
96. Rappuoli R: Bridging the knowledge gaps in vaccine design. Nat Biotechnol 25:1361-1366, 2007.
97. Mcmichael AJ: HIV vaccines. Annu Rev Immunol 24:227-255, 2006.
98. Serruto D, Rappuoli R: Post-genomic vaccine development. FEBS Lett 580:2985-2992, 2006.
99. Desrosiers RC: Prospects for an AIDS vaccine. Nat Med 10:221-223, 2004.
100. Apetoh L, Mignot G, Panaretakis T, Kroemer G, Zitvogel L: Immunogenicity of anthracyclines: moving towards more personalized medicine. Trends Mol Med 14:141-151, 2008.
101. Apetoh L, Tesniere A, Ghiringhelli F, Kroemer G, Zitvogel L: Molecular interactions between dying tumor cells and the innate immune system determine the efficacy of conventional anticancer therapies. Cancer Res 68:4026-4030, 2008.
102. Li Y, Wang LX, Yang G, Hao F, Urba WJ, Hu HM: Efficient cross-presentation depends on autophagy in tumor cells. Cancer Res 68:6889-6895, 2008.
103. Zitvogel L, Apetoh L, Ghiringhelli F, Andre F, Tesniere A, Kroemer G: The anticancer immune response: indispensable for therapeutic success? J Clin Invest 118:1991-2001, 2008.
104. Kepp O, Tesniere A, Schlemmer F, Michaud M, Senovilla L, Zitvogel L, Kroemer G: Immunogenic cell death modalities and their impact on cancer treatment. Apoptosis 2009.
105. Schmid D, Pypaert M, Munz C: Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity 26:79-92, 2007.
106. Crotzer VL, Blum JS: Autophagy and Its Role in MHC-Mediated Antigen Presentation. J Immunol 182:3335-3341, 2009.
107. Schmid D, Dengjel J, Schoor O, Stevanovic S, Munz C: Autophagy in innate and adaptive immunity against intracellular pathogens. J Mol Med 84:194-202, 2006.
108. Paludan C, Schmid D, Landthaler M, Vockerodt M, Kube D, Tuschl T, Munz C: Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science 307:593-596, 2005.
109. Craiu A, Akopian T, Goldberg A, Rock KL: Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. Proc Natl Acad Sci U S A 94:10850-10855, 1997.
110. Berti DA, Morano C, Russo LC, Castro LM, Cunha FM, Zhang X, Sironi J, Klitzke CF, Ferro ES, Fricker LD: Analysis of intracellular substrates and products of thimet oligopeptidase (EC 3.4.24.15) in human embryonic kidney 293 cells. J Biol Chem 284:14105-14116, 2009.