ANALYSIS OF THE ROLE OF CYTOSOLIC AMINOPEPTIDASES IN THE GENERATION OF MHC-CLASS I PRESENTED PEPTIDES

A Dissertation Presented

By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 27, 2006

IMMUNOLOGY AND VIROLOGY

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Parts of this dissertation have been presented in the following publications:

Towne CF, York IA, Neijssen J, Karow ML, Murphy AJ, Valenzuela DM, Yancopoulos GD, Neefjes JJ, Rock KL. (2005) "Leucine aminopeptidase is not essential for trimming peptides in the cytosol or generating epitopes for MHC class I antigen presentation." J Immunol **175** (10): 6605-14.

Towne CF, York IA, Neijssen J, Karow ML, Murphy AJ, Valenzuela DM, Yancopoulos GD, Neefjes JJ, Rock KL. (2006) "Puromycin-sensitive aminopeptidase is important for normal MHC class I peptide presentation in dendritic cells" (manuscript in progress)

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(February 27, 2006)

ACKNOWLEDGEMENTS

First and foremost, I thank my wonderful wife. Maxine has supported me at all times. Even when I had to go to the lab in the middle of the night, she did not mutter a word of complaint. She has consistently offered words of encouragement and love, while managing to sustain an ever-growing household on a graduate student's stipend. What an unselfish person and wonderful companion.

I thank my son Nathan who started graduate school with me, as well as Rhiannon, and Morgan, and Caelyn who joined us along the way. They have been a constant reminder to me of what is truly important, and why I want to make the world a better place to live through science. How fortunate am I to have such beautiful reminders!

I would like to thank Ken for allowing me to express my own opinions about my data, allowing me the freedom of trying new things, advising me to stay on track with the project when I felt like taking a attractive tangent, pointing out flaws in my experimental design, and encouraging me in general. Few students have the privilege of being able to work so independently while simultaneously feeling supported by their mentor. I admire how he handles all the responsibilities of Department Chair, and supervises his lab personnel in a way that the department and his people are both able to flourish and grow.

Finally, I thank Ian York who has consistently been available to me for questions and for stimulating discussion of my data. He has laid much of the groundwork upon which my dissertation work is built. His kind and thoughtful manner has been beneficial in discussions when my opinions were strong or conflicting, and it makes him an

outstanding Canadian! I thank him for connecting with me on a personal level as well, and teaching me more about the strength that can be found in gentleness.

ABSTRACT

To detect viral infections and tumors, CD8 T lymphocytes monitor cells for the presence of antigenic peptides bound to MHC class I molecules. The majority of MHC class I-presented peptides are generated from the cleavage of cellular and viral proteins by the ubiquitin-proteasome pathway. Many of the oligopeptides produced by this process are too long to stably bind to MHC class I molecules and require further trimming for presentation. Cytosolic aminopeptidases such as leucine aminopeptidase (LAP), which is IFN-inducible, Bleomycin Hydrolase (BH), and puromycin-sensitive aminopeptidase (PSA) can trim precursor peptides to mature epitopes and have been thought to play an important role in antigen presentation. To examine the role of these aminopeptidases in generating MHC class I peptides *in vivo*, we generated mice deficient in LAP or PSA, as well as cell lines deficient in LAP, PSA, or BH. LAP mutant mice and cells are viable and grow normally, whereas PSA mutant mice are smaller than their wild-type and heterozygote littermates, are subfertile as adults, and are subviable as embryos.

The trimming of peptides in LAP-deficient cells is not reduced under basal conditions or after stimulation with IFN. Similarly, there is no reduction in presentation of peptides from precursor or full length antigen constructs or in the overall supply of peptides from cellular proteins to MHC class I molecules, even after stimulation with IFN. After viral infection, LAP-deficient mice generate normal CTL responses to seven

epitopes from three different viruses. Similarly, PSA deficient mice and BH deficient mice generate normal CTL responses to viral epitopes. These data demonstrate that LAP, BH, and PSA are not essential enzymes for generating most MHC class I-presented peptides and reveal redundancy in the function of cellular aminopeptidases in most cell types.

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CHAPTER I

INTRODUCTION

MHC class I molecules present peptides to cytotoxic T-lymphocytes (CTLs) that survey the body for infected or otherwise aberrant cells. CTLs recognize MHC class I molecules via their T-cell receptor (TCR). Recognition of an MHC class I molecule containing an agonistic peptide activates CTLs to proliferate and kill the "offending" cell(s). Upon activation, CTLs may also secrete cytokines such as IFNγ; a cytokine that enhances and alters MHC class I presentation in a most cell types.

Peptides bound to MHC class I are derived from intracellular proteins which may initially be normal full length sequences. They may be degraded because they are abnormal, e.g., mistranslated and/or misfolded polypeptides (DRiPs), or as part of the turnover of normal proteins. Regardless of the initial form of the antigen, the generation of a majority of the class I-presented peptides requires the proteasome. However, the proteasome generates some peptides that are longer than the 8, 9, or 10 residues required to bind MHC class I molecules, and that must be trimmed by aminopeptidases located in the cytosol or ER before they can be presented. This trimming of peptides by aminopeptidases is the focus of my thesis studies.

1 The classical MHC class I presentation pathway

1.1 The sources of MHC class I peptides

Theoretically, any protein that is degraded by the proteasome can produce peptides for MHC class I presentation. Proteins from the cytoplasm, the nucleus, the ER, viruses, long-lived proteins, short-lived proteins, and **d**efective **ri**bosomal **p**roducts (DRiPs) can all contribute to the MHC class I peptide repertoire.

An unresolved issue is how much each protein source contributes to the resulting peptide repertoire. Upwards of 30% of MHC class I peptides has been suggested to come from newly synthesized proteins that are mistranslated and/or misfolded (1). These **D**efective **Ri**bosomal **P**roducts (DRiPs) do not pass the "quality control" of chaperone proteins and are shuttled to the proteasome for degradation, thereby generating MHC class I epitopes (1-3). This conclusion was largely based on radiolabeling of cells (S^{35}-Met) under conditions of inhibited or uninhibited proteasomal activity (1). However, this approach has been contested by recent data which suggests that the observed increase in radiolabeling of newly synthesized proteins after proteasomal inhibition, which supported the initial DRiP hypothesis, resulted from a lack of intracellular amino acids that would have been present and used in new protein sythesis under normal proteolytic conditions (i.e. functional proteasome) (4). This, combined with computational analyses of the original DRiP data by another group, suggest that DRiPs do not contribute a large portion of MHC class I peptides (5).

However, there is other evidence that may support the DRiP hypothesis. Treating cells with cycloheximide (which blocks translation of mRNA) has indirectly been shown to reduce peptide transport into the ER (6). The reduction of peptide transport by treatment with cycloheximide (CHX) was comparable to the reduction observed upon treatment with lactacystin (a proteasome inhibitor), suggesting that a large fraction of peptides transported by TAP into the ER are derived from newly synthesized proteins with a short half-life (e.g. DRiPs) (6). However, other data suggests that newly synthesized proteins are largely protected from proteasomal degradation, and that pre-existing proteins are preferentially degraded as a source of amino acids for new translation (4).

Using viral proteins as a model, Reits, et al. demonstrated that the inhibition of RNA-dependent RNA transcription of viral RNA with ribavirin during an influenza infection of cells results in the measurable reduction of peptide transport into the ER by TAP within a short amount of time (30 min) (6). This suggests that newly synthesized viral proteins can be a major contributor to the MHC class I peptide pool, but it does not differentiate between the contributions of functional short-lived proteins (or peptide precursors (7)) and non-functional DRiPs.

In an effort to quantitate the contribution of DRiPs to the MHC class I peptide pool, an inducible Tet-on/off system was used to express LCMV nucleoprotein which has a long half-life in cells (>3 days) (8). Presentation of the NP 118 epitope derived from this protein was measured over time after translation was repressed by the addition of tetracycline to the culture. Presentation of NP118 steadily decreased over a period of

three days to levels of negative control cells, suggesting that this peptide is generated from short-lived translational products of LCMV-NP protein (possibly DRiPs), rather than long-lived, mature, functional protein (8).

Although this study demonstrates that DRiPs are one source of MHC class I peptides, it does not eliminate the possibility that stable long-lived proteins also contribute to the MHC class I peptide pool. Full-length OVA and β-galactosidase have both been loaded into the cytosol of cells, and then degraded into peptides that were efficiently presented to CTLs (9, 10), which suggests that functional proteins contribute to the MHC class I peptide pool. By varying the N-terminal residue of full-length β-gal, the rates of degradation of β-gal protein and presentation of β-gal peptides can be altered (9). This model demonstration of the N-end rule could suggest that the half-life of intracellular functional proteins is influenced by the residue on the N-terminus. It may also suggest that short-lived functional proteins contribute more to the peptide pool than longer-lived proteins, although this possibility has not been experimentally tested.

The intracellular localization of proteins also affects their level of contribution to the MHC class I peptide pool. Sequence analysis of an array of peptides eluted from secreted HLA-B*1801 molecules suggests that 53% of presented peptides are derived from cytoplasmic proteins, 43% are from nuclear proteins, and 18% are derived from membrane-bound proteins (some proteins can be found in multiple compartments) (11). These calculations seem reasonable since proteasomes are localized in the cytoplasm and the nucleus, and proteins in these compartments are the most highly represented. Other

minor amounts of presented peptides (≤8% each) were derived from ribosomes, the ER, the plasma membrane, mitochondria, the nucleolus, chromosomes, and the Golgi (11).

In order to determine if this pattern is also true for other HLA alleles, MHC class I peptides in the SYPEITHI database (12) that are known to uniquely bind HLA-A2 were cross-referenced with an online database containing information on the subcellular localization of proteins (13). Roughly 1150 unique peptides that bind HLA-A2 were successfully localized and determined to be from cellular proteins. From this cursory screen we have determined that, 31% of HLA-A2-presented peptides are derived from cytoplasmic proteins, 37% are from nuclear proteins, and 24% are derived from membrane-bound proteins (Ian York, personal communication). Other minor contributors (≤8%) were the ER, and mitochondria. In addition, some cellular compartments were represented here (albeit at very low levels) that were not represented in Hickman's study such as peroxisomes, endosomes, lysosome, late endosomes, and secretory vesicles.

Despite these minor differences, the data from this brief HLA-A2-peptide screen are in general agreement with Hickman's findings concerning HLA-B*1801. However a more thorough examination of the array of MHC class I peptides is required to validate the idea that cytoplasmic and nuclear proteins contribute the most to the MHC class I peptide repertoire.

Along with intracellular location, protein abundance can also affect the level of contribution of peptides (and peptide precursors) by proteins, since peptides from overexpressed proteins in tumors have been shown to be presented at higher levels than

in normal tissue (14). The N-terminal residue of intracellular proteins may also influence the level of contribution of peptides from proteins (N-end rule), since proteins with a high rate of turnover would theoretically contribute more peptides than proteins with a long half-life (9). It is also likely that changes in the rate of protein turnover can account for changes in the contribution of certain proteins to the peptide pool (15).

However, protein abundance, rate of protein turnover, and intracellular location, are not the only factors that determine whether a peptide is presented by MHC class I. Many proteins contain peptide sequences that could theoretically bind MHC class I molecules and serve as epitopes, but do not stimulate immune responses even though some of these theoretical peptides bind MHC class I with high affinity (16). One potential explanation for this is that these peptides may never be generated *in vivo* due to cleavage sites within the peptide sequence.

Flanking sequences have also been shown to prevent or contribute to the generation of potential MHC class I peptides or peptide precursors (17-19). Some highly immunodominant epitopes require their natural flanking sequences in order to efficiently compete for transport into the ER by TAP (20). In contrast, some epitopes are destroyed or presented less efficiently due to their flanking sequences (19-21). In fact, by substituting the flanking sequences of an immunodominant epitope with those of a subdominant epitope, presentation of the immunodominant epitope is reduced (19). These data demonstrate that the amino acids around potential epitopes can influence the presentation of that peptide.

1.2 Intracellular proteins are degraded by the ubiquitin-proteasome pathway to generate MHC class I-presented peptides and peptide precursors

Most proteins that have reached the extent of their lifespan are polyubiquitinated by enzymes in the ubiquitin conjugation system (22). In most eukaryotic cells, there is a single E1 ubiquitin-activating enzyme, which forms a thiol-ester bond with ubiquitin (using ATP for energy) activating the C-terminus of ubiquitin for nucleophilic attack. Then one of roughly twenty (identified) E2 conjugating enzymes transfers the activated ubiquitin to one of possibly thousands of E3 ligases. The E3 ligase covalently attaches to the 76-amino acid ubiquitin molecule to lysine residues within the protein (or an already ligated ubiquitin). They do so by ligating the C-terminus of ubiquitin to the ε-amino group of the side chain of the lysine residue, although there have been reports of ubiquitin being conjugated to residues other than lysine by viral E3 ligases (23). Substrates marked with polyubiquitin chains are selectively targeted to the proteasome for destruction, however not all proteins require polyubiquitination in order to be degraded by the proteasome (24-26).

Although there are multiple forms of biologically-active proteasome complexes, the 26S proteasome is generally considered to be the most abundant under normal cellular conditions, and is composed of a 20S core with one 19S cap on each end of the core. The core contains four stacked rings (two α, and two β) with seven subunits per

ring. Due to nuclear localization sequences (NLS) in the α rings, proteasomes can be found both in the cytosol and in the nucleus (27, 28).

There are six catalytic sites residing in the two inner β-rings. Two of these sites are chymotrypsin-like, two are trypsin-like, and two have post-acidic cleaving activity (27, 29). However, replacement of some of the proteolytically active subunits with other subunits (such as those described below) can alter the catalytic activity of the proteasome, and thereby alter the peptide profile of the proteasomal products.

The proteasome degrades proteins into peptides that generally range from 3 to 25 amino acids (30). Some MHC class I molecules can bind 8mers, others 9mers or 10mers (31-34). Although the proteasome can generate peptides that are 8 to 10 amino acids long (35), only around 15% of proteasomal products fall within this size range, and most (around 66%) are shorter (36) and can not stably bind to MHC class I molecules. Studies with purified proteasomes (37), and in living cells (35, 37, 38) have shown that inhibition of proteasomal activity results in a loss of MHC class I peptide generation from full-length proteins. In addition, inhibiting the proteasome also blocks the generation of MHC class I peptides from peptide precursors with C-terminal extensions, suggesting that the proteasome is required for the generation of the proper C-terminus of MHC class I peptides (35, 39).

1.3 The enzymatic activity of the immunoproteasome differs from the proteasome

Extended incubation with IFNγ induces the expression of the β-ring subunits LMP2 (β1i), LMP7 (β5i), and MECL1 (β2i) which are incorporated into newly assembling proteasomes (29). Like their constitutively-expressed homologs, these three subunits are catalytically active. LMP2 replaces β1 (δ or subunit Y), LMP7 replaces β5 (ε or subunit X), and MECL-1 replaces β2 (subunit Z) (29, 40-42). The newly formed immunoproteasome is different in enzymatic activity from the original proteasome. For example, LMP7 can enhance cleavages after hydrophobic and basic residues (43), and LMP2 can enhance cleavage after basic residues, and decrease cleavages after acidic residues (27, 43). Such cleavages generate more peptides with hydrophobic and basic residues at the C-terminus, which is preferred by the TAP transporter for transport to the ER, and are essential for binding to MHC class I molecules (29).

With its different sequence preferences and its dependence on IFN for expression, the immunoproteasome has been suggested to be more suited for generating peptides for antigen presentation. This has been supported by evidence that LMP2-deficient mice have a decreased ability to present antigen to CTLs for positive selection, and for stimulation during viral infection (44, 45). In addition, the immunodominance hierarchy of flu epitopes is changed in LMP2-deficient mice (45) suggesting that LMP2 may be involved in generating specific epitopes.

LMP-7 deficient mice exhibit normal numbers of B and T cells, but MHC class I levels on all subpopulations of lymphocytes in these animals is reduced by 25 to 45% (46). Exogenous addition of peptide ameliorates this reduction suggesting that LMP7 is involved in the generation of MHC class I peptides.

LMP2 and LMP7 have been suggested to contribute to the low level of presentation of one LCMV epitope (47). However, a second study reports that the CTL response of this epitope is not altered in LMP-deficient mice during a bon a fide LCMV infection, and further reports that these immunoproteasome subunits are not necessary for clearance of LCMV (48). These data suggest that although these proteasomal subunits may be better suited for antigen presentation than those that are constitutively expressed, they are not essential.

Although data on the MECL-1 deficient mouse generated by Michael Basler and colleagues is as yet unpublished, it has been reported that MECL-1 deficient mice have 20% less CTLs than wild-type (49). More information on these mice will no doubt be forthcoming.

But is the immunoproteasome really better at generating MHC class I peptides? As mentioned earlier, immunoproteasomes generate peptides with hydrophobic C-termini, which have higher affinity for the TAP transporter and for MHC class I binding grooves. Yet more importantly than generating more (or better-binding) MHC class I peptide ligands, the immunoproteasome may generate more peptides with N-terminal extensions, which can be trimmed to size by other peptidases within the cell (50). In order to compare the ability of immunoproteasome and 26S constitutive proteasome to

generate antigenic peptides or peptide precursors, degradation products from *in vitro* digests of denatured ovalbumin from each proteasome were analyzed by immunoaffinity chromatography using a monoclonal antibody that recognizes SIINFEKL at the C-terminus of peptides, regardless of the N-terminal extension (50). Through chemical modification, these SIINFEKL-containing peptides could then be quantified. Immunoproteasomes did not generate peptides faster, more efficiently, or of different mean lengths (50). They also did not generate more SIINFEKL peptide, however the cleavage pattern of immunoproteasomes was different such that more N-terminally extended SIINFEKL peptides were generated by the immunoproteasome than by the constitutive proteasome (50).

It is possible that these peptides can be further trimmed *in vivo* to produce MHC class I peptides (50). Thus, while the immunoproteasome may not generate more antigenic peptides directly, they may generate more peptide precursors, which can be trimmed by aminopeptidases. Therefore, immunoproteasomes may indirectly contribute more to the MHC class I peptide pool than constitutive proteasomes, although it should be noted that both proteasomes generate MHC class I peptide precursors (50).

1.4 MHC class I peptide precursors can be trimmed by aminopeptidases

Many epitopes are generated by the proteasome as precursors with the correct C-terminus, but with additional residues on the N-terminus which need to be removed in

order to bind MHC class I molecules (29, 35, 39, 51, 52). *In vitro* experiments using soluble cell extracts that were cleared of proteasomes by ultracentrifugation and then treated with proteasome inhibitors have shown that there are enzymatic activities in the cell that can trim residues from the N-terminus, but not the C-terminus of peptide precursors (53). Treating live cells with a variety of proteasome inhibitors and then measuring epitope generation (via HPLC or CTL assay) from synthetic N-extended precursors, or precursors expressed from mini-genes demonstrated that enzymes other than the proteasome can generate peptides of the correct length. In one study, precursors with N-extensions of up to 25 residues were presented after proteasomal inhibition (39), suggesting that MHC class I epitopes can be generated from N-extended proteasomal products. However, inhibiting the proteasome blocked generation of a mature epitope from any C-terminally extended precursor, indicating that the proteasome is required for generating the correct C-terminus (39, 51).

Together these data suggest that although the proteasome may generate a large portion of MHC class I peptides, there are cellular enzymes that can further trim peptide precursors to produce suitable peptides for MHC class I binding. Aminopeptidases are characterized by their ability to trim N-terminal residues off peptides. In live cells, N-extended peptides with a blocked N-terminus are not trimmed and are not presented by MHC class I, suggesting that aminopeptidases can generate MHC class I peptides from N-extended precursors (28, 51, 54).

Pharmacological inhibitors of aminopeptidases have also been used to demonstrate that aminopeptidases are involved in trimming peptide precursors. Bestatin

is a common aminopeptidase inhibitor (55-59) that, when added to cytosolic extracts, partially inhibits the peptide trimming activity that generates MHC class I peptides (51, 53).

Amino acid-conjugated chloromethylketone, AAF-CMK (Ala-Ala-Phe-CMK) is an inhibitor that has been used in cell extracts to inhibit the trimming of synthetic peptide precursors (60, 61). It has also been shown to inhibit the presentation of epitopes to CTLs generated from full-length tumorigenic protein (RU1) (61), full-length viral protein (VSV-NP) (60), and certain flu epitopes (62). AAF-CMK also inhibits presentation of SIINFEKL in cells stably transfected with a full-length OVA construct (60).

However, AAF-CMK is not specific for any single aminopeptidase since it has been shown to inhibit three cytosolic aminopeptidases (TPPII, PSA, and BH) (60, 62, 63), and may inhibit others. Therefore, it is possible that other aminopeptidases responsible for trimming in live cells were inhibited by AAF-CMK in these studies. An additional caveat in these studies is that although AAF-CMK was shown to inhibit the presentation of MHC class I epitopes to CTL by live cells (60, 61), this lack of presentation was not directly linked to the ability of the AAF-CMK to prevent peptide trimming (i.e. increase in peptide half-life or peptide concentration). Therefore, the use of AAF-CMK (and other inhibitors) may affect other cell processes and/or enzymes that were not examined in these studies.

Butabindide is an inhibitor that has been suggested to be highly specific for TPPII (64), and has been used to study this enzyme in cell lysates (61) and in living cells (54, 62). However, butabindide does inhibit other peptidases, e.g. TPPI (65), and the initial

(and subsequent) claims for TPPII-specificity have not examined potential effects of butabindide on other aminopeptidases, particularly BH, PSA, and LAP.

In total, the studies using pharmacological inhibitors suggest that a few specific aminopeptidases can trim, generate, and/or destroy many MHC class I peptides. However, because evidence demonstrating inhibitor specificity is scarce, a more accurate conclusion from these studies is that aminopeptidases are involved in trimming MHC class I peptides.

1.5 Peptide trimming can occur in the cytosol

Because there are aminopeptidase activities present in the cytoplasm and the ER, an important question is how much do the activities from each compartment contribute to producing MHC class I peptides. At least one peptide (RU1) has been shown to be trimmed exclusively in the cytosolic fraction of cell lysate, and not by ER luminal proteins or ER-membrane proteins (61). This indicates that cytosolic aminopeptidases may be required for the presentation of at least some peptides. Other studies have also used cytosolic cell lysates and purified cytosolic aminopeptidases to demonstrate that cytosolic aminopeptidases can trim MHC class I peptides (51, 53, 60, 61). These biochemical studies demonstrate that cytosolic aminopeptidases can trim peptides, but they do not show that this is what occurs *in vivo*.

In living cells, TAP does not efficiently transport long peptides (>25AA) into the ER (66, 67). Therefore, peptide precursors of this length would likely require trimming

in the cytosol prior to transport. Indeed, peptides from precursors of this length have been shown to be efficiently presented by MHC class I (39), suggesting that cytosolic aminopeptidases can trim proteasomal products of this size. In addition, the half-life of microinjected peptides in the cytosol of living cells has been related to the activity of aminopeptidases in the cytoplasm (28, 54). Overexpression of LAP (a cytosolic aminopeptidase) results in the reduced half-life of endogenous peptides (28) and the reduced MHC class I presentation of certain epitopes (68). In contrast, peptide half-life in the cytoplasm is increased when the N-terminus of the peptide is blocked (28). Together, these data suggest that cytosolic aminopeptidases can trim MHC class I peptides *in vivo*.

1.6 MHC class I peptides can be trimmed by leucine aminopeptidase in the cytosol

Leucine aminopeptidase (EC 3.4.11.1) (LAP3 in humans) was one of the first aminopeptidases to be implicated in antigen processing. It is a Zn^{2+}-binding metalloprotease which was described in 1964 when it was isolated from swine kidney (69), but may have been discovered even earlier (70, 71). LAP has a number of other names such as cytosol aminopeptidase, leucyl aminopeptidase (72), and peptidase S (73). LAP levels in blood have been used as a diagnosis of breast cancer (74), and liver damage (75). It has also been implicated in the catabolism of glutathione (the main

cellular defense against oxidative stress) (72, 76). LAP is susceptible to bestatin (53), as are many aminopeptidases (55, 58, 77).

LAP was identified as a major enzymatic activity in cytosolic extracts from HeLa cells that was capable of trimming N-extended precursors of SIINFEKL, an antigenic peptide from OVA, to the mature presented epitope (53). And as previously mentioned, overexpressing LAP in cells leads to more rapid trimming of peptides (28) and a reduction in peptides available for binding MHC class I molecules (28, 68). These data suggested that LAP played a major role in trimming peptides for presentation by MHC class I. This may be particularly true during an immune response (such as during a viral infection) since the LAP gene is strongly upregulated both by type I (78), and type II IFN (53, 79).

1.7 MHC class I peptides can be trimmed by Puromycin-sensitive aminopeptidase and bleomycin hydrolase in the cytosol

PSA (EC 3.4.11.14), also known as MP100 or NPEPPS, is a Zn^{2+}-binding metalloprotease that was initially identified in 1981 (80) It is a highly conserved protein, sharing 98% identity between human and mouse (81), and is considered to be a housekeeping gene due to the high GC content and multiple Sp1-binding sites in its gene promoter region (81). The protein is mainly cytoplasmic although one membrane-associated form has been identified in the brain (82, 83). It has been suggested to play a role in Alzheimer's disease progression by generating β-amyloid peptide (84), although

this has been disputed (85). In addition, PSA has been suggested to be involved in neuropeptide degradation (86-88), although this has also been questioned since PSA is mainly an intracellular protein (83), and the expression and distribution of neuropeptides in the brains of PSA-deficient mice appears to be normal (89). PSA has also been suggested to associate with mitotic spindles, perhaps through microtubule binding sites which are similar to those found in tau protein (58, 90). This association has been implied to be important for cell cycle progression (58).

BH (EC 3.4.22.40) is a cysteine protease that has also been conserved through evolution (92% identity between rabbit and human) with broad tissue expression in humans (91), and a housekeeping gene structure (92). It derived its name from its ability to hydrolyze the antitumor agent bleomycin, and has been shown to be upregulated in a number of bleomycin-resistant tumors and cell lines (91, 93, 94). BH can exist as a homohexamer, making it roughly 300 kDa and barrel-shaped (similar to the proteasome) (95). It has been argued that a genetic polymorphism in BH is linked to susceptibility to Alzheimer's disease (96-99), however this is controversial (100-102).

PSA and BH were both suggested to play a role in MHC class I peptide processing based on 1) their ability to trim the VSV nucleoprotein epitope (NP52-59) in soluble cell extracts, 2) their sensitivity to the aminopeptidase inhibitor AAF-CMK, which was shown in living cells to inhibit presentation of NP52-59 generated from N-extended precursors, and 3) their resistance to the proteasome inhibitor lactacystin (60).

In this initial study, cells were lysed and the lysates were depleted of proteasomes and ER-fraction. The soluble cell lysate was separated into fractions by column

chromatography. Fractions that cleaved a fluorogenic substrate and were sensitive to the aminopeptidase inhibitor AAF-CMK were further purified. Then, after running these purified fractions on a polyacrylamide gel, in-gel digests and mass spectrometry revealed that two major bands in the active fractions were PSA and BH. Stoltze then showed that purified BH and PSA could trim precursors of the VSV-NP epitope, suggesting that these aminopeptidases are important for peptide processing (60). This study went further to demonstrate that pre-incubation of living cells with AAF-CMK could prevent MHC class I presentation of the VSV epitope (and SIINFEKL) in cells transfected with peptide precursors, again suggesting that BH and PSA were involved in the processing of this precursor.

Although this study was the first to implicate BH and PSA in antigen processing, it contained a few crucial caveats. First, AAF-CMK was used to detect or inhibit cytosolic aminopeptidases, however it is possible that AAF-CMK inhibited aminopeptidases in the ER as well. An experimental control that could have been included in Stoltze's experiments is the expression of an ER-targeted peptide precursor in TAP-deficient (or ICP47-expressing) cells that were also treated with AAF-CMK. This would have evaluated the possibility that an ER-aminopeptidase is responsible for the observed trimming activity *in vivo*. A second caveat is that treating the cells with AAF-CMK may have resulted in a non-specific inhibition of the class I processing pathway. The authors could have used a construct or recombinant virus that expressed the mature epitope (requiring no processing) as an appropriate control. Therefore, with these weaknesses in mind, only a weak correlation can be made between the *in vitro* peptide

trimming activity of purified PSA and BH, and the *in vivo* role of these enzymes in antigen presentation.

To extend the findings on PSA, another study using human cells suggested that PSA could process the RU1 epitope in tandem with TPPII, and that PSA was required for the presentation of this epitope to CTLs (61). In addition, Saric et al has suggested that PSA degrades the Sendai nucleoprotein epitope (FAPGYNPAL) in cell extracts better than any other peptidase (59). This preferential trimming of FAPGYNPAL *in vitro* is presumably due to the basic and hydrophobic residues in this peptide, which are preferred by PSA (103).

A further indication that BH may be involved in peptide trimming is that it (like all cysteine proteases) is susceptible to the cysteine protease inhibitor E-64, which was shown to block presentation of one MCMV epitope from an N-extended precursor expressed from a recombinant vaccinia minigene (104). However, E-64 inhibits many other proteases (105) and it did not inhibit the generation of the NP52-59 epitope in a different cell line (60). The difference in the ability of E-64 to inhibit antigen generation in one cell line, but not another may suggest that the role of specific aminopeptidases may vary in certain cell types, and/or that aminopeptidases prefer some peptide sequences over others.

1.8 TPPII and other aminopeptidases that might be involved in peptide trimming.

Although I have not included the following aminopeptidases in my studies, it is important to mention them here due to their potential role in MHC class I peptide processing. Tripeptidyl peptidase II (TPPII) is a cytosolic aminopeptidase that has been suggested to have a role in peptide trimming (61, 63). TPPII is an enormous protein complex with a mass of 5000 to 9000 kDa (61). It was originally thought to act similarly to (and perhaps to substitute for) the proteasome (106-108). Although later studies suggested that these findings were due to artifacts (109, 110), the idea that TPPII can compensate for reduced proteasome function has recently been revived (62). As its name implies, TPPII cleaves three residues at a time from the amino-terminus of peptides, however it requires a free N-terminus for this activity (54). It has a slight preference for hydrophobic residues, but cannot cleave before or after proline residues (110). It also has been reported to have a poorly characterized endopeptidase activity, however the physiological relevance of this activity remains to be determined (110).

TPPII has been suggested to act in concert with PSA to trim the RU1 peptide for presentation (61). This study used cytosolic extracts, inhibitors, and purified aminopeptidases to show that TPPII can trim the first three residues off a 19-mer precursor of the RU1 peptide, while PSA completed the final N-terminal trimming steps. This preference for longer peptides was also observed by another group that treated living cells with butabindide followed by microinjection of fluorogenic substrates (54). Upon

treatment of butabindide, degradation of peptides longer than 15 residues was no longer observed, suggesting that an enzyme susceptible to butabindide (presumably TPPII) is responsible for trimming these very long peptide precursors (54). Targeting of TPPII with siRNA revealed similar results (54). Based on these data, Reits, et al. suggest a model whereby proteasomal products that are longer than 15 residues and have the correct C-terminus, can initially be trimmed by TPPII. These peptides can then be trimmed by other cytosolic or ER-resident aminopeptidases in order to generate the proper antigenic peptide (54).

Aside from TPPII and the aminopeptidases that I am studying, there are a number of other aminopeptidases present in the cytosol. Although these aminopeptidases have not been reported to be involved in antigen presentation, it is nevertheless important to recognize them. They include methionine aminopeptidase and methionine aminopeptidase 2 (EC 3.4.11.18), which prefers (but is not limited to) cleaving N-terminal methionines, and are thought to be important for cleaving the starting methionine from nascent proteins (111). Prolyl aminopeptidase (EC 3.4.11.5) prefers N-terminal prolines (112). Arginyl aminopeptidase, or aminopeptidase B (EC 3.4.11.6) can cleave N-terminal arginines and lysines, and is thought to be involved in multiple processing mechanisms including spermatozoa formation (113), and may also be secreted (114). Aspartyl aminopeptidase (EC 3.4.11.21) can liberate aspartate or glutamate from peptides, and is thought to be important for cellular protein and peptide metabolism (115).

Currently, it is unknown what the relative contribution of each aminopeptidase is to the total intracellular aminopeptidase activity. Some of the factors that would be key to determining such roles are cell type (since aminopeptidase expression levels vary among cell types), the substrate used (since aminopeptidases have amino acid biases), and culture conditions.

1.9 Peptides are transported into the Endoplasmic Reticulum by TAP

After the cytosolic events of antigen processing, a fraction of the resulting peptides are transported to the ER lumen via the transporter associated with antigen processing (TAP). TAP is an ATP-binding cassette (ABC) transporter that specializes in transporting cytosolic peptides into the ER where they associate with MHC class I molecules. A functional TAP complex is composed of TAP1 and TAP2 that form a heterodimer (116). Tapasin, calreticulin, and ERp57 act as complex stabilizers/chaperones for the empty MHC class I/β2-microglobulin complex that is associated with TAP (117-119).

TAP selects peptides in the cytosol generally suitable for binding the species-specific MHC alleles based on C-terminal residues, but TAP is not very particular to internal sequences (120). Human TAP prefers peptides with hydrophobic or basic carboxyl termini (121, 122), whereas murine TAP prefers hydrophobic C-termini (122).

TAP generally prefers binding peptides that are 8-16 residues in length (123, 124), although it has been suggested that TAP can transport peptides of up to 40 residues in length, albeit with low efficiency (125). TAP's preferences for binding have been suggested to be a major limiting factor in the presentation of certain peptides (7).

If a peptide can bind with sufficient affinity to the TAP complex, an ATP-dependent reaction transports the peptide through the pore into the ER lumen (126), where it binds to empty MHC class I molecules held in the peptide-loading complex (PLC). The PLC is made up of a number of chaperone proteins that aid in retention of the class I molecule until it binds a peptide. These chaperones include calreticulin, ERp57, and Tapasin. In mice, calnexin can also be considered part of the PLC, since it remains associated with the MHC molecule during peptide loading (127, 128), whereas calnexin is replaced by calreticulin during MHC class I assembly in human cells (117, 129). Calreticulin (CRT) has been shown to associate only with peptide-receptive forms of MHC class I/ β2m complexes (117, 130), and to dissociate from Tapasin and MHC-I/β2m/peptide complex after peptide binding (131), although there may be some data that conflicts with this model (119). The exact function of calreticulin in the PLC remains under investigation (119). Tapasin (TPN) acts as a bridge between TAP and MHC class I, and is one of the chaperones responsible for retaining MHC in the ER until an appropriate peptide is bound (119, 132). TPN has been suggested to serve as a "peptide editor" by selecting peptides of high affinity for binding empty MHC class I (119, 133).

Once the peptide is bound in the groove, the MHC class I/peptide complex is released from TAP (134, 135). If an MHC class I molecule cannot bind a peptide, then it

is eventually retro-translocated to the cytoplasm for proteasomal degradation (136, 137). This retro-translocation is thought to occur by way of the Sec61 translocon, and may also be the means by which unbound peptides make their way back to the cytosol for further degradation (138).

1.10 ER-localized aminopeptidases can trim MHC class I peptides

There is a growing body of evidence to suggest that peptides may be trimmed after transport by TAP, and before (or perhaps during) MHC class I peptide loading. As mentioned earlier, some immunodominant peptides may require natural flanking sequences in order to be transported into the ER (20, 139). The fact that these peptides cannot be presented with those flanking sequences suggests that they undergo trimming in the ER after their transport by TAP (7, 20, 21, 139). In TAP-deficient cells, large peptide precursors of influenza nucleoprotein epitope that are targeted to the ER via a hydrophobic signal sequence can be trimmed to the correct 9-mer as detected by RPLC and CTL assay (140). A number of other studies have also used peptides preceded by a signal sequence in order to study ER-localized trimming of peptides (39, 141-143).

In TAP-deficient cells, ER-targeted expression of peptides composed of two tandem epitopes results in the presentation of the C-terminal, but not the N-terminal epitope, suggesting that the N-terminal epitope is destroyed before it can bind MHC class I (140, 144, 145). This trimming activity was localized to the ER, rather than in the late *trans*-Golgi or post-Golgi vesicles (144). Together, these data indicate that there are ER-resident enzymes that can trim MHC class I peptides and peptide precursors.

ER-localized aminopeptidase 1 (ERAP1) is a ubiquitous (146) Zn^{2+} metalloprotease that was simultaneously reported by two groups as a major peptide-trimming activity localized in the ER lumen. One group reported its role in murine cells (141), while the other reported its role in human cells (142, 147). Serwold, et al. reported that reducing mouse ERAP1 led to a reduction of overall MHC class I presentation and the presentation of a few specific epitopes, whereas the presentation of other epitopes was enhanced (141). York et al. reported that reducing human ERAP1 with siRNA decreased presentation of ER-targeted LEQLESIINFEKL almost completely (142). Therefore ERAP1 may be the only enzyme that can trim N-extended SIINFKEL in the ER. In contrast to these results, when MSIINFEKL was expressed in the cytosol, loss of ERAP1 only reduced antigen presentation by 70-80%. The residual presentation of the cytosolic precursor indicated that there is some contribution to trimming by cytosolic aminopeptidases (142).

The findings by York, et al. are in conflict with a recent study, which shows that the presentation of three ER-targeted precursors in HEK293 cells is only minimally affected (reduced by 10-30%) by ERAP1 knockdown, even after stimulation with IFNγ (148). The presentation from these precursors is further reduced (over 50%) only when the ERAP1 knockdown cells express the TAP inhibitor ICP47, suggesting that a TAP-dependent mechanism, which may involve peptide recycling to the cytoplasm, can compensate for the loss of ERAP1 (143, 148).

The observation that these ER-targeted peptides are presented in the absence of ERAP1 may conflict with conclusions made by York, et al. (142), however both studies

demonstrate that ERAP1 can trim peptides in the ER, and that cytosolic aminopeptidases can trim peptides to the final epitope independently of ER-localized trimming activity. Presumably the difference between the York and Saveanu studies relates to the use of different cell lines. The HeLa cells used by Saveanu express a second ER aminopeptidase ERAP2, which can substitute for ERAP1; this enzyme is absent in mice and the HeLa cells used by York (I. York, personal communication).

Another point of conflict is the effect of ERAP1 on overall MHC class I presentation. York, et al. reported that knockdown of ERAP1 in HeLa cells enhances overall MHC class I presentation, and overexpression of ERAP1 in COS cells reduces presentation, suggesting that in humans, ERAP1 generally destroys potential peptides under normal conditions (142). York, et al. further concluded that HeLa cells treated with IFNγ after knockdown of ERAP1 presented less MHC class I than control cells, suggesting that ERAP1 may contribute to the peptide pool under these conditions (142).

In contrast, Saveanu, et al. has reported that knockdown of ERAP1 in HeLa cells reduces overall presentation of MHC class I by roughly 10% (143, 148), and has no effect on MHC class I presentation in HEK293 cells (148) under normal conditions. In addition, ERAP1 knockdown has no effect on MHC class I presentation in IFNγ-treated cells (143, 148). Since this data is in agreement with the observations of ERAP1 knockdown in murine cells (141), the authors suggest that the siRNA used by York, et al. may have caused non-targeted effects that are responsible for the observed phenotype (148).

Lymphocytes from ERAP1 knockout mice also demonstrate a measurable reduction in MHC class I presentation (149)(York, et al. manuscript in preparation), and peptides from ER-targeted precursors are presented poorly in ERAP1-/- fibroblasts also deficient in TAP (149). ERAP1 mice also demonstrate a skewed immunodominance of epitopes from some viruses,(York et al. manuscript in preparation), a reduced immune response to HY antigens, and an altered peptide repertoire (149), suggesting that ERAP1 plays a major role in MHC class I peptide processing in the ER.

ERAP1 has also been suggested to act in concert with a newly identified luminal aminopeptidase ERAP2 (143), which has variable expression in human tissues (150) and is not present in mice (143), but may play a role in MHC class I peptide generation (150). These two IFN-inducible proteins were shown to cooperatively trim model HIV antigens based on sequence preference in microsomal extracts, and in living cells treated with siRNA (143).

Other peptidases in the secretory pathway have also been associated with antigen processing. Furin is a *trans*-Golgi network protease that is involved in the processing of a number of pro-proteins, and has also been suggested to play a role in the processing of MHC class I peptides derived from the secreted form of the HBV core protein (151, 152) and from the HIV gp160 protein (152).

When signal sequences were found to be presented by HLA-A2 molecules, investigators thought that signal peptidase might play a role in generating these peptides for MHC class I presentation (153, 154), however studies directly addressing this possibility have not been forthcoming.

Other ER-localized or membrane bound aminopeptidases that have not been linked to antigen presentation but merit consideration are PLAP/IRAP/oxytocinase (EC 3.4.11.3), aminopeptidase N (CD13) (EC 3.4.11.2), glutamyl aminopeptidase (EC 3.4.11.7), and aminopeptidase P (EC 3.4.11.9).

1.11 Peptide destruction can prevent presentation by MHC class I

Aside from their ability to generate MHC class I peptides, most aminopeptidases can trim peptides down to the size that they can no longer bind MHC class I molecules. For example, PSA has been shown to degrade the Sendai virus nucleoprotein epitope FAPGNYPAL in cytosolic extracts (59). Purified BH and PSA can both destroy the VSV nucleoprotein epitope (60). Overexpression studies have shown that LAP can reduce the MHC class I peptide pool (28, 68). TPPII is currently thought to trim longer peptides, but it has been shown to efficiently degrade short fluorogenic substrates *in vitro* (107), demonstrating that it can degrade short peptides as well. TPPII is also thought to be responsible for the destruction of some MHC class I peptide precursors in living cells (54). Together, these data suggest that cytosolic aminopeptidases can destroy MHC class I peptides.

Other cytosolic peptidases can destroy MHC class I peptides as well. Thimet oligopeptidase (TOP) (EC 3.4.24.15) is an endopeptidase that hydrolyzes peptides that are 6-17 amino acids in size (155). It was initially reported to have a role in antigen processing in 1999 when Portaro et al. used recombinant TOP to show that TOP endopeptidase activity is inhibited by antigenic peptides (156). They further suggested

that TOP acts as a peptide chaperone in the cytosol rather than an endopeptidase that degrades antigenic peptides (156). Treatment of cells with a TOP inhibitor reduced activation of epitope-specific CTLs, suggesting that the chaperoning activity of TOP was required for CTL activation (157).

However, these conclusions are not without conflict. Portaro et al. also showed that the majority of tested peptides (including SIINFEKL precursors), were stable in the cytosol for long periods of time (156). Yet Saric, et al. has shown that incubation of antigenic peptides in cytosolic extracts leads to rapid degradation of the peptides, mainly by TOP (59). Upon immunodepletion of TOP from HeLa cell lysates, degradation of a decapeptide library was reduced by roughly 33% (57). In addition, reducing TOP protein via siRNA leads to increased levels of SIINFEKL presentation (158), and increases overall MHC class I presentation (158, 159), suggesting a role for TOP in MHC class I peptide destruction. Overexpression of TOP led to suppression of MHC class I presentation (159). It also led to decreased presentation of SIINFEKL and other antigenic peptides from minigenes expressed in the cytosol (158), peptide precursors (68), and full length proteins (158), but not ER-targeted minigenes (158). Together, these data suggest that TOP limits the size of the MHC peptide pool by degrading peptides in the cytosol.

2 Object of this work

The goal of this work has been to define the role(s) of cytosolic aminopeptidases in the generation of peptides for antigen presentation. There was evidence to suggest that a number of aminopeptidases might be involved in this process. The questions that have been addressed by my work relate specifically to three aminopeptidases: LAP, PSA, and BH. These aminopeptidases were chosen because they are all localized in the cytosol, and were all identified previously as candidates for having a role in peptide generation. Theoretically, aminopeptidases might play unique roles. Alternatively, some aminopeptidases may act in concert as has been suggested by two previous studies (61, 143). And finally, the broad specificity that these aminopeptidases exhibit *in vitro* may allow them to be functionally redundant *in vivo* for some or all peptide trimming. The work that I present here suggests that the latter is true, and that the functions of LAP, BH, and PSA in generating peptides for MHC class I presentation is mostly redundant.

To demonstrate this, a series of experiments were performed involving antigen presentation assays in cultured cells that are aminopeptidase-deficient and/or are knock-down cells (RNAi) with reduced aminopeptidase expression. In addition, MHC class I antigen presentation was examined in a series of aminopeptidase-deficient mice.

Of course, one of the main functions of the MHC class I presentation pathway is to help the immune system detect infection, and we hypothesized that LAP, PSA and/or BH could contribute to the generation of antigenic viral epitopes. To address this hypothesis, the presentation of specific CTL epitopes was examined under the conditions

of viral infection in mice. The conclusion is that these aminopeptidases are redundant in generation of viral epitopes (or at least those that have been examined).

CHAPTER II

MATERIALS AND METHODS

Plasmids, primers, and PCR

The plasmids used to make the HL3F.1 and control cell lines were generated by designing shRNA inserts using RNAiOligoRetriever software and were based on the NCBI sequence for human LAP NM_015907. Synthetic oligos (IDT, Coralville, IA) were inserted into BseRI and BamHI digested pSHAG-1 plasmid (160)(a gift of Dr. G. Hannon, CSH Labs). The oligos are listed in Table 1.

Kanamycin-resistant bacterial clones were selected and the plasmids were sequenced. pSHAG+shRNA plasmids were digested with PvuII and the fragment including the U6 promoter and shRNA sequence were subcloned into pTracer-CMV2 (Invitrogen, Carlsbad, CA) which had been digested with NruI and EcoRV, removing the CMV promoter.

Primers used for identification of shRNA knock-down clones and for real-time PCR are included in Table 2.

The SIINFEKL mini (MSIINFEKL), N5+SIINFEKL (LEQLESIINFEKL), N25+SIINFEKL (LPFASGTMSMLVLLPDEVSGLEQLESIINFEKL), and full-length

OVA genes were all subcloned from other vectors into pTracer-CMV2 (Invitrogen, Carlsbad, CA), a plasmid containing a GFP/Zeocin-resistance fusion protein by restriction digest and ligation. The plasmids were then sequenced to confirm correct sequences and reading frames. The pcDNA3.1-N5+SIINFEKL plasmid, which was used in some experiments, does not contain a GFP gene and has been described previously.

To express mature SIINFEKL with no N-terminal residues, we constructed pUG-SIINFEKL, and other pUG plasmids as listed in Table 3. These plasmids consist of ubiquitin with SIINFEKL (or SIINFEKL precursor) fused to the C terminus; C-terminal ubiquitin hydrolases efficiently release peptides thus fused to ubiquitin (163). An internal ribosome entry site (IRES) downstream of the ubiquitin-SIINFEKL fusion was followed by GFP.

mRNA was isolated from 10^6 cells using an RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized using Superscript II enzyme (Invitrogen, Carlsbad, CA) and real-time PCR was performed on an iCycler machine (BioRad, Hercules, CA) with Sybergreen buffer (Applied Biosystems, Foster City, CA), or a MyIQ machine (BioRad, Hercules, CA) with BioRad 2X Master mix (BioRad, Hercules, CA).

For screening of ES cells that contained an LAP knock-out allele, Taqman® primers were used and are listed in Table 2. Each Taqman® primer sequence spanned the point of recombination between the reporter gene and endogenous genomic sequence. Three-

primer PCR protocols were used to screen for LAP-deficient and PSA-deficient mice. Primers are listed in Table 2. LAP-WT(R1) (or mPSA-R2) lies downstream of the knock-out insert in the genomic sequence, and therefore amplifies both the wild type and knock-out alleles. LAP-KO(F5) (or mPSA-KO(F1)) lies within the knock-out allele, and amplifies only the knock-out allele. LAP-WT(F1) (or mPSA-WT(F3)) lies within the wild-type allele and amplifies only the wild type allele.

Generation of LAP-/- mice

The mouse LAP gene (GeneID: 66988) was deleted using VelociGene® technology (164). Briefly, a large targeting vector (BACvec) was constructed by bacterial homologous recombination in which the 19.2 kb LAP gene was replaced by a lacZ-neo cassette. An 129xC57BL/6 F1 ES cell line was electroporated with the BACvec and selected for G418 resistance. Drug-resistant clones were screened for loss of one copy of the LAP gene by quantitative PCR using probes at either end of the deletion. Two independent targeted ES cell clones were microinjected into C57BL/6 blastocysts, which were implanted in C57BL/6 females to generate chimeras. Chimeras were bred back to BL/6 to generate F1 heterozygote mice. Both ES cell clones produced healthy appearing knockout mice in proper Mendelian proportions. A line derived from one of these clones was used for subsequent experiments.

Generation of PSA-/- mice

The mouse PSA (NPEPPS) gene (GeneID: 19155) was deleted using VelociGene® technology (164), just as the LAP mice. Briefly, a large targeting vector (BACvec) was constructed by bacterial homologous recombination in which 18.1 kb of the PSA gene (Exons 3-9) were replaced by a lacZ-neo cassette. An 129xC57BL/6 F1 ES cell line was electroporated with the BACvec and selected for G418 resistance. Drug-resistant clones were screened for loss of one copy of the LAP gene by quantitative PCR using probes at either end of the deletion. Two independent targeted ES cell clones were microinjected into C57BL/6 blastocysts, which were implanted in C57BL/6 females to generate chimeras. Chimeras were bred back to BL/6 to generate F1 heterozygote mice. A line derived from one of these clones was used for subsequent experiments

Poly I:C treatment of mice

For LAP studies, mice were injected interperitoneally with 200μg of poly I:C (Amersham Pharmacia, Piscataway, NJ) in a total volume of 200μL PBS (Gibco, Logan, UT). Spleens from the mice were harvested after 24 hours and then stained for flow cytometric analysis.

Virus infection of mice

Mice were injected IP with 5×10^4 pfu/ mouse of LCMV Armstrong (a gift from Dr. Ray Welsh, University of Massachusetts Medical School, Worcester, MA), or with 5×10^6 pfu/ mouse of recombinant vaccinia (provided by Drs. Jon Yewdell and Jack Bennink, NIH,

Bethesda, MD), containing chicken ovalbumin (21). Mice were infected IV with 5×10^6 pfu/ mouse of Vesicular Stomatitis Virus (VSV) (a gift from Dr. Ray Welsh), or with 50-100 HAU of Sendai virus (a gift from Dr. Kate Fitzgerald). Eight or nine days (LCMV), 7 days (Vac-OVA and VSV), or 6 days (Sendai) post-infection, splenocytes were harvested and incubated for 5 hours with the appropriate peptide (5μM for LCMV, Vac-OVA, and Sendai; 2-5μM for VSV), or with anti-CD3ε (BD Biosciences, San Jose, CA) in the presence of GolgiPlug (BD Biosciences, San Jose, CA), and recombinant IL-2 (BD Biosciences, San Jose, CA). Vaccinia peptides that were used to stimulate IFNγ production were B8R (TSYKFESV) (165), SIINFEKL, and P10 (STLNFNNL) (166). LCMV peptides included gp33 (KAVYNFATC), gp276 (SGVENPGGYCL), NP205 (YTVKYPNL), and NP396 (FQPQNGQFI). The VSV peptide (RGYVYQGL), and Sendai peptide (FAPGNYPAL) were also used. All peptides were synthesized (Anaspec, Inc., San Jose, CA). Cells were then stained for CD8, CD44, and intracellular IFNγ using commercial antibodies (BD Biosciences, San Jose, CA), and analyzed by flow cytometry

Construction of stable shRNA cells

HeLa-K^b cells (142) were transfected with the shRNA+GFP/Zeocin resistance-expressing plasmids using HeLa Monster (Mirus, Madison, WI). 48 hrs after transfection, the cells were serially diluted in DMEM containing 100 μg/mL of Zeocin (Invitrogen, Carlsbad, CA) to select for plasmid transfectants and seeded in 96-well plates. After 14 days of incubation in selection media, clones were isolated and tested for expression of LAP

mRNA by RT-PCR and GFP expression by FACS. Clones used in these studies were selected based on comparable expression of GFP.

Cells and tissue culture

shRNA knock-down cell lines were incubated at 37°C and 10% CO_2 in DMEM+10% FCS, 100 μg/mL Zeocin to select for cells expressing the shRNA, and 100 μg/mL G-418 (Invitrogen, Carlsbad, CA) to select for cells expressing H-2K^b. Mouse Embryonic Fibroblasts (MEFs) were generated from 12-14 day embryos and cultured at 37°C and 10% CO_2 in DMEM+20% FCS. Cells were cultured in flasks, and transfections were performed in 6-well plates (Corning-Costar, Acton, MA). During transfection periods cells were cultured in DMEM+10%FCS.

For incubations in IFNγ, MEFs or shRNA knock-down cells were transfected first with the indicated construct. Then 8 hrs later, the transfection media was removed and DMEM+FCS+IFNγ was added to each well. The IFNγ concentrations used were 250 Units/ml recombinant human IFNγ for HeLa cells (Biogen, Cambridge, MA), or 50 Units/mL recombinant murine IFNγ for MEFs (BD Biosciences, San Jose, CA). The cells were incubated in IFNγ until the cells were analyzed.

Antibodies, immunoblotting, and flow cytometry

The mAb 25.D1.16 (anti-K^b+SIINFEKL) (167), AF6-88.5 (anti-K^b) (168), Y3 (anti-K^b) (169), M1/42 (anti-H2) (170), or H36.4.5 (anti–influenza HA) (a gift of W. Gerhard, The

Wistar Institute, UPENN) were used as primary antibodies in staining HeLa cells and MEFs for flow cytometry. After incubation in one of the primary antibodies, the cells were washed with PBS and stained with donkey anti-mouse (or donkey anti-rat) F(ab')2 fragments conjugated to Cy5 (Jackson Immunoresearch, West Grove, PA). For staining cells isolated from spleen, AF6-88.5 (H-2K^b) and KH95 (H-2D^b) antibodies conjugated to a fluorophore were used according to the manufacturer's directions (BD Biosciences, San Jose, CA). The cells were then analyzed by flow cytometry on a FACSCalibur apparatus (Becton Dickinson, San Jose, CA) with FlowJo software (Tree Star, San Carlos, CA).

For immunoblotting HeLa cells, cells were lysed in a 1mL Dounce homogenizer on ice in Dounce buffer 10mM Tris-HCL, 0.5mM $MgCl_2$, pH 7.6. Complete mini EDTA-free protease inhibitor cocktail tablets (Roche, Nutley, NJ) were added according to manufacturer's instructions. After 50 strokes in the Dounce homogenizer, stabilization buffer was added (10mM Tris-HCL, 0.5mM MgCl2, 0.6M NaCl, and complete mini tablets) to the lysate to stabilize the nuclei. After a 5-minute spin in a mini-centrifuge at 4°C at full speed, the resulting supernatant was centrifuged at 4°C in an ultracentrifuge (Beckman, Fullerton, CA) with a 70.1Ti rotor (Beckman) at 35,000 rpm for 1hr. 3X SDS sample buffer + DTT (NEB) was added to the resulting supernatant and the samples were heated to 95°C for 5min.

For immunoblotting MEFs, cells were lysed by adding NP40-lysis buffer (1% NP40, 300mM NaCl, 50mM NaH_2PO_4) with complete mini EDTA-free protease inhibitor cocktail tablets added (Roche, Nutley, NJ) according to manufacturer's instructions. After a 5-minute incubation on ice, they were spun in a mini-centrifuge at 4°C at full speed for 5 minutes. 3X SDS+DTT buffer was added to the resulting supernatant and the samples were heated to 95°C for 5min.

1.5×10^5 HeLa cell equivalents (or 3×10^5 for MEFs) were run on a 12% SDS gel followed by protein transfer to a nitrocellulose membrane (Schleicher & Schuell Bioscience, Keene, NH). After transfer, the membrane was rotated overnight in PBS+5% milk+ 0.2% Tween20 to block. After 18 hrs, the membrane was stained with rabbit anti-LAP polyclonal antibody (a gift from Dr. Alfred Goldberg, Harvard Medical School) diluted 1:10,000 in PBS+0.2% Tween+0.02% NaN_3. After 2 hrs, the blot was washed three times with PBS+0.2%Tween for 30 min. The blot was then stained with horseradish peroxidase–conjugated goat anti-rabbit diluted 1:50K. The blot was developed with enhanced chemiluminescence (Pierce, Rockford, IL). The same protocol was followed using two different polyclonal anti-BH antibodies that were diluted 1:25,000 (a gift from Dr. Paul O'Farrell, Cold Spring Harbor Laboratories) or 1:500 (a gift from Dr. Jacques Neefjes, The Netherlands Cancer Research Institute), and the HRP-conjugated antibody diluted 1:25,000.

CHAPTER III

RESULTS

3.1 Leucine aminopeptidase is not essential for trimming peptides in the cytosol or generating epitopes for MHC class I antigen presentation

3.1.1 Generation of LAP deficient mice

LAP deficient mice were generated by and purchased from Regeneron using VelociGene® technology (164). Homologous recombination resulted in a loss of all exons and introns of the LAP genomic sequence (Fig 1A), beginning 6 bp downstream of the LAP start codon, and ending 624 bp after the LAP stop codon. In total, approximately 19.2 kb was deleted. Presence of the neogene and subsequent loss of the entire LAP gene was confirmed by PCR (Fig 1B). LAP -/- mice express no LAP protein in any of the organs tested (Fig 1C), even after IFN stimulation (Fig 1D).

3.1.2 Lymphocyte ratios and MHC class I levels in LAP -/- mice are normal

Since MHC class I surface expression is dependent on peptide supply, changes in peptide supply can be detected by measuring surface MHC class I levels. Elimination of LAP could in principle reduce peptide supply to MHC class I (if LAP predominately trims peptides longer than 9-10 amino acids) or increase peptide supply (if LAP preferentially trims peptides 9 to10 amino acids in length that could otherwise bind to MHC class I). We therefore evaluated the expression of MHC class I on cells in the LAP deficient animals. The levels of H-2D^b and H-2K^b as well as the overall amount of total MHC class I on splenocytes from LAP-/- mice were similar to those on splenocytes from wild-type C57BL/6 mice (Fig 2). The small reduction in K^b expression on LAP-/- cells in this experiment was not statistically significant and was not observed in other experiments. LAP is normally induced by IFN (53, 78), and in wild-type mice LAP protein levels increased after intraperitoneal injection of the type I IFN-inducer poly I:C. However, even under these conditions surface levels of H-2D^b and H-2K^b were not significantly different in wild type and LAP -/- mice (Fig 2).

Mice lacking important components of the MHC class I antigen presentation pathway sometimes have reduced levels of CD8+ T lymphocytes due to reduced positive selection in the thymus (44, 171, 172). Therefore, we examined the levels of CD8 T cells in the LAP-deficient mice. Inguinal, mesenteric, and cervical lymph nodes and spleens were harvested from C57BL/6 and LAP -/- mice and their CD4 and CD8 T cells were

enumerated by flow cytometry. CD4+ B220- cells, and CD8+ B220- cells were present in similar ratios and numbers in both strains, suggesting that T cell generation and maturation is grossly normal in LAP -/- mice (Fig 3).

3.1.3 LAP -/- MEFs present peptide as well as wild type MEFs

Several independent lines of mouse embryonic fibroblasts (MEFs) were generated from WT and LAP-/- embryos. As with lymphocytes, no significant differences in surface H-2D^b, H-2K^b or total MHC class I levels were detected either under normal conditions or after stimulation with IFNγ (Fig 4). Using real time PCR, we detected a large amount of variation in the expression of BH, PSA, and ERAP1 mRNAs among all MEFs (Fig 5), however there was certainly no pattern of increased expression of these aminopeptidases in the LAP-/- MEFs. Therefore, these other aminopeptidases were not upregulated and compensating for a lack of LAP.

Cleavage of full-length ovalbumin by the proteasome *in vitro* generates SIINFEKL, as well as SIINFEKL extended at the N-terminus by up to 12 residues (50). Similarly, N-extended precursors of SIINFEKL are generated from full-length ovalbumin constructs and require trimming for presentation *in vivo* (142). To examine the role of LAP in this process, we transfected wild type or LAP -/- MEFs with plasmids expressing full-length ovalbumin and GFP, so that transfected cells could be identified by GFP expression. Wild type and LAP -/- MEFs (gated for comparable GFP expression)

generated similar amounts of SIINFEKL-K^b complexes (Fig 6A) as detected by flow cytometry using the H-$2K^b$-SIINFEKL-specific antibody 25.D1.16 (167).

To more thoroughly examine the dependence of the processing of N-extended peptides on aminopeptidases, we expressed in these MEFs N-extended forms of SIINFEKL from minigenes. In this situation, all of the peptides are produced in the cytosol as precursors that require trimming for presentation (39, 51). SIINFEKL generation from precursors extended by 1 (the initiating methionine) or 5 residues, was identical in wild-type and LAP-/- MEFs (Fig 6A). Since these 9-13 residue antigenic precursors can be transported into the ER and trimmed by ERAP1 (142), we also examined the presentation of a 33 residue precursor that is too long to be efficiently transported into the ER by TAP (125). This construct, which has a 25 residue N-terminal extension, was trimmed and presented similarly by wild type and LAP-/- MEFs (Fig 6A). Therefore, under constitutive conditions, LAP is not essential for the trimming of N-extended SIINFEKL precursors.

Aminopeptidases can also destroy antigenic peptides by trimming them below the size required for stable binding to MHC class I molecules. To test whether LAP might be destroying some presentable peptides, MEFs were transfected with a construct encoding a ubiquitin-SIINFEKL fusion protein. When expressed in cells, the N-terminal ubiquitin is cleaved, thereby producing the mature epitope SIINFEKL. If LAP were to trim SIINFEKL the resulting products would not bind stably to H-$2K^b$. However, LAP-/- and wild type cells presented equivalently SIINFEKL generated from the ubiquitin fusion

construct (Fig. 6A). Therefore, LAP is not limiting the amount of SIINFEKL available for presentation.

Interferon treatment of cells alters the composition and enzymatic activities of the proteasome, resulting in relatively more N-extended precursors of antigenic epitopes (50), as well as increased levels of LAP (53). However, even though IFN treatment increased overall presentation, the presentation of SIINFEKL from each precursor was the same in wild-type and LAP -/- MEFs (Fig 6B) suggesting that even at induced levels, LAP is not essential in the generation of some MHC class I peptides.

3.1.4 Presentation of viral epitopes is not altered in LAP -/- mice

To test antigen processing *in vivo*, we examined the CTL response to several viruses. Wild type and LAP -/- mice were infected with vesicular stomatitis virus (VSV), LCMV, or a recombinant vaccinia virus expressing ovalbumin. At the peak of each infection, splenic lymphocytes were isolated, stimulated *in vitro* with the appropriate antigenic peptide, and stained for intracellular IFNγ levels. The frequency of responding (IFNγ-positive) CTL (Fig. 7) and their level of production of IFNγ (data not shown) to the VSV peptide (Fig 7A), to four antigenic LCMV peptides (Fig 7B), and to two recombinant Vac-OVA peptides (Fig. 7C) in LAP -/- mice was equal to that of wild-type mice, suggesting that neither the quantity nor the quality of the CTL response *in vivo* was dependent on LAP.

3.1.5 LAP mRNA expression and protein levels are reduced in shRNA stable transfectants of HeLa cells

The findings that elimination of LAP does not alter antigen presentation in mouse cells, both *in vivo* and *in vitro* was unexpected, since LAP has been shown to process N-extended SIINFEKL peptides in cell lysates (53). Because the previous studies used HeLa cell extracts, it seemed possible that LAP might play a more important role in human cells or in particular cell types. To investigate this issue, HeLa-K^b cells (142) were stably transfected with a plasmid encoding a short-hairpin RNA (shRNA) targeting LAP, or encoding a control shRNA that does not recognize human sequences. Clone LAP-KD showed a reduction in LAP of 90-95% by real time PCR (Fig 8A) and western blot (Fig 8B), as compared to the control cell line. Real-time PCR analysis showed a slight increase in BH and PSA mRNA in LAP-KD cells, however this may be due to clonal variation between cell lines or to a difference in β-actin mRNA levels between these cells since β-actin mRNA was used for normalization (Fig 9).

LAP is inducible by IFNγ in HeLa cells (53). Therefore, we examined the silencing of LAP in LAP-KD cells treated with IFNγ. After 48 hr of IFNγ treatment, LAP mRNA was increased approximately 10-fold in the control cell line (Fig 8A), but LAP mRNA levels in the LAP-KD cell line, while increased, were still approximately 95-99% lower than in the control (IFN-stimulated) cell line. Analysis of protein levels by semi-quantitative immunoblotting gave similar results (Fig. 8B). LAP-KD constitutively had approximately 5-10% as much LAP as the control cell line. While treatment with

IFNγ increased the levels of LAP protein approximately 10-fold in both the LAP-KD and control cell lines, LAP levels in LAP-KD were about 10-fold lower than in control cells treated with IFNγ.

3.1.6 Stable shRNA does not induce IFN-sensitive genes

Expression of shRNA from plasmids has been suggested to induce transcription of IFN-sensitive genes (173, 174). Because MHC class I levels are also affected by IFN, such induction could skew any phenotype observed in the LAP-KD cell line. To determine if either of these shRNAs constitutively induces IFN-sensitive genes in LAP-KD cells, real-time PCR assays were used to measure mRNA levels of protein kinase R (PKR), and interferon-sensitive gene 15 (ISG15), which are activated by IFN (175).

In LAP-KD and shRNA control cells, expression of IFN-sensitive genes were the same even after incubation with poly I:C, suggesting that low level expression of these shRNAs does not induce transcription of IFN-sensitive genes (Fig 10).

3.1.7 Reduction of LAP has no effect on surface presentation of H-2K^b + SIINFEKL in HeLa cells.

Since LAP was originally described as an enzyme in HeLa cell extracts that trimmed N-terminally extended precursors of the OVA peptide SIINFEKL, we tested such peptides in the LAP-KD cells. Therefore, LAP-KD cells and control cells were transiently transfected with plasmids encoding ovalbumin or N-extended SIINFEKL

precursors, as described above. For each of the constructs, the generation of H-2K^b-SIINFEKL was the same in control and LAP-KD cells (Fig. 11A). There was also no difference between the control and LAP-KD cells when they were treated with IFNγ (Fig 11B). Therefore, LAP is not required for generation of MHC class I peptides in HeLa cells.

N-terminally extended peptides may be transported to the ER by TAP, where they can be trimmed to mature epitopes by ERAP1. To determine if ERAP1 was masking an effect of LAP deficiency, we examined the phenotype of cells in which both LAP and ERAP1 were silenced. LAP-KD and control cells were treated with an siRNA targeting ERAP1, or with control siRNA (142) and then transiently transfected with plasmids encoding N-terminally extended SIINFEKL precursors, as described above. As previously reported (142), presentation of SIINFEKL from N-extended precursors is reduced in HeLa cells treated with ERAP1 siRNA (Fig 12). In comparison, SIINFEKL presentation was not further reduced in LAP and ERAP1 double-KD cells. Presentation of the SIINFEKL precursor was also not different in ERAP1-deficient versus LAP + ERAP1-deficient cells after stimulation with IFNγ for 24 hours post-transfection (Fig 12).

3.1.8 Reduction of LAP has no effect on the rate of peptide trimming in cells.

The quantitation of antigen presentation is an indirect measure of the generation of antigenic peptides. It is possible that a contribution of LAP to this process could be

missed if the trimming of peptides was not a rate-limiting step in the pathway. Therefore, we sent the LAP-KD cells and control cells to Joost Neijssen and Jacques Neefjes so that they could directly measure the rate of peptide degradation in these cells by microinjection of peptides containing fluorescein and quencher adducts (28, 54). These substrates generate a fluorescent signal when aminopeptidases cleave and thereby separate the residues containing the fluorophore and quencher moieties. Peptides with different amino acids in the P1 position were microinjected into cells, and the fluorescence resulting from trimming the peptides was measured.

Although there was some variation in the rate of trimming of different peptide sequences, there was no statistically significant difference in the half-life of any of the tested peptides between the two cell lines (Fig. 13A). Treating the cells with IFNγ prior to microinjection increased LAP expression (Fig. 13B), but did not alter the rate of processing of the peptides (Fig. 13A & C). These data demonstrate that LAP activity does not influence the rate of peptide trimming in the cytosol.

3.2 Bleomycin Hydrolase is not essential for trimming peptides in the cytosol or generating epitopes for MHC class I antigen presentation

3.2.1 siRNA reduction of BH may not affect MHC class I presentation

siRNA is a powerful tool that has been used by a number of groups to examine the effect of transient loss of aminopeptidases in living cells (54, 141, 142, 158)(York, et

al. submitted). To examine the role of BH in human cells, two siRNA sequences were designed to target BH mRNA. Both siRNAs reduced BH mRNA levels in HeLa-K^b cells compared to mTOP control siRNA (which targets murine TOP) (Fig 14). However, they had different effects on endogenous MHC class I presentation in HeLa-K^b (Fig 15). Transfection of BH siRNA#1 had little effect on levels of MHC class I presentation, whereas transfection of BH siRNA#2 caused an overall increase in MHC class I.

Because BH is a processive enzyme, a small amount of enzyme could account for the observed difference, and it is possible that some residual protein not knocked-down by BH siRNA#1 could account for the discrepancy between phenotypes. An alternative explanation is that one of the BH siRNA sequences increases transcription of type-I IFN. Since many components of the MHC class I pathway are sensitive to IFN expression, this could be influencing the MHC class I presentation pathway independently of BH activity.

siRNAs, originally designed as 21mers, were thought to be too short to bind Toll-like receptor 3 (TLR3) or activate enzymes like Protein Kinase R (PKR) and Oligoadenylate Synthase (OAS) (176, 177). They were also thought to target only mRNAs with 100% homology for degradation. However, others have shown that siRNAs can activate IFN-sensitive genes (173, 178, 179) and bind TLR3 (173, 180, 181). In addition, siRNAs have also been shown to alter gene expression of mRNAs with less than 100% sequence homology to the siRNA (off-target effects) (182, 183). To address whether either of these postulated siRNA "side-effects" could explain the contrast in phenotypes of cells transfected with BH#1 or BH#2 siRNA, a series of experiments were designed and performed.

HeLa cells were transfected with BH#1, BH#2, mTOP, or Luc1 which is a siRNA sequence that has been shown to increase transcription of IFN-sensitive genes, but does not have a target mRNA in human cells (173). After siRNA transfection, mRNA was isolated at varying time points to measure transcriptional induction of OAS2, PKR, and interferon-sensitive gene 15 (ISG15). Transcription of all of these genes is stimulated by IFN, and they are often used to measure IFN-signaling (175).

Results from real-time PCR suggest that siRNAs can affect these genes at different levels (Fig 16). As expected, Luc1 siRNA increased expression of ISG15, PKR, and OAS as compared to mTOP control siRNA (Fig 16A). Transfection of BH#1 did not induce any of the genes tested and had lower amounts of all transcripts, even compared to the mTOP control (Fig 16B). In contrast, BH#2 siRNA induced expression of ISG15 to levels higher than Luc1, but did not significantly enhance transcription of PKR or OAS2 (Fig 16). Together these data suggest that BH#2 siRNA has physiological effects other than the reduction of BH mRNA, and that like Luc1, BH#2 may upregulate IFN-sensitive genes such as proteins involved in MHC class I machinery, and thereby enhance surface presentation. In support of this conclusion, HeLa cells transfected with Luc1 or BH#2 siRNA show comparable increases in MHC class I presentation (Fig 17), and COS-K^b cells transfected with these siRNAs show similar decreases in SIINFEKL presentation when expressing SIINFEKL peptide precursors (Fig 18).

Although it is possible that an increase in MHC class I presentation may be due to an intracellular IFN response, it is still formally possible that transfection of BH#2 siRNA demonstrates the accurate phenotype, whereas BH#1 transfection inefficiently

reduces BH protein. To address the question of whether the observed phenotype was specifically due to the ability of BH#2 to knock down BH mRNA, other siRNAs were designed to target the 3' untranslated region (UTR) of the BH mRNA, which is located after the stop codon in the mRNA transcript. Such an siRNA will target endogenous BH mRNA that has been properly processed from pre-mRNA, but will not be able to target an open reading frame (ORF) that lacks a 3'UTR. By real time PCR, this new siRNA (BH-UTR) was shown to knock down BH mRNA as well as BH#2 (Fig 19). In addition, it was just as effective (or more effective) than BH#2 and Luc1 in reducing SIINFEKL presentation from peptide precursors in HeLa-K^b (Fig 20A) and COS-K^b cells (Fig20B & 21B).

COS-K^b cells were transfected, with Luc1, mTOP, or BH-UTR siRNA. Then 24 hours later (day 1), the cells were transfected with a plasmid expressing a SIINFEKL precursor, and BH-ORF or vector control. On day 3, the cells were counted, and analyzed by flow cytometry for presentation of SIINFEKL, and by real-time PCR for expression of BH mRNA. If the reduction of SIINFEKL presentation was due to the knockdown of BH mRNA by BH#2, then overexpression of BH-ORF (which is not targeted by BH-UTR) would ameliorate the phenotype. Overexpression of BH-ORF resulted in high levels of BH mRNA (indicating that the BH-ORF, was not reduced by BH-UTR) (Fig 21A). However, this increase did not ameliorate the decrease in SIINFEKL presentation (Fig 21C), suggesting that the reduction of SIINFEKL presentation by BH#2, BH-UTR, and Luc1 is likely the result of an effect other than their efficiency at knocking down BH.

These studies demonstrate that although RNAi (siRNA) is a powerful experimental tool, care should be taken in the interpretation of data since off-target effects may account for phenotypes that may initially be attributed to the reduction of the target. To ensure that observed phenotypes are due to silencing of the target gene, proper experimental controls (such as multiple siRNAs) are essential. In the case of BH, it is clear that knockdown by siRNA demonstrated phenotypes that were inconclusive, and emphasized the desirability for an alternative system of study.

3.2.2 BH -/- MEFs present peptide as well as wild type MEFs

To examine antigen presentation in cells deficient in BH, BH-deficient and wild type control MEFs were generated (Fig 22A). Levels of MHC class I were measured by flow cytometry to determine if there were any defects in antigen presentation. Overall, expression of H-2K^b (Y3 and B8) and all MHC class I (M142) were comparable between BH-deficient and wild type MEFs (Fig 22B).

To compare the ability of BH-/- MEFs to present one specific model peptide (SIINFEKL), MEFs were transfected with a series of constructs encoding GFP and ubiquitin-fusion minigenes. In these plasmids, the C-terminus of ubiquitin is fused to an N-extended form of the SIINFEKL peptide. These minigenes mimic an *in vivo* ligation of ubiquitin via its C-terminus which can be cleaved by ubiquitin C-terminal hydrolases (9, 163). Such a cleavage results in the peptide of interest with no starting methionine, thus generating peptides more similar to the peptides generated by the proteasome. Using this system, it is possible to generate a peptide with virtually any N-terminal residue by

varying the amino acid at the ubiquitin-linker junction. Cleavage of ubiquitin from the peptide, and subsequent trimming of the N-terminal residues can be detected by 25.D1.16 staining of transfected cells. Two series of N-terminal extensions were generated in order to address the possibility that BH is important for trimming some sequences, but not others. First, the natural upstream sequence of SIINFEKL was used since it has been used in previous experiments and is known to be trimmed in live cells (39). Second, the upstream sequence of the VSV nucleoprotein epitope was used since this sequence has previously been shown to be trimmed by BH and PSA (60) (Table 3).

MEFs were transfected with the constructs listed in Table 3 and analyzed by flow cytometry after 48 hrs. Cells were gated based on comparable GFP expression, and then compared for SIINFEKL presentation. Although SIINFEKL preceded by the natural upstream sequence was better presented than SIINFEKL preceded by the VSV NP sequence, no statistical difference was detected between BH-/- and wild type MEFs, although there was consistently a slight decrease in presentation by the BH-/- MEFs (Fig 23). This suggests that BH does not play an essential role in generating MHC class I peptides *in vivo* either from peptide precursors or full length protein. In addition, the fact that there was no difference (or only a minor decrease) in presentation of the 0+S8L construct suggests that BH does not destroy MHC class I peptides either, since any degradation of this peptide would result in reduced presentation (Fig 23).

3.2.3 Lymphocyte ratios and MHC class I levels in BH -/- mice are normal

To examine more carefully the role of BH *in vivo*, MHC class I levels were measured on splenocytes from wild type and BH-deficient mice. CTLs (CD8+, B220-), T-helpers (CD4+, B220-), B-cells (B220+, CD4-, and CD8-), and dendritic cells (CD11c+, CD86+, CD8+ or CD8-) from BH-/- mice presented MHC class I as efficiently as wild type mice. T-cells and B-cells in the peripheral lymph nodes were also normal in number and MHC class I expression (data not shown), suggesting that BH is not required for MHC class I presentation in these cell compartments, or for maturation of T-cells (Fig 24).

3.2.4 Presentation of viral epitopes is not altered in BH -/- mice

BH has previously been shown to act on viral epitope precursors to generate the correct peptide *in vitro* (60), so it possible that BH may have a role in generating MHC class I peptides from viral proteins during an infection *in vivo*. If BH is essential for trimming viral epitopes for presentation, then BH-/- mice would theoretically mount a reduced CTL response to these epitopes. To investigate, BH mice were infected with VSV, LCMV, or rVV+OVA, and the CTL response to known viral epitopes was analyzed at the peak of each infection via intracellular cytokine staining for IFNγ. BH-deficient mice responded to all eight viral epitopes tested as efficiently as wild type (Fig 25), indicating that BH is not required for presentation of these viral peptides *in vivo*.

3.2.5 Lack of BH has no effect on the rate of peptide trimming in cells

MHC class I surface expression is an indirect measure of the generation of antigenic peptides. It is possible that a contribution of BH to this process could be missed if the trimming of peptides is not a rate-limiting step in the pathway. We sent the BH-/- MEFs (and wild type controls) to Joost Neijssen and Jacques Neefjes so that they could measure the rate of peptide degradation in these cells by microinjection of peptides containing fluorescein and quencher adducts (28, 54). These substrates generate a fluorescent signal when aminopeptidases cleave and separate the residues containing the fluorophore and quencher moieties. Peptides with different amino acids in the P1 position were microinjected into cells, and the fluorescence resulting from trimming the peptides was measured.

Although there was some variation in the rate of trimming of different peptide sequences particularly with proline and glycine (Fig 26), there was no statistically significant difference in the half-life of any of the tested peptides between the two cell lines (Fig 26B). These data demonstrate that BH activity does not influence the rate of peptide trimming in the cytosol.

3.3 Puromycin-sensitive aminopeptidase is not essential for trimming peptides in the cytosol or generating epitopes for MHC class I antigen presentation

3.3.1 siRNA reduction of PSA may not affect MHC class I presentation

To examine the role of PSA in human cells, two siRNA sequences were designed to target human PSA mRNA (PSA#1 and PSA#2). Transfection of HeLa-K^b cells resulted in the efficient and equal reduction of PSA mRNA by both siRNAs, as detected by real-time PCR (Fig 27A). By transfecting PSA siRNA into COS-K^b cells, and then overexpressing human PSA in the same cells, a reduction of protein could be observed by western blot (Fig 27B), suggesting that PSA siRNA could specifically target PSA mRNA, resulting in the reduction of protein. COS-K^b cells were used for this experiment due to their highly transfectability and the inability of the anti-PSA antibody to detect endogenous levels of PSA

Next, MHC class I levels were examined on HeLa-K^b cells transfected with PSA#1 or PSA#2. Overall levels of MHC class I levels did not change significantly in cells transfected with PSA#1, but were increased in cells transfected with PSA#2 (Fig 28). In addition, COS-K^b cells transfected with PSA#1 siRNA presented SIINFEKL from peptide precursors as well as cells treated with control siRNA, whereas cells treated with PSA#2 siRNA presented lower amounts of SIINFEKL (Fig 29). If PSA#2 was presenting an accurate phenotype, then this would suggest that PSA plays a generally

destructive role in MHC class I presentation with the exception of some peptides, such as SIINFEKL.

3.3.2 Some siRNAs may cause off-target effects unrelated to type I IFN

In order to determine if one of the PSA siRNAs was causing an increase in type-I IFN, like BH#2, stimulation of IFN-sensitive genes was measured by real-time PCR. Real-time PCR on mRNA harvested from cells transfected with PSA#1, PSA#2, mTOP, or Luc1 siRNA indicated that neither PSA siRNA was eliciting a type I IFN-mediated response (Fig 30). However, the reason why gene silencing with PSA#1 and PSA#2 manifested different phenotypes was still unknown.

A potential explanation as to the reason for the discrepancy came inadvertently in an experiment examining the role of BH. COS-K^b cells transfected with a variety of siRNAs were later transfected with a CMV plasmid expressing human BH. After two days, the cells were analyzed by western blot and real-time PCR for levels of BH mRNA and protein. PSA#2 and mTOP siRNAs were both used in this experiment as controls since neither of them share sequence homology with BH. Surprisingly, PSA#2 siRNA knocked-down BH mRNA as effectively as BH#1 siRNA (Fig 31A), and reduced translation of BH mRNA as well as BH#2 siRNA (Fig 31B). Importantly, this ability to limit overexpression of BH was not shared by PSA#1 siRNA (Fig 31C). Therefore, barring the possibility that PSA protein plays a role in transcription and/or translation

(which has not been reported), these data suggest that PSA#2 siRNA can affect the expression of genes unrelated to PSA. This brings into question the relatedness and relevance of the observed phenotypes.

The previous experiment demonstrates that PSA#2 has effects in the cell other than the silencing of PSA, however it does not show that these unrelated effects are the cause of the increase in MHC class I presentation, or of the decrease in SIINFEKL presentation. One or both of these phenotypes may be directly linked to the reduction of PSA protein, and unaffected by the side-effects of PSA#2. To address this question, new siRNAs were designed that targeted the 3'UTR of human PSA (PSA-UTR). Upon transfection of HeLa-K^b cells, PSA-UTR siRNA was as effective as PSA#2 at reducing PSA mRNA (Fig 32A). It also did not appear to activate type-I IFN genes by real-time PCR (Fig 32B), and demonstrated a reduction of K^b+SIINFEKL presentation in HeLa-K^b cells (Fig33A) and in COS-K^b cells (Fig 33B) comparable, but not identical to PSA#2 and control siRNA (Fig 33B).

To determine if the gene-specific silencing of PSA could explain the phenotype elicited by PSA#2 and PSA-UTR, COS-K^b cells were transfected with PSA-UTR siRNA or control siRNA. The next day (day 1), these cells were transfected with a SIINFEKL peptide precursor construct, and a plasmid expressing the ORF region of human PSA or vector. On day 3, the cells were analyzed for PSA mRNA expression by real-time PCR, and ability to present SIINFEKL by flow cytometry. If PSA protein is required to ameliorate SIINFEKL presentation in cells treated with PSA-UTR, then cells transfected

with PSA-ORF plasmid should present SIINFEKL as well as mTOP control siRNA since mRNA from the PSA-ORF is not a target of PSA-UTR siRNA.

Compensatory overexpression of PSA from PSA-ORF was confirmed by real-time PCR (Fig 34A), but it did not ameliorate the reduction in K^b+SIINFEKL presentation caused by transfection with PSA-UTR siRNA (Fig 34B), suggesting that the reduction in K^b+SIINFEKL presentation in COS-K^b cells, and the increased MHC class I presentation in HeLa-K^b cells are due to unrelated effects of PSA#2, rather than the specific gene-silencing capabilities of this siRNA.

The fact that PSA#1 siRNA was as effective at silencing PSA gene expression as the others, but did not demonstrate a phenotype, suggests that PSA does not play an essential role in MHC class I presentation (at least in HeLa-K^b and COS-K^b cells). However, because the reliability of the assay was brought into question, this conclusion can not be validated using only siRNA.

3.3.3 Generation of PSA-deficient mice

PSA-deficient mice were generated using VelociGene® technology (164) resulting in a loss of exon 4 through exon 9 of the PSA genomic sequence (Fig 35A). In total, approximately 18.1 kb was deleted. Presence of the neogene and loss of the PSA exons 4-9 was confirmed by PCR (Fig 35B). This deletion includes exon 9, which encodes half of the active site.

In contrast to previous reports (184, 185), we find that PSA-deficient mice can reproduce but are subfertile. At weaning, PSA-deficient mice are smaller than wild-type

and heterozygote littermates (Fig 35C), suggesting that PSA is required for normal growth. As adult breeders, PSA-/- mice delivered an average litter size from of 3.27 pups, whereas heterozygous breeding pairs delivered an average of 6.28 pups per litter suggesting that PSA-/- mice are subfertile (Table 4). Of the mice born to PSA+/- breeding pairs, only 14.1% were PSA-/- which is less than normal Mendelian ratios, and indicates that PSA-/- mice are subviable as embryos (Table 4).

3.3.4 Dendritic cells express more MHC class I in PSA-deficient mice

Elimination of PSA could in principle reduce peptide supply to MHC class I (if PSA predominately trims peptides longer than 8-10 amino acids) or increase peptide supply (if PSA preferentially degrades peptides 8 to10 amino acids in length that can bind to MHC class I). We therefore evaluated the expression of MHC class I on lymphocytes in the PSA-deficient animals. There were no significant differences in surface levels of MHC class I on CTLs (CD8+, B220-), T-helper cells (CD4+, B220-), or B-cells (B220+, CD4- and CD8-) isolated from spleen and lymph nodes of PSA-/- and control mice (Fig 36 and data not shown). However, splenic CD11c+ dendritic cells from PSA-/- mice express more MHC class I than wild type cells. The magnitude of this effect was small (11-25% increase) but was reproducible and statistically significant ($p<0.05$). In contrast, the expression of MHC class II molecules was equivalent on dendritic cells from control and PSA-deficient mice. These results suggest that in dendritic cells, PSA normally destroys a small fraction of peptides that could otherwise be presented on MHC class I.

3.3.5 The CD8+ T-cell response to viral epitopes is not altered in PSA-/- mice

To more specifically examine effects of PSA knockout on MHC class I antigen presentation, we analyzed the response of PSA -/- mice to various viral infections. PSA mice were infected with LCMV (Fig 37A), VSV (Fig 37B), rVV+OVA (Fig 37C), or Sendai (Fig 37D), and the frequencies of CTL specific for viral peptides was analyzed by intracellular cytokine staining.

PSA-deficient mice mounted comparable CTL responses to all the viral epitopes tested a total of 9 peptides, including the VSV nucleoprotein epitope and the Sendai nucleoprotein epitope, which have both been reported to be substrates for purified PSA (59, 60), suggesting that PSA is not required for presentation of these viral peptides *in vivo*. Therefore, although PSA may destroy some potential MHC class I peptides in DC's, this does not affect the ability of PSA-/- mice to mount a normal CTL response to many viral epitopes epitopes.

3.3.6 PSA -/- MEFs present peptide as well as wild type MEFs

To analyze antigen presentation in more detail, we generated PSA-deficient or control mouse embryonic fibroblast (MEF) cell lines as previously described. These cells do not express truncated forms of PSA mRNA, as determined by real-time PCR for transcripts encoding exons 10 and 11 (downstream of the neogene insertion) suggesting that they do not express truncated forms of PSA mRNA that could be translated in to

functional protein (Fig 38A). Surface levels of H-2K^b, H-2D^b, and total MHC class I were consistently higher in PSA-deficient MEFs, but this difference rarely reached statistical significance (Fig 38B), indicating that PSA may not be essential for the generation of MHC class I-presented peptides in these cells. LAP, BH, and ERAP1 expression was somewhat variable in these MEFs as determined by real-time PCR, but increases were not limited to the PSA MEFs, indicating that these enzymes are not upregulated to compensate for the loss of PSA (Fig 39).

However, MEFs express very low levels of cell-surface MHC class I, and peptide may not be limiting for cell surface expression of MHC class I in these cells. To more closely examine the processing of specific peptide precursors in PSA-/- cells, MEFs were transfected with a series of constructs encoding N-extended SIINFEKL precursors fused to the C terminus of ubiquitin, so that ubiquitin C-terminal hydrolases cleave the SIINFEKL precursor from ubiquitin (9, 163). Two series of N-terminal extensions were generated in order to address the possibility that PSA is important for trimming some sequences, but not others. First, the natural upstream sequence of SIINFEKL was used since it is well characterized and is known to be trimmed in live cells (39) (Table 3). Second, the sequence upstream of the VSV nucleoprotein epitope was used, since this sequence has previously been shown to be trimmed by PSA in cell lysates (60) (Table 3). Full length OVA was used because it is a substrate for the proteasome and would generate an array of N-extended peptides that might be dependent on PSA for trimming (Table 3).

MEFs were transfected with the constructs listed in Table 3 and analyzed by flow cytometry after 48 hrs (Fig 40). Cells expressing similar levels of GFP were compared for SIINFEKL presentation. No statistically significant difference between PSA-/- or wild-type MEFs was detected in the presentation of SIINFEKL, whether the peptide was preceded by the natural upstream sequence or by the VSV-NP sequence (Fig 40). This suggests that PSA is not required for trimming the VSV-NP upstream sequence, and does not play an essential role in generating MHC class I peptides *in vivo* either from peptide precursors or full length protein. In addition, the fact that there was no difference in presentation of the SIINFEKL minigene construct suggests that PSA does not destroy MHC class I peptides either, since any degradation of this peptide would result in reduced SIINFEKL presentation (Fig 40).

3.3.7 Lack of PSA has no effect on the rate of peptide trimming in cells

MHC class I surface expression is an indirect measure of the generation of antigenic peptides. It is possible that a contribution of PSA to this process could be missed if the trimming of peptides is not a rate-limiting step in the pathway. We sent the PSA-/- MEFs (and wild type controls) to Joost Neijssen and Jacques Neefjes so that they could measure the rate of peptide degradation in these cells by microinjection of peptides containing fluorescein and quencher adducts (28, 54). These substrates generate a fluorescent signal when aminopeptidases cleave and thereby separate the residues

containing the fluorophore and quencher moieties. Peptides with different amino acids in the P1 position were microinjected into cells, and the fluorescence resulting from trimming the peptides was measured.

Although there was some variation in the rate of trimming of different peptide sequences (particularly with Pro, Gly, and Asp) (Fig 41), there was no statistically significant difference in the half-life of any of the tested peptides between the two cell lines (Fig 41B). These data demonstrate that PSA activity does not influence the rate of peptide trimming in the cytosol.

CHAPTER IV

DISCUSSION

There is considerable evidence that aminopeptidases are important in trimming antigenic precursors for presentation. Proteasomes have been shown *in vitro* (50, 186) and in living cells (142) to generate many N-extended precursors. Other experiments have shown that if N-extended peptide precursors are expressed or injected into cells, they are trimmed and presented by MHC class I on the cell surface (28, 39). Processing of such peptides can occur in the presence of proteasome inhibitors (35, 39, 187), indicating that other peptidases in the cell can cleave N-terminal residues. Aminopeptidases can trim N-extended precursors that have a free amino terminus, but not if the N-terminus is blocked (28, 51). And eliminating or inhibiting aminopeptidases in cells reduces antigen presentation (54, 60, 61, 142). Together, these data suggest that N-extended precursors are generated during protein degradation and then trimmed by aminopeptidases.

N-extended peptides that are preceded by an ER-localizing signal sequence are efficiently trimmed to mature epitopes in TAP-deficient cells, demonstrating that aminopeptidases localized in the ER can generate peptides for presentation (39, 144, 188). In cells lacking ERAP1, N-extended peptides targeted to the ER are not presented as well (141-143) indicating that ERAP1 plays an important role in trimming in the ER. However, several lines of evidence indicate that N-extended peptides can also be trimmed in the cytosol prior to their transport into the ER. First, cytosolic N-extended

SIINFEKL precursors expressed in ERAP1-deficient cells are trimmed and presented (albeit in reduced amounts), whereas when the same constructs are targeted to the ER, SIINFEKL is not presented (142). Second, peptides preceded by N-extensions of up to 25 amino acids are too long to be efficiently transported by TAP, but when expressed in the cytosol, are still trimmed and presented by MHC class I in the absence of proteasome activity (39). Third, cytosolic extracts devoid of ER contaminants can trim N-extended peptides (53, 60). Fourth, treatment of cells with inhibitors of cytosolic aminopeptidases reduces the presentation of some peptides (54, 60). A major unresolved issue in the field is the identity and extent of contribution of the cytosolic aminopeptidases that participate in the generation of MHC class I-presented peptides.

TPPII is a cytosolic peptidase that has been shown to play an important role in trimming peptides that are longer than about 16 residues (54). However, it remains unclear which cytosolic aminopeptidase(s) trims shorter peptides of 15 amino acids or less. It has also been unclear how much redundancy exists among the cytosolic aminopeptidases.

4.1 LAP is not necessary for mouse viability or generation of MHC class I peptides

In this study, I show (i) that LAP is not essential for viability or normal development of mice, and (ii) that LAP does not play an indispensable role in generating

peptides presented by MHC class I under constitutive conditions or after stimulation with IFN.

LAP is a cytosolic aminopeptidase that was thought to be a major contributor to the MHC class I peptide pool for a number of reasons. First, it was shown to be a major peptidase in cytosolic extracts that generated SIINFEKL from N-extended precursors. Second, transcription of LAP is inducible by both type-I and type-II IFNs. Since IFNs enhance MHC class I presentation and upregulate most components of this pathway, the IFN induction of LAP suggested it might have a particularly important role during inflammation. Third, overexpression of LAP results in a shorter half-life of peptides in living cells (28). Given these findings, our present results are very surprising.

We have found no detectable defect in the ability of LAP-deficient mice to generate or present MHC class I peptides, even after treatment with the type-I IFN inducer polyI:C. In these experiments, we have measured the presentation of a number of peptides to CTL's, including the antigenic model epitope SIINFEKL, which is a well characterized substrate of LAP. No qualitative or quantitative difference was detected in the priming of CTL responses to any of these epitopes in LAP-deficient mice. In addition, we have shown in MEFs and in HeLa cells, that LAP was not required for the trimming of peptide precursors from various N-extended SIINFEKL constructs, even in the presence of IFNγ.

One possibility is that in the absence of LAP, peptides that would normally be trimmed into the cytosol are transported into the ER (either in their original form, or as an intermediate precursor that has been partially trimmed by other cytosolic

aminopeptidases). ERAP1 in the ER can rapidly process N-extended SIINFEKL precursors to SIINFEKL (142), and it was possible that even if LAP deficiency led to an increased amount of N-extended precursors entering the ER that ERAP1 activity could mask this effect. However, although reducing ERAP1 dramatically reduced the presentation of SIINFEKL generated from these peptides, there was no further reduction seen when LAP was also knocked down (Fig 12). Therefore, ERAP1 is not masking a contribution of LAP to trimming precursor peptides.

Although we examined a number of different peptides in this study, it is still possible that a subset of peptides exists for which LAP is required for presentation. LAP has been shown to hydrolyze different amino acid residues (from amino-acid-AMC substrates) at different rates, so LAP may potentially play a more dominant role in trimming certain sequences. However, when peptides with different N-terminal residues were injected into LAP-deficient HeLa cells no difference in peptide half-life was detected, even after stimulation with IFNγ. Moreover, LAP-deficient mouse cells express normal levels of MHC class I indicating that the overall supply of peptides to MHC class I is not changed. Therefore, if there are LAP-dependent epitopes, they must constitute a minority of peptides.

Together these findings demonstrate that LAP does not play an essential role in producing the majority of peptides for MHC class I presentation. The fact that SIINFEKL was presented from N-extended precursors, even in LAP + ERAP1 double-deficient cells indicates that there are other cytosolic aminopeptidases that can substitute for LAP. Although it is possible that other aminopeptidases could be upregulated to

compensate for the absence of LAP, we have not found any consistent increase in BH, PSA, and ERAP1 expression that is correlated with the loss of LAP, in the MEFs (Fig 5). Although BH and PSA had slightly higher expression in LAP-KD cells (Fig 9), this may be due to clonal variation or a difference in β-actin expression between these two cell lines, and is likely not responsible for the lack of an effect in these cells, particularly under conditions of IFNγ induction since BH and PSA expression is not sensitive to IFN. In any case, our data indicate that there must be considerable redundancy in the trimming function of cytosolic aminopeptidases *in vivo*.

It is possible that the contribution of LAP to peptide supply is not apparent because the trimming step is not rate limiting. This would explain why peptides with one, two, or three extra residues on the N-terminus are presented with similar kinetics to one another and to the mature epitope itself, at least in some systems (39). Under these conditions, other aminopeptidases would be sufficient to trim antigenic precursors to mature epitopes.

Surprisingly, we also find no evidence that LAP destroys mature epitopes, thereby limiting their presentation. There was no increase in peptide supply in the absence of LAP. It is possible that this is because LAP destroys as many peptides as it produces, however our data argue against such a possibility. First, LAP-deficient cells show no defect in generating and presenting the mature SIINFEKL epitope from the ubiquitin-SIINFEKL fusion construct, even though trimming of just one residue would generate IINFEKL, which is too short to bind H-2K^b (Fig. 6). Second, the hydrolysis rates of individually injected peptides are not different in LAP-KD cells. For these reasons, we

believe that LAP does not play a major role in the destruction of antigenic peptides under physiological conditions. However, it should be noted that when LAP is expressed at supraphysiological levels, peptides are hydrolyzed more rapidly in cells (28).

However the question remains, if LAP is not essential for antigen presentation then why is it inducible by IFN? It is known that IFN stimulation upregulates many components of the antigen processing pathway including the immunoproteasome. Immunoproteasomes can generate distinct MHC class I presented peptides that are not produced by the constitutive proteasome (189-192). In addition, the immunoproteasome has also been shown to generate longer peptides than the constitutive proteasome, for at least one antigen (50) and there is indirect evidence that suggests that this might be generally true (142). It is possible that in some cell types the immunoproteasome generates antigenic precursors that are more efficiently processed by IFN-inducible aminopeptidases like LAP. Alternatively, it is possible that LAP is more essential in a pathway not involved in antigen presentation such as the breakdown of some other component involved in an immune response, or in the breakdown of viral peptides during infection. The LAP deficient mice and cells should be useful in future studies to help answer these questions. In addition, further investigations into the role of other cytosolic aminopeptidases will help to define the redundancy and/or unique roles of these various peptidases relative to LAP.

4.2 BH is not essential for the generation of MHC class I peptides

BH is a cytosolic cysteine protease that was thought to contribute to MHC class I peptide presentation for two main reasons. First, it was identified along with PSA as a major trimming activity of the VSV-NP epitope in living cells and cell lysates treated with proteasome inhibitors (60). Second, the cysteine protease inhibitor E-64 was shown to inhibit the presentation of an MCMV epitope precursor to CTLs in combination with a proteasome inhibitor (104).

Initially, BH was targeted by siRNA in order to determine its role in living cells. Unfortunately, I have found that certain siRNAs can have side-effects in addition to their ability to silence gene expression, and that these side effects can cloud any discernable effect on MHC class I presentation. siRNA can induce type-I interferon expression by signaling through TLR3 in a sequence independent fashion (173). By real-time PCR, one siRNA targeting BH (BH#2) was found to induce ISG15 to levels similar to Luc1, an siRNA previously reported to induce IFN effects. By flow cytometry, Luc1 and BH#2 siRNAs increased overall MHC class I presentation, and reduced levels of SIINFEKL presentation from peptide precursors. Because these phenotypes are shared between siRNAs that do and do not reduce BH mRNA, it is difficult to attribute them to the reduction of BH alone. In addition, another BH siRNA targeting the 3' UTR induced similar phenotypes that could not be rescued by overexpression of the BH open reading frame (ORF) despite the increased levels of BH expression. This suggests that the

reduction of BH protein was not the main contributing factor to the overall increase in MHC class I presentation, or the reduction of presentation of SIINFEKL from peptide precursors.

Although these findings have little to do with the role of BH in antigen presentation, it is important to notice since there are a continuously growing number of reports that use RNAi to study intracellular and even intercellular processes that can be affected and altered by type-I IFN. RNAi is certainly a powerful tool which can generate good preliminary data, however it is not without its drawbacks and limitations.

With preliminary data from siRNA studies, and knowing that a BH knock-out mouse had already been generated, we examined antigen presentation in BH-deficient MEFs that were generated for this purpose. Overall presentation of MHC class I was the same in BH-deficient MEFs and control MEFs, suggesting that BH does not play an essential role in generating the majority of peptides for MHC class I in these cells. In addition, BH-deficient MEFs show no defect in presenting SIINFEKL from peptide precursors containing the natural upstream sequence of SIINFEKL.

Since flanking sequences have been shown to influence presentation of particular sequences, and because BH was specifically shown to trim the N-terminal sequence of the VSV NP epitope, I examined the presentation of SIINFEKL when it was preceded by the upstream sequence of the VSV-NP epitope. The lack of a defect in the presentation of SIINFEKL from these precursors suggest that BH is not essential for trimming the VSV NP sequence, which is not really surprising since purified PSA has also been shown to trim this sequence (60), suggesting redundancy between these two aminopeptidases.

However, it was possible that the redundancy between BH and PSA was limited to the trimming of a few particular sequences. In addition, one could envision a greater role for specific aminopeptidases in specific cell types, such as professional APCs and other immune cells.

For these reasons, BH-deficient mice were examined for their ability to present MHC class I. Total numbers and percentages of B-cells, CTLs, and CD4 T-cells appeared normal in the spleen and the lymph nodes of BH-deficient mice. This suggests that BH is not required for T-cell generation in the thymus, or maintenance in the periphery. BH knock-out mice also have normal numbers of CD11c+ dendritic cells, which present MHC class I and MHC class II as well as wild type, suggesting that it is not required for DC maturation, or antigen presentation by DC's.

We found no defect in the ability of BH-deficient mice to respond to a number of viral epitopes. These viral peptides represent a wide array of sequences, therefore these findings suggest that BH is generally not required for the trimming of specific sequences. However, the tested viral peptides are only a small portion of a comprehensive list, and it is possible that BH may play an essential role in generating a minor population of peptides. Reducing presentation of a minor population of peptides may be compensated by a reciprocal increase in presentation of peptides that are not normally presented abundantly. Such a change would not be detectable by flow cytometry.

The ability of BH to degrade and/or trim short peptides may be of physiological significance, but it is clear that BH is not an essential gene. This is manifest in the viability of BH-deficient mice, and in the ability of BH-deficient mice to mount a normal

immune response to viral infection from a number of viruses. Theoretically, it is possible that BH only degrades peptides in order to recycle amino acids, but sufficient redundancy exists to allow viability in the absence of BH which argues against an essential role for BH in this process. Alternatively, because BH is a cytosolic aminopeptidase, it is possible that it generates some peptides in the cytosol, but trimming activities in the ER compensate for the loss of BH in the cytosol. If this is true, then a deficiency in BH could be eclipsed by the function of ER-trimming activities. This speaks to the redundancy of the system. Since aminopeptidases are present on both sides of the ER membrane, a much clearer picture will arise when suitable reagents are available (i.e. BH/ERAP1 double-deficient mice).

4.3 PSA is not essential for the generation of MHC class I peptides

In this study, I show (i) that PSA does not play an indispensable role in generating peptides presented by MHC class I, and (ii) that PSA is not essential for reproduction in male or female mice, which contradicts previous reports (184, 185).

PSA is a cytosolic metalloprotease that is thought to influence the MHC class I peptide pool for a number of reasons. First, it was identified along with BH as a major trimming activity of the VSV-NP epitope in living cells and cell lysates treated with proteasome inhibitors (60). Second, an analysis of cell lysates and purified enzymes suggested that PSA degrades the Sendai NP epitope better than any other peptidase (59).

Third, presentation of the RU1 peptide from N-extended precursors was suggested to be dependent on PSA, in tandem with TPPII (61). Fourth, Puromycin can inhibit the trimming of some, but not all peptides (59), indicating that PSA may preferentially trim/degrade some peptide sequences. Together, these data implicate PSA in both destructive and constructive processes in MHC class I peptide generation.

Because these previous studies lacked proper controls, or focused on the use of aminopeptidase inhibitors, the role of PSA as an individual aminopeptidase in peptide generation was still unclear. To specifically study the role of PSA, siRNAs were designed that target the PSA open reading frame (ORF). Two siRNAs were shown by real-time PCR to be equally effective in reducing PSA mRNA, but resulted in very different effects on MHC class I presentation and SIINFEKL presentation from N-extended precursors. In contrast to BH, these phenotypic differences did not appear to be attributable to type-I IFN effects.

In an effort to determine which of the siRNA phenotypes was correct, another siRNA was designed which targeted the 3' UTR of endogenous PSA mRNA. This siRNA was similar to PSA#2 in that it reduced PSA mRNA, caused an increase in overall MHC class I, and a decrease in SIINFEKL presentation. However, these changes in MHC class I presentation could not be attributed to PSA knockdown since overexpression of the PSA ORF (which is not targeted by PSA-UTR) did not ameliorate the phenotypes. Because the phenotypes are not related to the reduction of PSA, or type I IFN effects, they must be due to other effects attributable to the siRNA sequence. It is important to point out that PSA #1 siRNA reduced PSA mRNA, but did not affect MHC

class I or SIINFEKL presentation, which suggests that PSA is largely redundant in MHC class I peptide generation, and correlates with my findings with PSA-deficient mice and PSA-deficient MEFs.

But how does PSA#2 influence MHC class I presentation if not by IFN-induction? Two main possibilities exist. First, it is possible that PSA#2 influences MHC class I through an unknown pathway that is independent of IFN stimulation and off-target silencing effects (discussed below). Such a pathway would be independent of TLR3 and PKR activation, since these ultimately lead to IFN expression. In this scenario, a protein that binds short double-stranded RNA molecules might directly or indirectly influence (i.e. increase) the levels of ER chaperoning, and/or protein degradation. This hypothetical dsRNA-binding protein could act as 1) a chaperone that binds and protects the antigenic peptides, 2) a transcription factor that increases transcription of proteins involved in MHC class I processing, or 3) a cofactor that alters proteasome function or TAP selectivity.

However, data presented here would indicate that the pathway that allows PSA#2 to have a different effect on MHC class I presentation would also allow a reduction in non-homologous mRNA levels (Fig 31). And these data would be more easily explained by the reported the off-target silencing effects of some siRNAs. There are two possible mechanisms for how such off-target silencing may occur. First, siRNAs can act as microRNAs (miRNAs) by binding sequences that share less than 100% homology, and sequestering them to prevent translation (182, 193). Such a microRNA (miRNA) mechanism may require as few as 8 nucleotides of homology (194).

The second mechanism by which siRNAs can mediate off-target mRNA silencing is binding mRNAs with high, but not 100% homology, and then targeting them for destruction. siRNAs have been shown to target and destroy mRNAs with as many as four mismatched nucleotides (195). The apparent promiscuity of siRNA gene-silencing suggests that there is a much larger number of mRNA sequences that could be silenced by any given siRNA sequence. And in reference to the work presented here, it implies that the siRNA PSA#2 may have another (or multiple other) mRNA targets that code for proteins directly or indirectly involved in MHC class I antigen presentation, possibly even mRNAs encoding another protease. Such possibilities made it essential to examine the role of PSA through a different experimental system, therefore PSA-deficient mice were generated.

PSA-deficient MEFs were generated in order to specifically examine the influence of PSA on antigen presentation in non-lymphoid cells. Overall MHC class I presentation is normal in these cells, which suggests that PSA is not essential for the generation of MHC class I peptides. Transfection of MEFs with a construct encoding SIINFEKL suggested that PSA does not degrade this epitope prior to presentation. This data is in agreement with previous work indicating that PSA does not effectively degrade the SIINFEKL epitope (59). Microinjection of PSA MEFs with fluorescent peptides shows no difference in the half-life of these peptides (data not shown), which also corroborates evidence that PSA does not contribute to the degradation of cytoplasmic peptides.

However studies with other epitopes have suggested that PSA trims the VSV NP epitope and epitope precursors (60), and is required for trimming some N-extended precursors of RU1 peptide presumably due to length and sequence (61). While both these studies demonstrate that PSA trimming activity may be redundant for some precursors and peptides, the latter study suggests that PSA is required for the generation of epitopes from some peptide precursors (61).

Our studies suggest that PSA is not required for trimming peptide precursors when they are preceded by the natural OVA sequence, or the VSV NP upstream sequence. These findings may not be surprising since purified LAP has been shown to trim QLESIINFEKL (53), and the VSV NP upstream sequence can be trimmed by purified BH (60). However, these findings unambiguously extend previous work into living cells without the use of aminopeptidase inhibitors, and suggest that there is sufficient redundancy in the cytoplasm to trim even very long precursors (up to 16-mers) in the absence of PSA. It also demonstrates that hydrophobic and basic residues thought to be preferred by PSA (103) can be adequately trimmed from peptides by other aminopeptidases in living cells. Measurements of the half-life of microinjected peptides into BH MEFs also support these conclusions (data not shown).

All splenic cell populations were present at normal frequencies in PSA-deficient mice. And except for DC's, all splenic lymphoid cells presented normal levels of MHC class I. Both CD8+ and CD8- DC's expressed more H-2K^b, and H-2D^b than wild type, however this increase in MHC class I presentation did not influence the activation of

CTLs in response to a variety of viral epitopes as measured by intracellular cytokine staining.

In summary, PSA is not essential for the general trimming or degradation of MHC class I peptides in mice. Some data from siRNA experiments suggests that the same is true in human cells as well.

Defining the exact amount of redundancy in aminopeptidases is necessary in order to determine which aminopeptidases are involved in generating MHC class I peptides in living organisms. Double knock-out mice should help in defining the level of redundancy. Such elucidation may help in designing more effective peptide vaccines.

4.4 Other models that might be considered

Heat shock proteins are molecular chaperones expressed in eukaryotic and prokaryotic cells that bind the hydrophobic regions of nascent proteins to aid in proper folding (196). In addition to this role, certain groups have suggested that HSPs may bind peptides that could serve as MHC class I peptides, thereby protecting them from trimming and/or degradation by cytosolic aminopeptidases (197). However, exogenously added HSP-bound peptide precursors have been shown to require proteasomal processing, and transport into the ER by TAP in order to be presented by MHC class I (198), suggesting that HSPs do not protect peptides from these intracellular processes.

Immunoprecipitation of HSP70 and HSP90 followed by elution of bound peptides indicates that antigenic peptides do not associate with these HSPs (199), and HSPs have

not been reported to specifically associate with TAP or the proteasome. These data (or the lack thereof) argue against the idea of HSPs protectively "shuttling" peptides from the proteasome to the TAP complex. In addition, peptides have been shown to be trimmed in cytosolic cell lysates (53) and living cells (28, 54), which presumably include cytosolic HSPs. These data indicate that HSPs do not protect peptides from trimming and/or destruction by aminopeptidases *in vivo*.

However HSP's are not the only proteins suggested to bind peptides *in vivo*. A recent report using a modified SIINFEKL substrate (KOVAK) suggests that instead of binding HSP's, proteolytic peptide intermediates can bind a cytosolic chaperonin TRiC which protects them from degradation by cytosolic proteases (199). However, this report does not describe an association between the TRiC complex and TAP, or TRiC and the proteasome (199). Thus, many of the same arguments can be made against TRiC functioning in peptide binding as in HSP's binding peptides.

Other chaperones that reside in the ER including gp96, calreticulin, and protein disulfide isomerase have also been suggested to protect peptides from degradation and increase presentation of specific epitopes (200). However, because these chaperones reside in the ER, it is unlikely that they play a role in protecting peptides from cytosolic proteolytic events, but possibly may sequester them and prevent them from being retro-translocated into the cytoplasm for degradation.

4.5 Concluding remarks

The redundancy of aminopeptidases is likely to be useful to the cell in some way. It is possible that there is so much redundancy in this system that the elimination of any single aminopeptidase would be insignificant in reference to MHC class I antigen processing. It is currently unknown how much of total aminopeptidase activity in cells is attributable to each individual aminopeptidase. Such studies may be unfeasible since expression levels of aminopeptidases vary in different cell types and under different conditions (53). In addition, the amino acid preferences of aminopeptidases vary such that the use of a single peptide or fluorogenic substrate may lead to an inaccurate conclusion as to the individual contributions of various aminopeptidases to the overall aminopeptidase activity.

The potential redundancy that may exist in aminopeptidase activity is illustrated by a study that measured the rate of degradation of six different antigenic peptides in cell lysates treated with a variety of aminopeptidase inhibitors. Treatment with bestatin was shown to inhibit LAP and PSA, but this inhibition only slightly reduced the degradation rates of six antigenic peptides (59). No reduction in peptide degradation was observed after treatment with E-64 (a cysteine protease inhibitor) or butabindide. Together, these data suggest that there are other aminopeptidases that can degrade these peptides.

Of equal importance is the observation that each peptide in this study was degraded at a different rate, indicating that some sequences are more quickly degraded by aminopeptidases than others (59). This susceptibility may be attributable to the sequence preferences of one or more aminopeptidases.

However, the redundancy in aminopeptidases may be necessary for other cellular processes. Aminopeptidases have recently been suggested to be required for recycling of essential amino acids to make them available for new protein synthesis (4), and aminopeptidase redundancy would ensure optimization of this process. It would also prevent the build-up of proteasomal products, which could interfere with protein-protein interactions or be toxic in other ways. Perhaps it is for these reasons that LAP-deficient, BH-deficient, and PSA-deficient mice are viable although the dwarfism manifested by the BH and PSA mice may suggest a role for these aminopeptidases in general growth rates.

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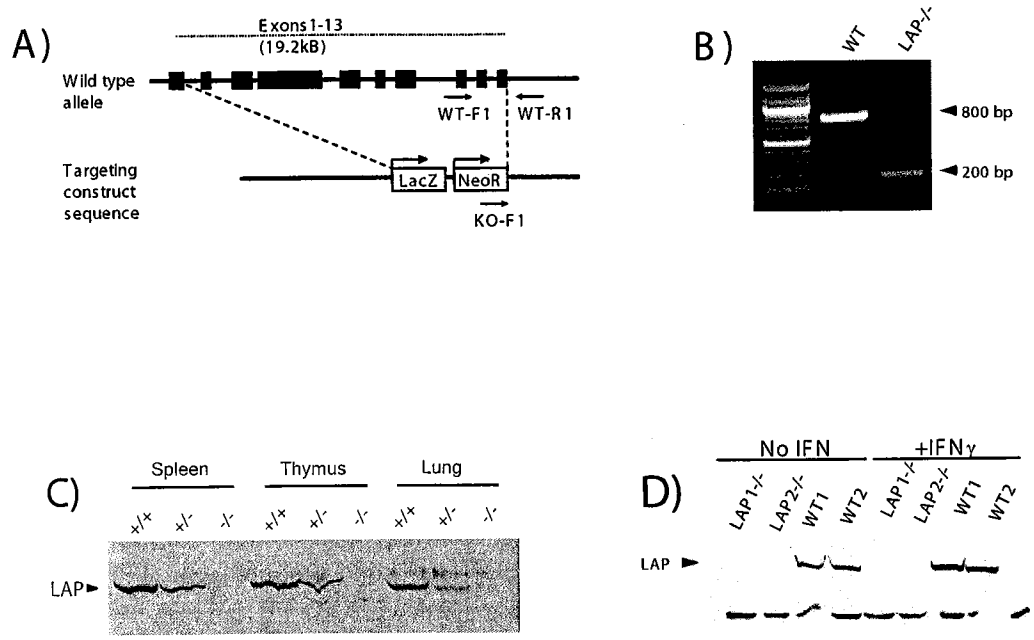


Figure 1 - Generation of LAP-deficient mice. A) Genomic organization of the mouse LAP gene (upper) and structure of the targeting vector (lower). Exons 1-13 are shown as boxes with the coding regions in black. The location of the primer sequences used for PCR geontype analysis are shown with arrows. B) PCR genotype analysis of wild-type and LAP-deficient animals. Amplification of the wild-type allele results in a 800bp fragment, whereas the disrupted allele produces a 200bp fragment. C) Western blot analysis of cell lysates prepared from Mouse Embryonic Fibroblasts (MEFs) with or without IFNγ-treatment.

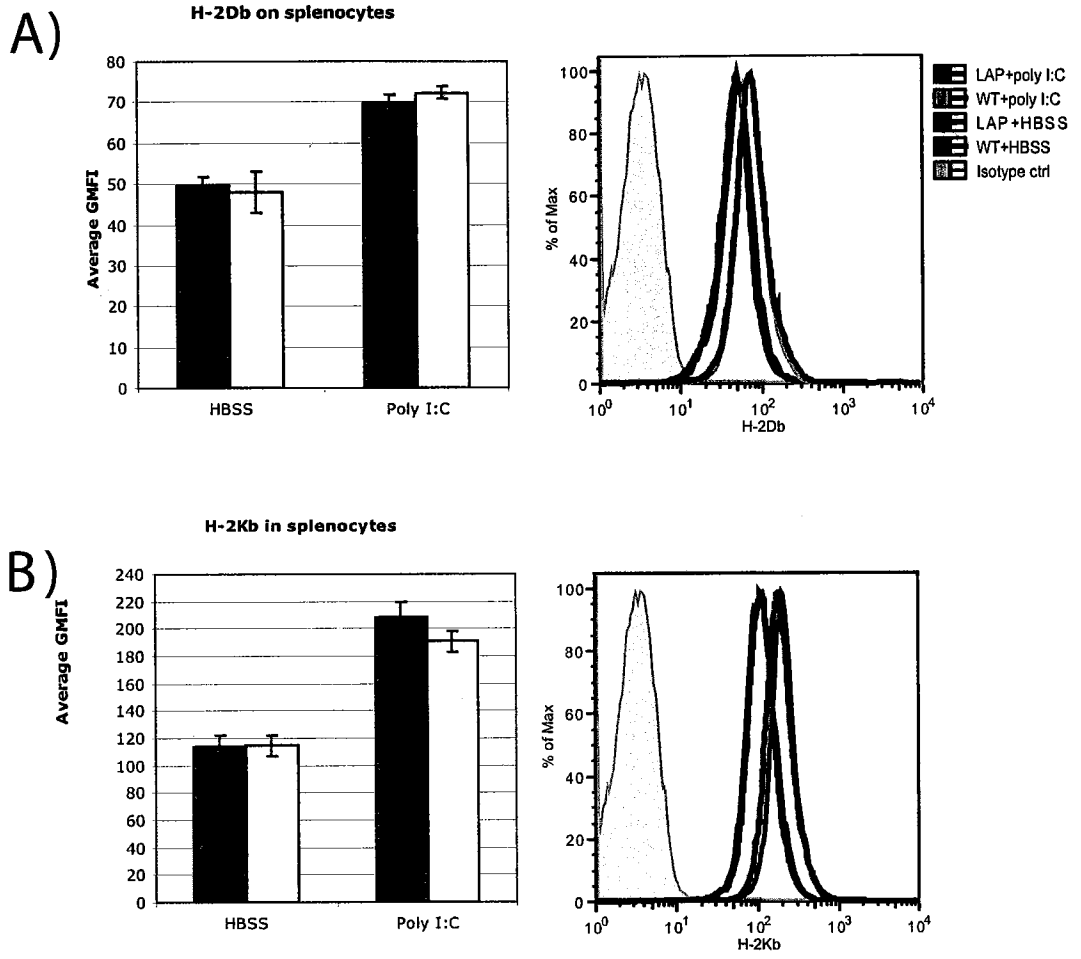


Figure 2 - MHC class I presentation in LAP-deficient and wild-type mice. FACS analysis of A) H-2K^b (AF6-88.5) and B) H-2D^b (KH95) expression on splenocytes 24 hrs after intraperitoneal injection with poly I:C or with Hanks Buffered saline solution (HBSS) as compared to staining with isotype control. Graphs represent the average Geometric Mean Fluorescence (GMFI) of 2 mice (HBSS) or three mice (poly I:C) from each genotype. Gray bars = C57BL/6, and white bars = LAP-/-. Error bars represent the standard deviation within each group. FACS traces represent individual mice treated with HBSS (red = BL6, and blue = LAP-/-), or with poly I:C (green = BL6, black = LAP -/-) and stained for A) H-2D^b, or B) H-2K^b. Data is representative of three independent experiments.

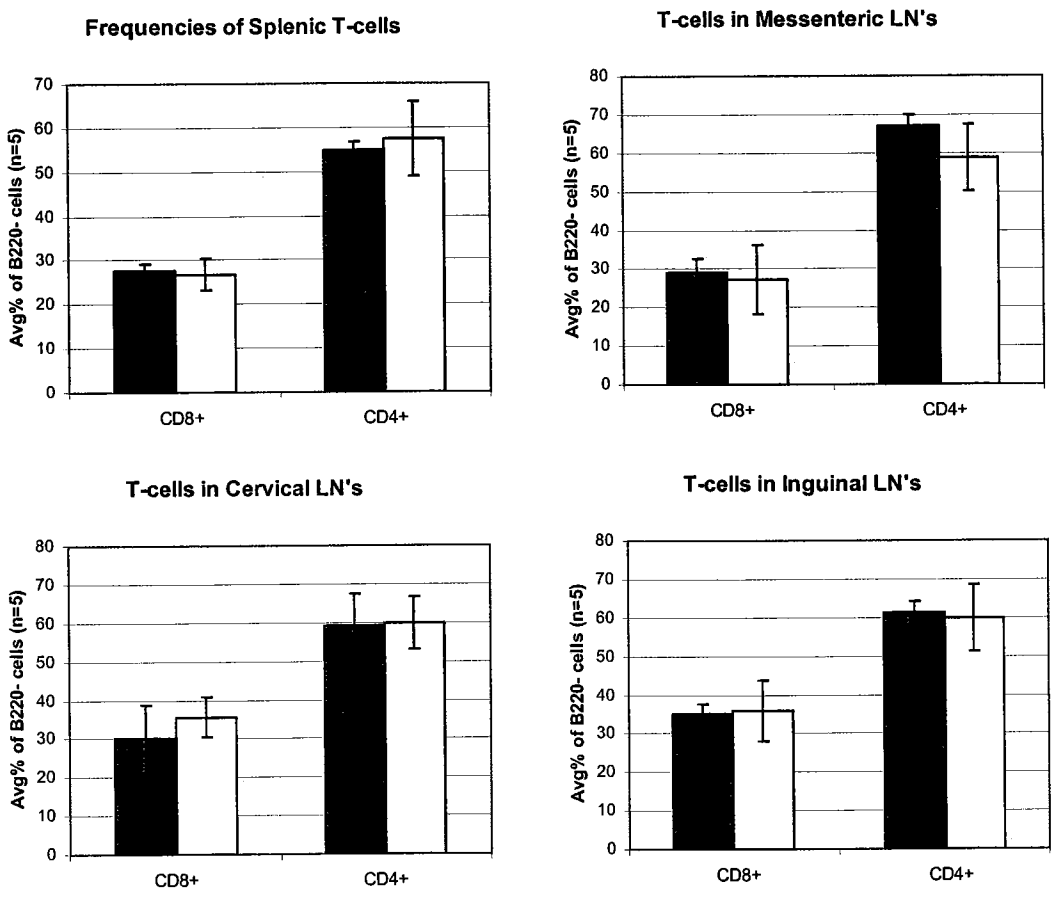


Figure 3 - T-cell frequencies in wild-type and LAP-/- mice. FACS analysis of T-cells from lymph nodes and spleens collected from wild-type and LAP-/- mice. Graphs represent the average % of CD8+, B220- or CD4+, B220- cells present in each lymph node. Five mice were used per genotype (gray bars = C57BL/6, white bars = LAP-/-). Error bars represent the standard deviation within each group.

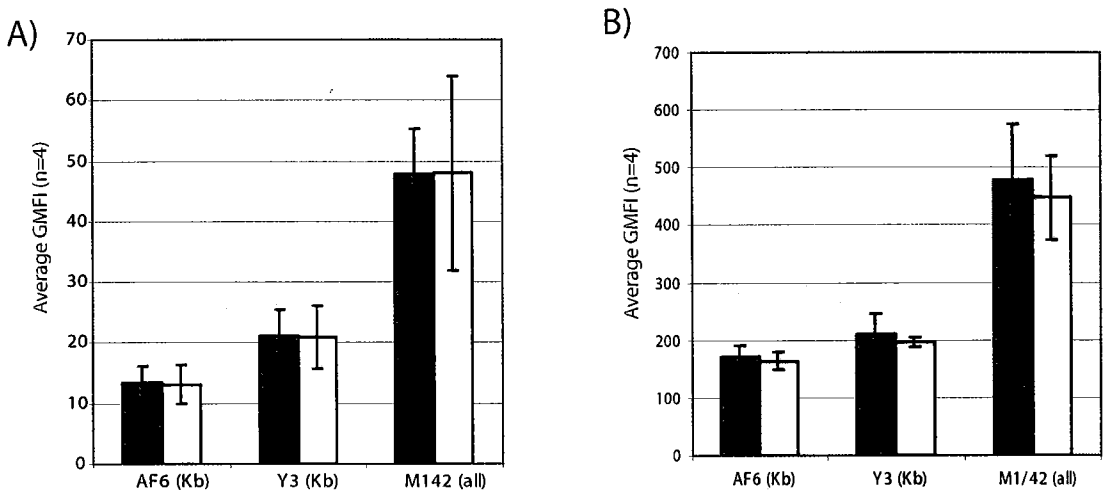


Figure 4 - MHC classs I presentation on Mouse Embryonic Fibroblasts (MEFs). A) MEFs, or B) MEFs treated with IFNγ were analyzed by flow cytometry after staining for H-2K^b with AF6 or Y3 mAbs, or for total MHC class I with the M142 mAb. Error bars represent the average geometric mean fluorscence (GMFI) of four independent MEF lines for each genotype. Gray bars = wild-type MEFs, and white bars = LAP-/- MEFs. Error bars represent standard deviation within each group.

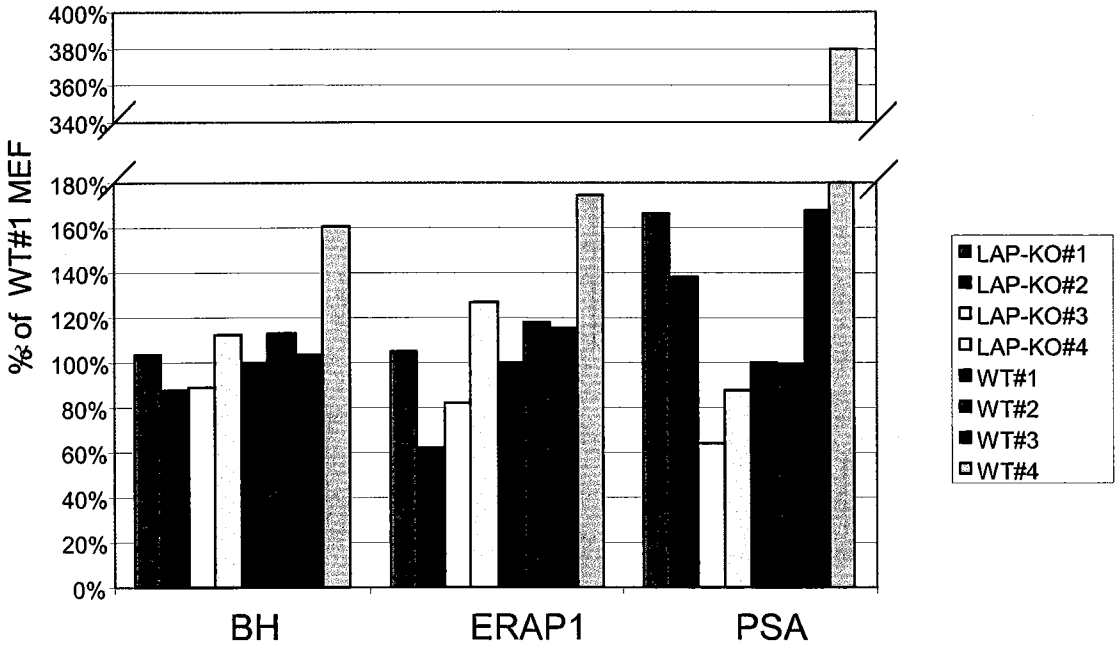


Figure 5 - Real-time PCR analysis of other aminopeptidases in LAP MEFs. 10^6 cells were used from each of four independent MEF lines (from each genotype) to prepare mRNA and cDNA as previously described. Results were normalized to β-actin as previously described. Results are representative of two independent experiments.

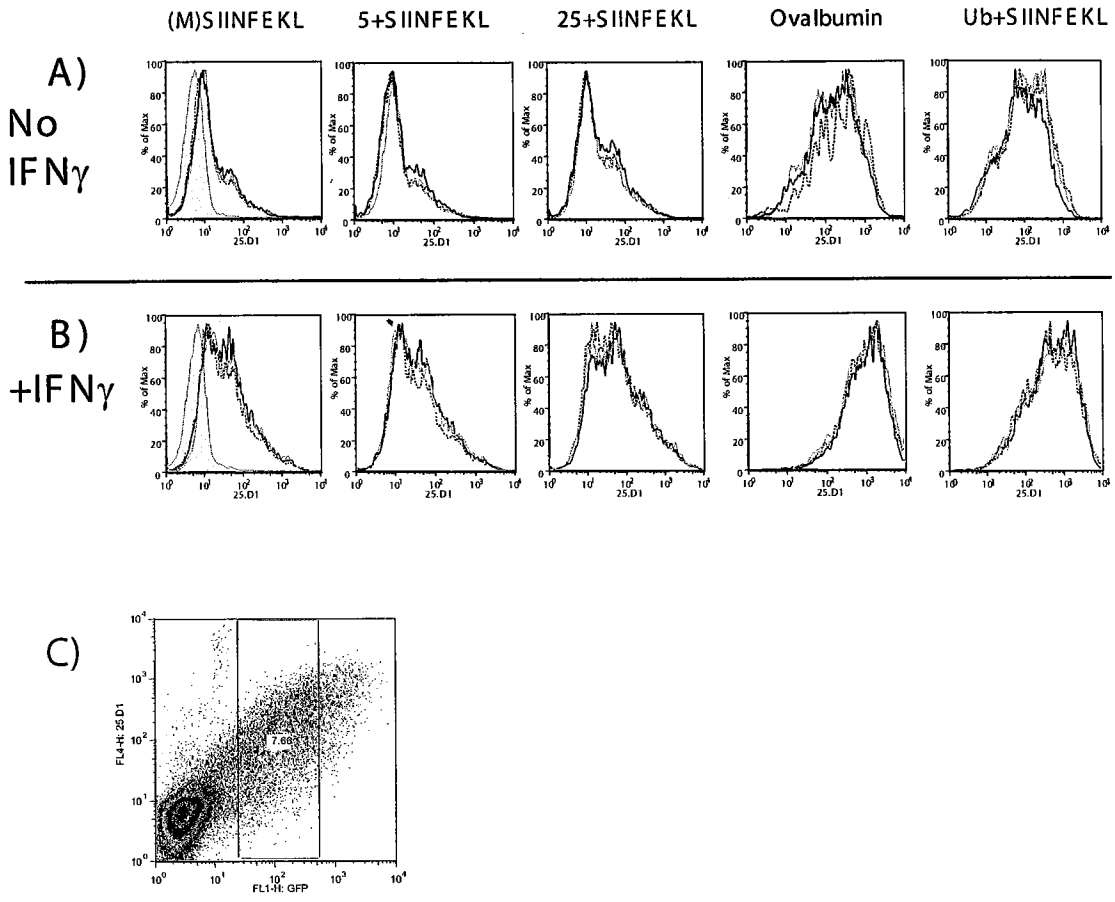
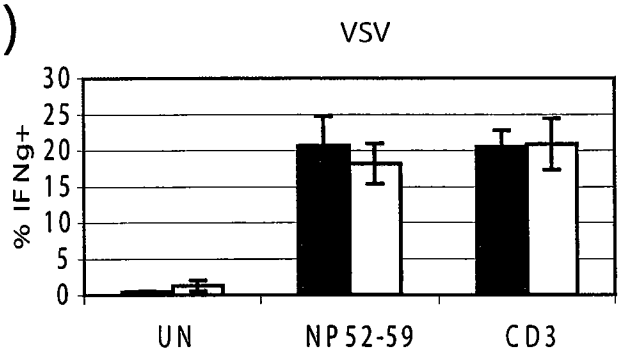
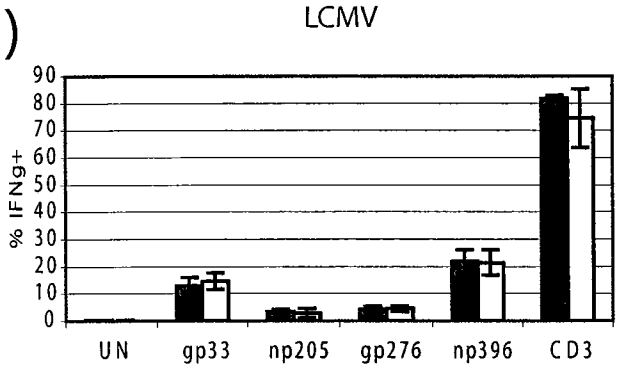


Figure 6 - SIINFEKL presentation by MEFs. 48 hours after transfection with the indicated peptide construct, A) MEFs, or B) MEFs treated with IFNγ were analyzed by flow cytometry. FACS traces represent gated cells expressing comparable amounts of GFP, as shown in C). SIINFEKL presentation by H-2Kb was determined using 25.D1.16. SINFEKL presentation by two independent LAP-deficient MEFs (LAP KO1 & 2, gray line, black dotted line respectively) and wild-type MEFs (black solid line) were compared to background staining (ctrl, gray filled line) with an isotype control antibody.

A)



B)



C)

Vac-OVA	UN	P10	SIINFEKL	anti-CD3
LAP	0.06 ± 0.02	0.19 ± 0.08	4.06 ± 0.81	20.44 ± 2.31
WT	0.07 ± 0.02	0.35 ± 0.16	4.72 ± 1.44	27.60 ± 4.66

Figure 7 - Intracellular IFNγ staining of peptide-specific T-cells. Spleen cells from LAP -/- and wild-type mice infected with virus were harvested and stimulated on, A) day 7 for VSV, B) day 9 for LCMV, or C) day 7 for recombinant vaccinia. After isolation, splenocytes were stimulated for five hours with anti-CD3ε, as a control for CTL viability, or A) VSV peptide NP52-59, B) LCMV peptides gp33, np205, gp276, and np396, or C) vaccinia peptide p10, or with SIINFEKL peptide as described in the materials and methods. They were then surface stained with anti-CD8 and anti-CD44, and intracellularly stained with anti-IFNγ. Graphs and table represent the average percentages of CD8+ T-cells that were IFNγ-positive (n=5 mice). Gray bars = C57BL/6, white bars = LAP-/-. Error bars represent the standard deviation within each group. There was no significant difference between LAP-/- and wild-type mice in their response to any of the epitopes tested.

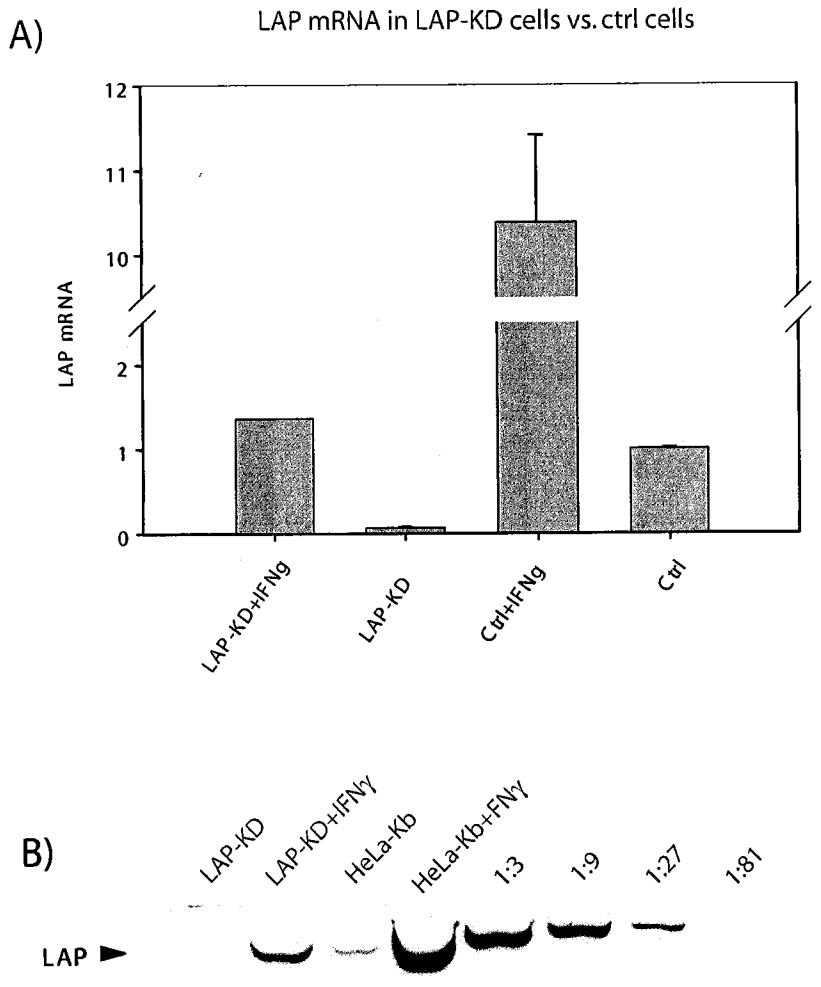


Figure 8 - LAP expression in the LAP-KD clone. A HeLa-Kb cell clone, stably transfected with a shRNA construct targeting LAP (LAP-KD) was compared to control cell lines. A) Results from real-time PCR for LAP mRNA in LAP-KD cells and control cells with or without treatment with IFNγ, all normalized to β-actin mRNA. Data is presented as a percent of total LAP mRNA from the control cell line under constitutive conditions (1 = 100%). Error bars represent variation between triplicate wells. B) A western blot for LAP of LAP-KD cells and the parental cell line with or without treatment with IFNγ. Serial dilutions (1:3) of the HeLa-K^b+IFNγ cell lysate were run on the gel in order to determine relative amounts of LAP protein between samples (lanes 5-8). According to the semi-quantitative immunoblotting and real-time PCR, LAP-KD cells have approximately 90-95% less LAP mRNA and protein, with or without IFNγ treatment.

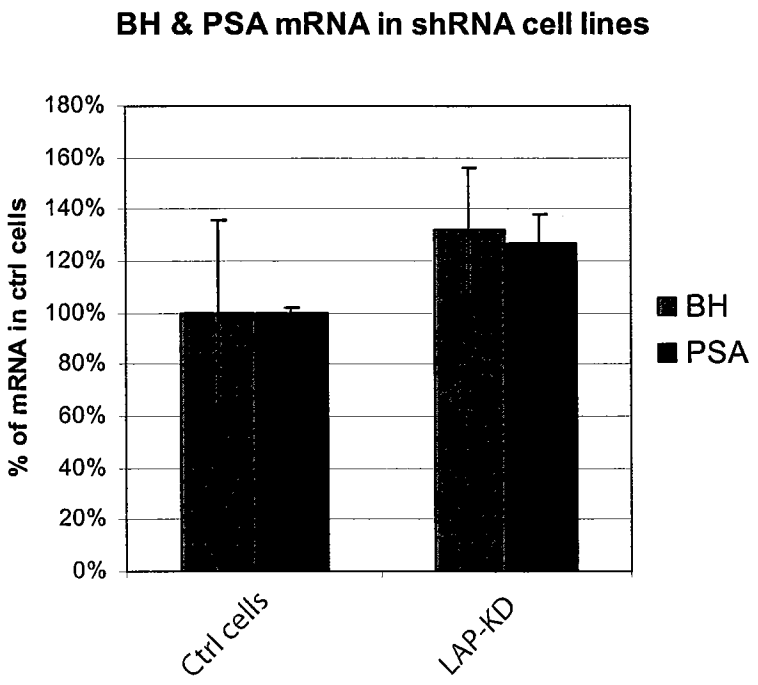


Figure 9 - Real-time PCR analysis of other aminopeptidases in LAP-KD and control cell lines. Cells stably transfected with a non-specific shRNA (Ctrl), or LAP-KD cells were used to collect mRNA for real-time analysis as previously described. Blue bars = levels of BH mRNA, and red = levels of PSA mRNA in both cell lines. All data is normalized to levels of β-actin. Error bars represent standard deviation between duplicate wells. Data is representative of at least two independent experiments.

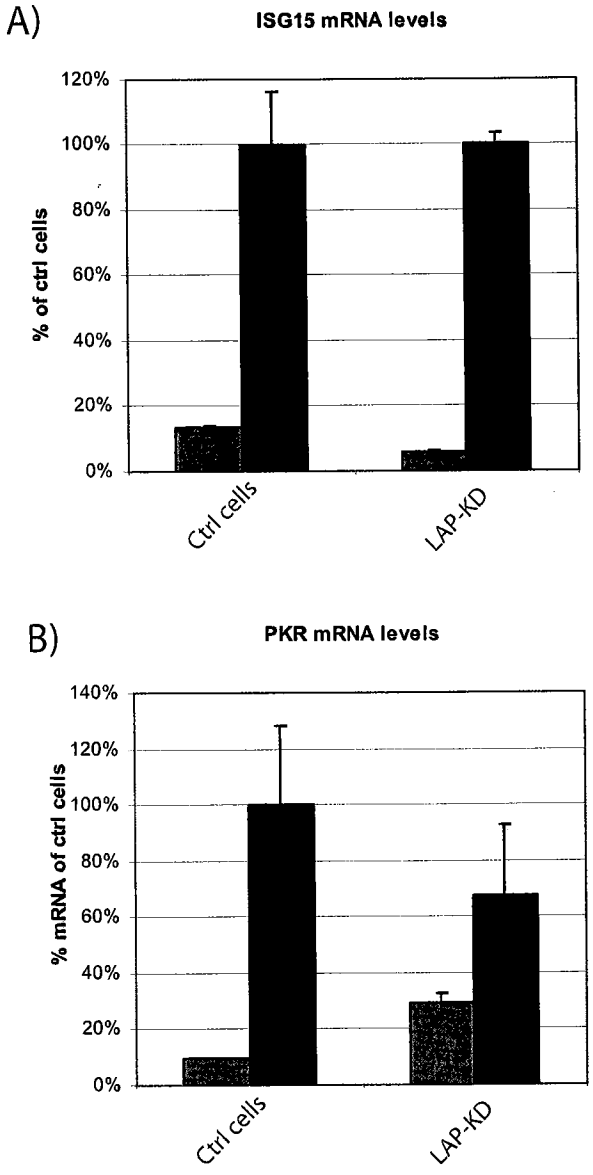


Figure 10 - Real-time PCR analysis of IFN-sensitive genes in LAP-KD and ctrl cells. Cells stably transfected with a non-specific shRNA (Ctrl), or LAP-KD cells were used to collect mRNA for real-time analysis as previously described. Blue bars = levels of (A) ISG15 or (B) PKR mRNA under constitutive growth conditions. Red bars = levels of (A) ISG15 or (B) PKR mRNA after incubation with IFNγ. All data is normalized to levels of β-actin. Error bars represent standard deviation between duplicate wells. Data is representative of at least two independent experiments.

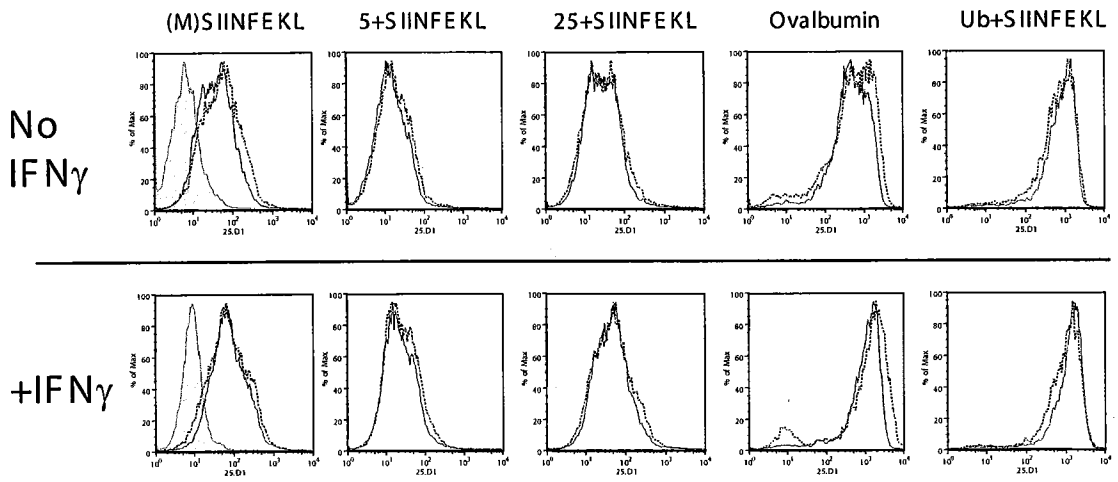


Figure 11 - SIINFEKL presentation by LAP-KD cells. 48 hours after transfection with the indicated peptide construct, A) LAP-KD and control cells, or B) LAP-KD and control cells treated with IFNγ were analyzed by flow cytometry. FACS traces represent gated cells expressing comparable amounts of GFP. SIINFEKL presentation by H-2Kb was determined using 25.D1.16 Ab. SIINFEKL presentation by LAP-KD cells (gray line) and control cells (black dotted line) were compared to background staining (gray filled line) using an isotype control antibody.

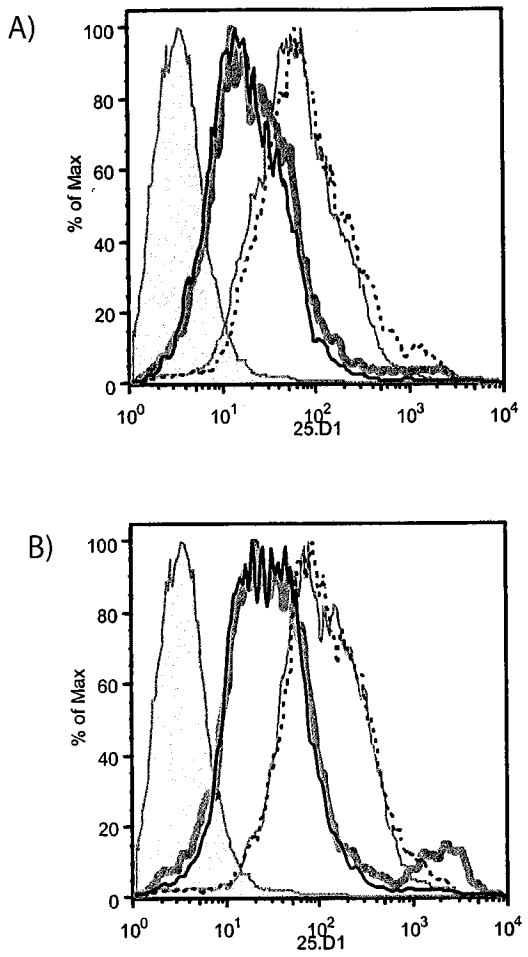


Figure 12 - SIINFEKL presentation on shRNA-stable cells treated with ERAP1 siRNA. LAP-KD or control cells were treated with siRNA targeting ERAP1 or ctrl siRNA. 24 hrs later (day 1), all cells were transfected with N25+SIINFEKL as previously described. On day 3, all cells were analyzed by FACS. For the cells in B) IFNγ was added on day 2. FACS traces represent gated cells expressing comparable amounts of GFP. SIINFEKL presentation by H-2Kb was determined using 25.D1.16 Ab. SIINFEKL presentation by LAP-KD+ctrl siRNA (gray thin line), LAP-KD+ERAP1 siRNA (black solid line), ctrl cells+ctrl siRNA (black dashed line), and ctrl cells+ERAP1 siRNA (gray thick line) were all compared to cells transfected with vector and stained with 25.D1.6 (gray filled). Although reduction of ERAP1 reduced presentation of SIINFEKL, this reduction was not enhanced or decreased by the loss of LAP, either under constitutive conditions (A), or after 24 hrs incubation with IFNγ (B).

A)

Peptide	Hela Kb		LAP-KD	
	t(1/2)	stdv.	t(1/2)	stdv.
PKNKTERCY	2.8	0.7	2.2	0.2
GKNKTERCY	3.2	1.0	2.4	0.5
LKNKTERCY	2.9	0.1	3.1	0.4
DKNKTERCY	2.0	0.4	3.0	0.6
KKNKTERCY	2.2	1.3	3.6	1.5
FKNKTERCY	2.8	0.4	1.9	0.1
KDLGPFKDLGPYCRETKNT	14.5	6.6	7.8	4.1

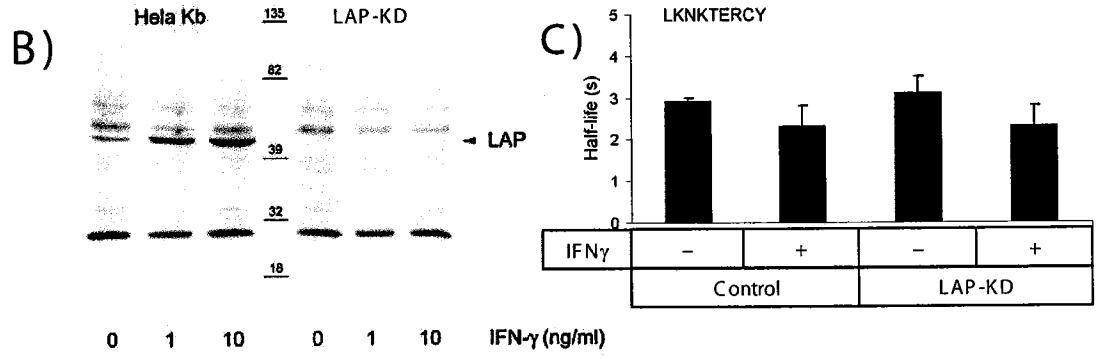


Figure 13 - Half-life of microinjected peptides in LAP-KD cells. A) Peptides with different residues in the P1 position were microinjected into LAP-KD or control cells. Half-life of peptide was determined by following the generation of fluorescence. B) LAP western blot of LAP-KD and control cells after incubation with IFNγ, C) peptidehalf-life measured in seconds of a microinjected fluorescent peptide with leucine in the P1 position, with or without incubation with IFNγ. In all these experiments, no difference in half-life was detected in any of the tested peptides between the two cell lines.

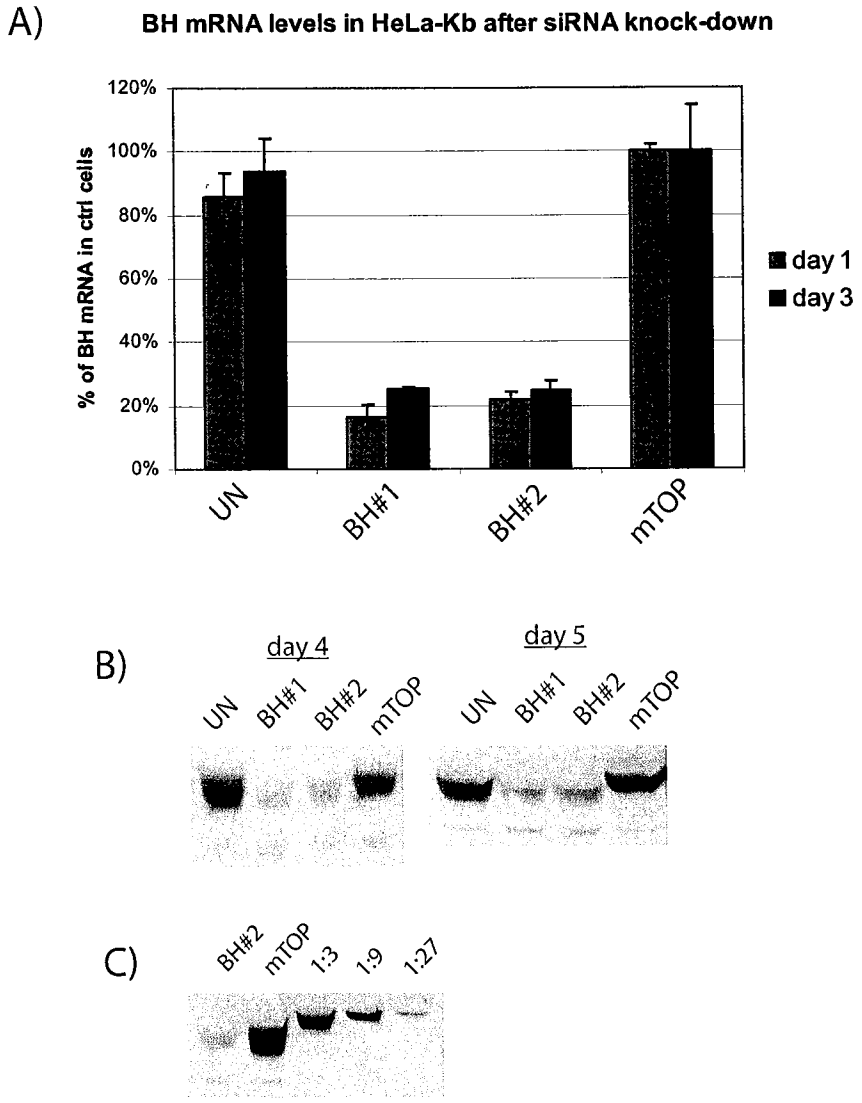


Figure 14 - BH expression in HeLa-Kb cells after siRNA knock-down. HeLa-Kb cells were transiently transfected with one of two siRNA's targeting BH mRNA, with control siRNA (mTOP), or untransfected (UN). A) Results from real-time PCR for BH mRNA on day 1 or day 3 after transfection. Data is normalized to β-actin, and is presented as a percent of total BH mRNA in cells transfected with control siRNA (mTOP). Error bars represent variation between duplicate wells. Data is representative of at least two independent experiments. B) A western blot for BH from soluble extracts. Extracts were taken from cells on day 4 and day 5 after transfection with the indicated siRNA. Previous days had similar levels. C) Serial dilutions of BH#2 and mTOP cell lysates from day 5 were run on a gel in order to determine relative amounts of BH protein. According to semiquantitative immunoblotting and real-time PCR, BH siRNAs can knock-down BH protein to ≤ 20% of normal levels.

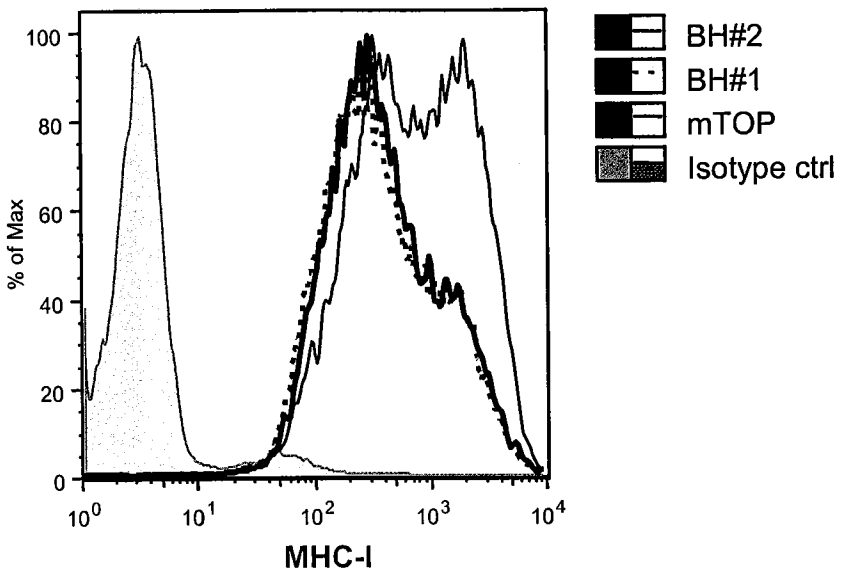


Figure 15 - MHC class I expression on HeLa-Kb cells after transfection with various siRNAs. 3 days after transfection with the indicated siRNA, HeLa-Kb cells were stained with PA2.6 and analyzed by flow cytometry. Live cells were gated and compared. Red line = ctrl siRNA (mTOP), blue dashed = BH#1 siRNA, black = BH#2 siRNA. Data is representative of at least 3 independent experiments.

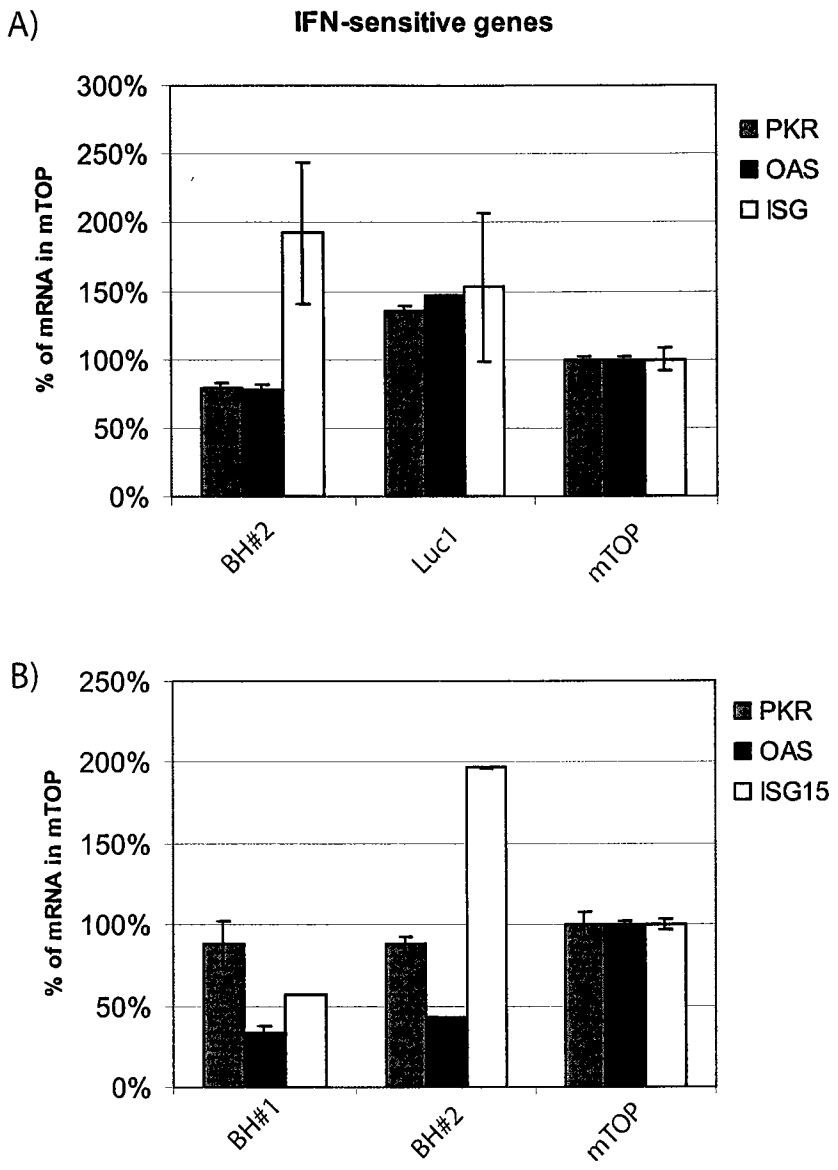


Figure 16 - Real-time PCR analysis of IFN-sensitive genes in HeLa-K^b cells treated with siRNA. Three days after transfection with the indicated siRNA, mRNA was isolated from HeLa-K^b cells and used for real-time analysis as previously described. A) includes BH#1 compared to BH#2 and mTOP. B) Luc1 is compared to BH#2 and mTOP on a different day. Blue = PKR, red = OAS, and yellow = ISG15. All data is normalized to levels of β-actin. Error bars represent standard deviation between duplicate wells. Data is representative of at least two independent experiments.

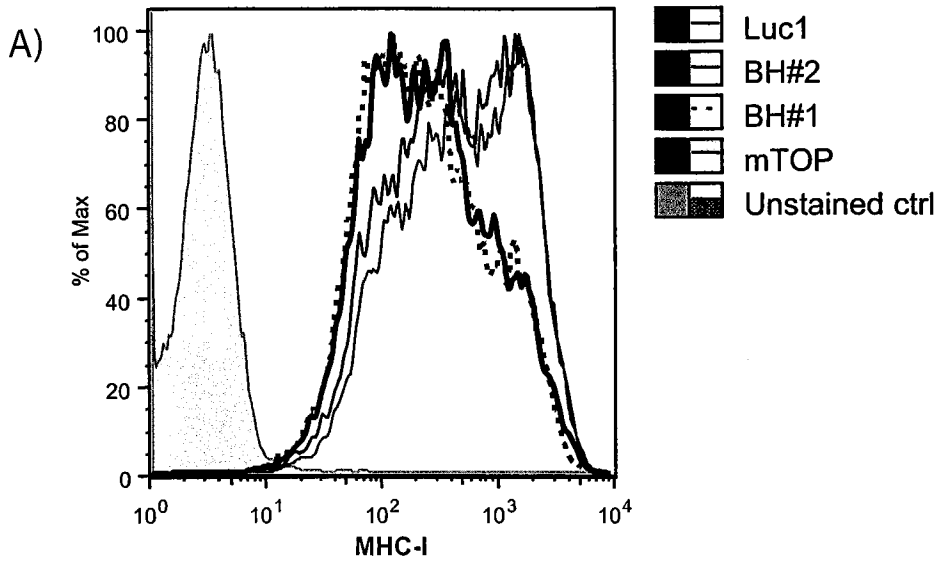


Figure 17 - MHC class I presentation on HeLa-Kb cells after transfection with various siRNAs. Two days after transfection with the indicated siRNA, HeLa-Kb cells were stained with PA2.6 and analyzed by flow cytometry. Live cells were gated and compared. Red line = ctrl siRNA (mTOP), blue dashed = BH#1 siRNA, black = BH#2 siRNA, yellow = Luc1. Non-specific Luc1 siRNA increases MHC class I presentation similarly to BH#2 siRNA. Data is representative of at least 3 independent experiments.

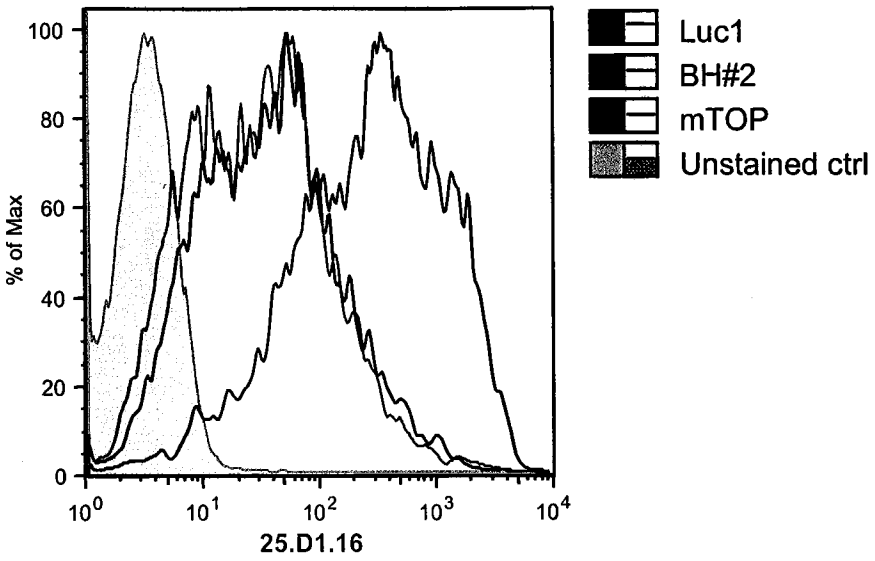


Figure 18 -SIINFEKL presentation from a peptide precursor on COS-Kb cells after transfection with various siRNAs. 1 day after transfection with the indicated siRNA, COS-Kb cells were transfected with a plasmid expressing a SIINFEKL peptide precursor and GFP. On day three (post siRNA transfection), the cells were stained with 25.D1.16 and analyzed by flow cytometry. Cells were gated based on comparable GFP expression. Red line = ctrl siRNA (mTOP), black = BH#2 siRNA, yellow = Luc1. Data is representative of at least 2 independent experiments.

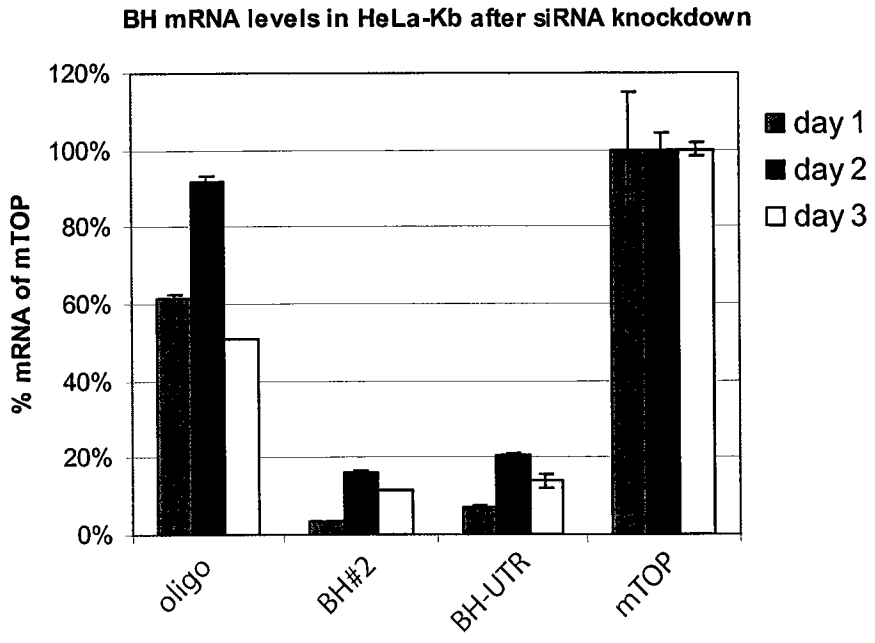


Figure 19 - BH expression in HeLa-K^b cells after siRNA knockdown. HeLa-Kb cells were transiently transfected with one of two siRNA's targeting BH mRNA, with control siRNA (mTOP), or with oligofectamine alone (oligo). A) Results from real-time PCR for BH mRNA on day 1 (blue), day 2 (red), or day 3 (yellow) after transfection. Data is normalized to β-actin, and is presented as a percent of total BH mRNA in cells transfected with control siRNA (mTOP). Error bars represent variation between duplicate wells.

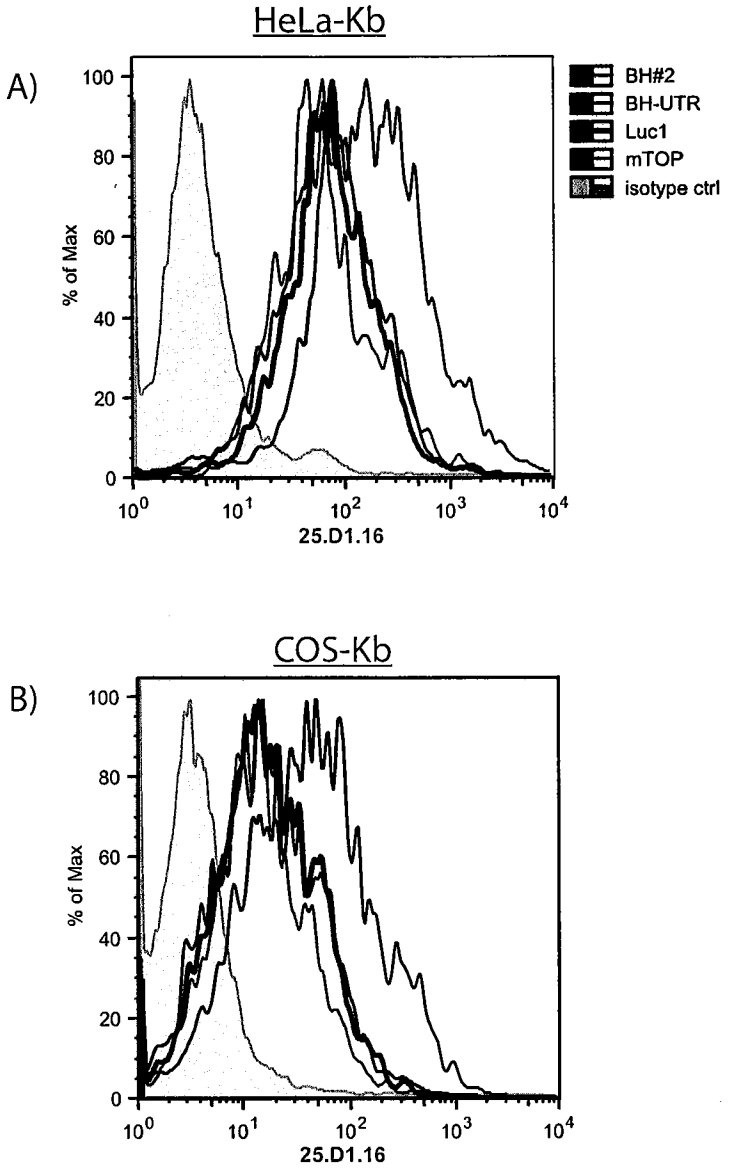


Figure 20 - Presentation of SIINFEKL on cells treated with siRNA. A) HeLa-K^b cells were transfected with the indicated siRNA as previously described. 24 hrs later, all cells were transfected with a GFP construct expressing an N25+SIINFEKL peptide precursor. Three days post-siRNA transfection, the cells were stained for SIINFEKL+K^b presentation with 25.D1.16. Cells were gated based on comparable GFP expression. B) The same experiment was performed in COS-K^b cells and analyzed as in A) with similar results. BH-UTR reduces SIINFEKL presentation as well as Luc1 and BH#2 siRNAs.

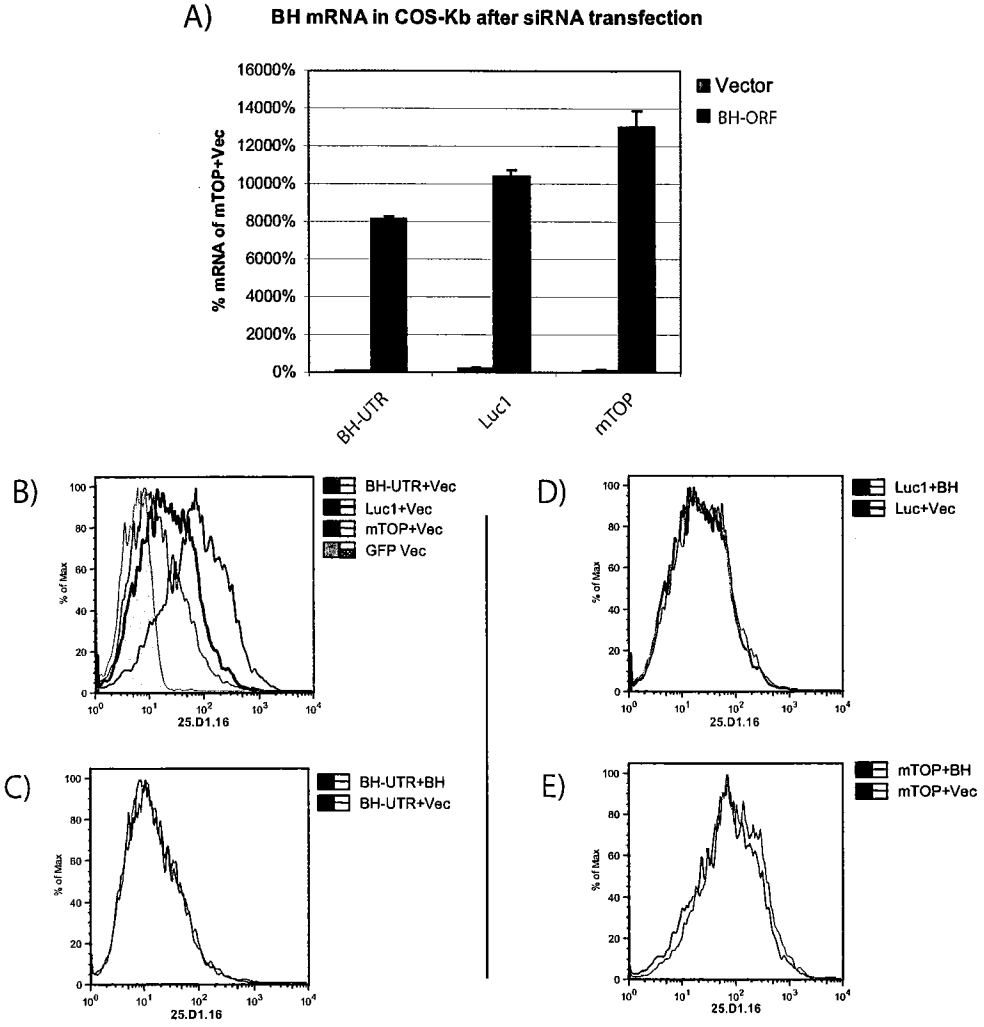


Figure 21 - BH siRNA effects on SIINFEKL presentation is not due to its ability to silence endogenous BH. COS-Kb cells were tranfected with the indicated siRNA. Then 24 hrs later, all cells were transfected with pcDNA3.1-N5+SIINFEKL plasmid (N-extended precursor), and a GFP-expressing plasmid encoding BH-ORF or GFP-vector. Three days after siRNA transfection the cells were analyzed for A) mRNA content by real-time PCR, and B) SIINFEKL presentation. A) Real-time PCR for BH of cells that were transfected with BH-ORF (green), or vector (blue). Data is normalized to β-actin, and is presented as a percent of total BH mRNA in cells transfected with mTOP siRNA and vector. B) The same cells (transfected with the SIINFEKL precursor and GFP-vector) were stained with 25.D1.16 and analyzed for SIINFEKL presentation by FACS. Cells were gated based on comparable GFP expression. C) Cells received BH-UTR siRNA, N5+SIINFEKL, and either BH plasmid or vector were analyzed as in B). C) Cells received Luc1 siRNA and the same plasmids as in C) and were analyzed as in B). D) Cells received mTOP ctrl siRNA and the same plasmids as in C) and were analyzed as in B).

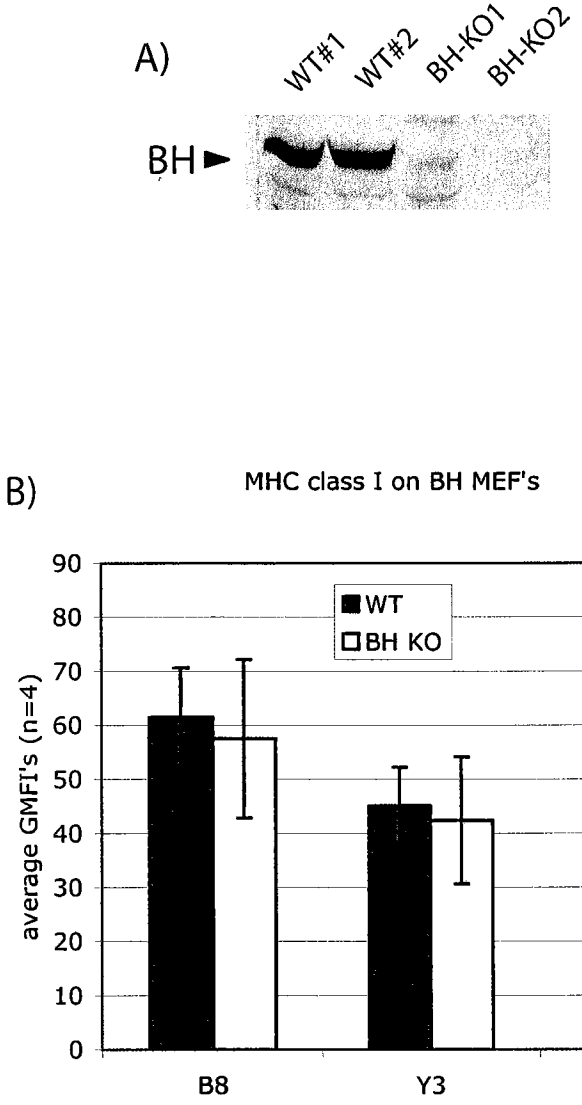


Figure 22 - MHC-class I presentation on BH-/- and wild-type MEFs. A) Mouse embryonic fibroblasts were generated from embryos as previously described and tested by western blot for BH expression. B) Four independent lines from each genotype were stained for MHC class I with B8 (H-2K^b), Y3 (H-2K^b), or M142 (all) and analyzed by FACS. Bars represent the average Geometric Mean Fluorescence of the independent MEF lines. Error bars represent the standard deviation within each group. Data is representative of at least three independent experiments.

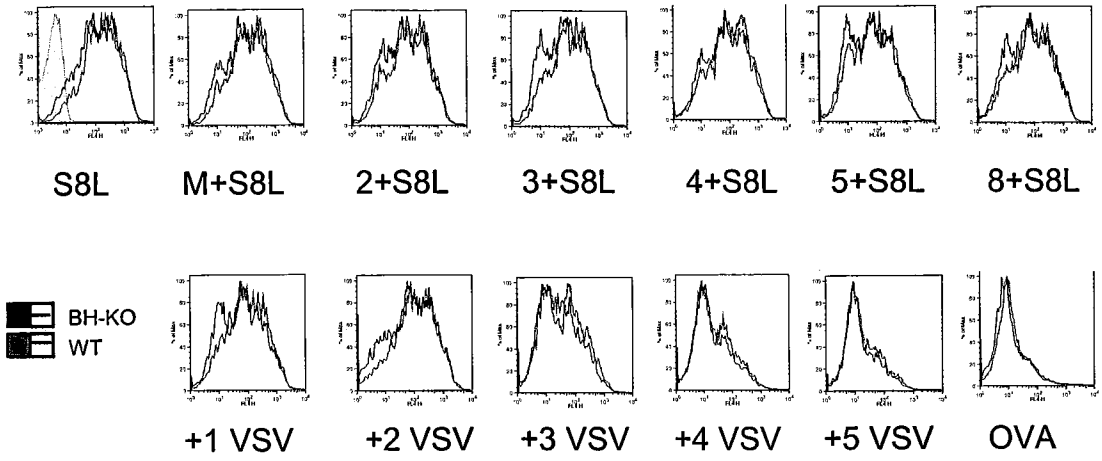


Figure 23 - SIINFEKL presentation in BH MEFs from peptide precursors. MEFs were transfected with the indicated GFP-expressing construct and analyzed 48 hrs later. Cells were stained with 25.D1.16 and analyzed by FACS. Gray = wild type MEFs, black = BH-/- MEFs. Cells were gated based on GFP expression, and SIINFEKL+K^b presentation was compared. Data is representative of at least three independent experiments.

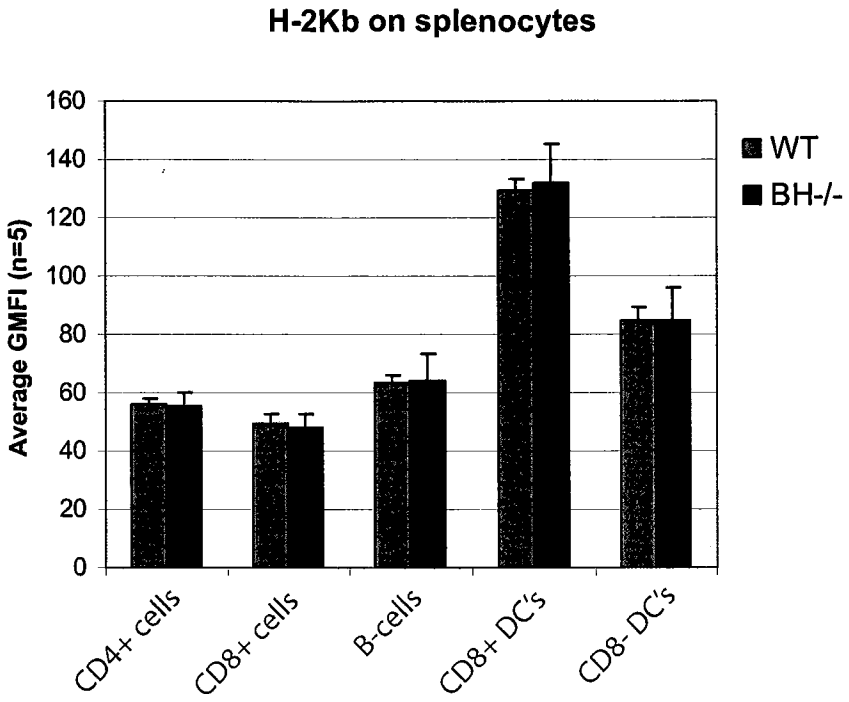


Figure 24 - MHC-class I presentation on splenocytes from BH-/- and wild type mice. Splenocytes were stained with appropriate antibodies and analyzed by FACS. CD4+ cells were gated based on B220-, CD8-, CD4+. CD8+ cells are B220-, CD4-. B-cells are B220+, CD4-, CD8-. Dendritic cells (DC's) are CD11c+, CD86hi, and CD8+ or CD8-. Data represent 5 mice per genotype and is representative of at least three independent experiments. Error bars represent standard deviation within each genotype.

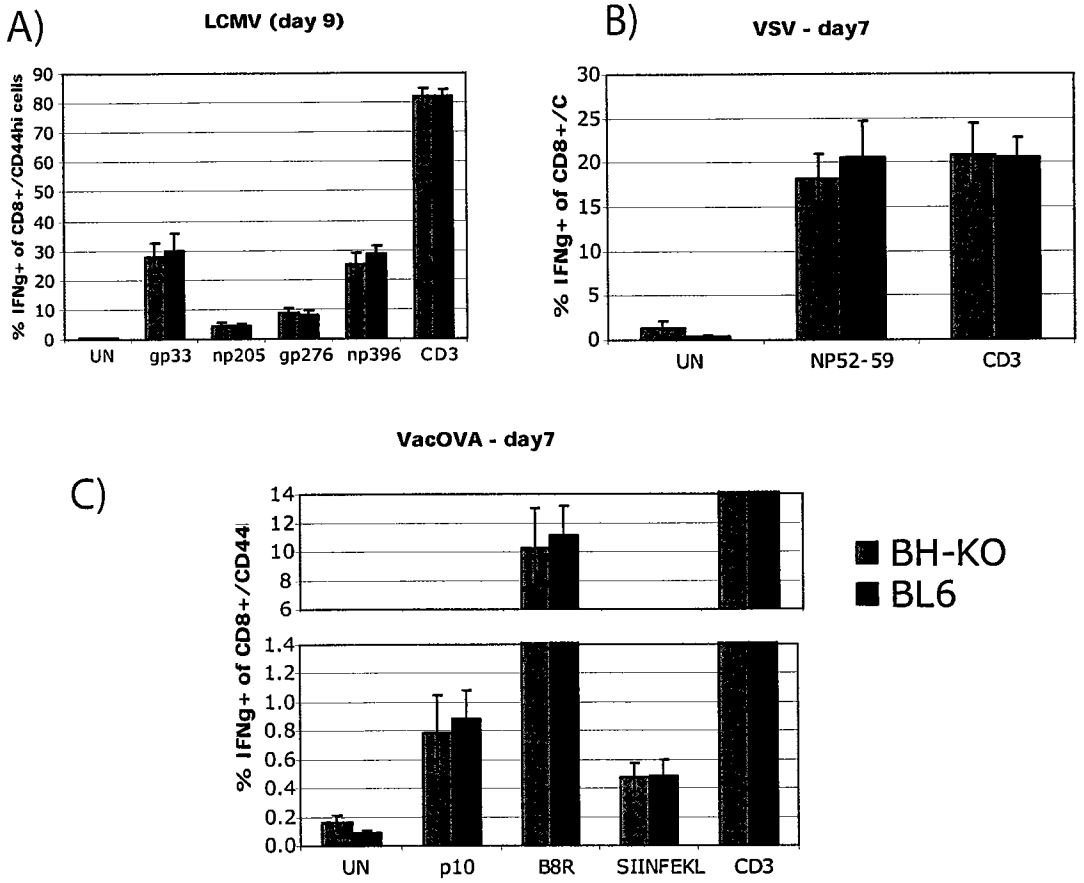
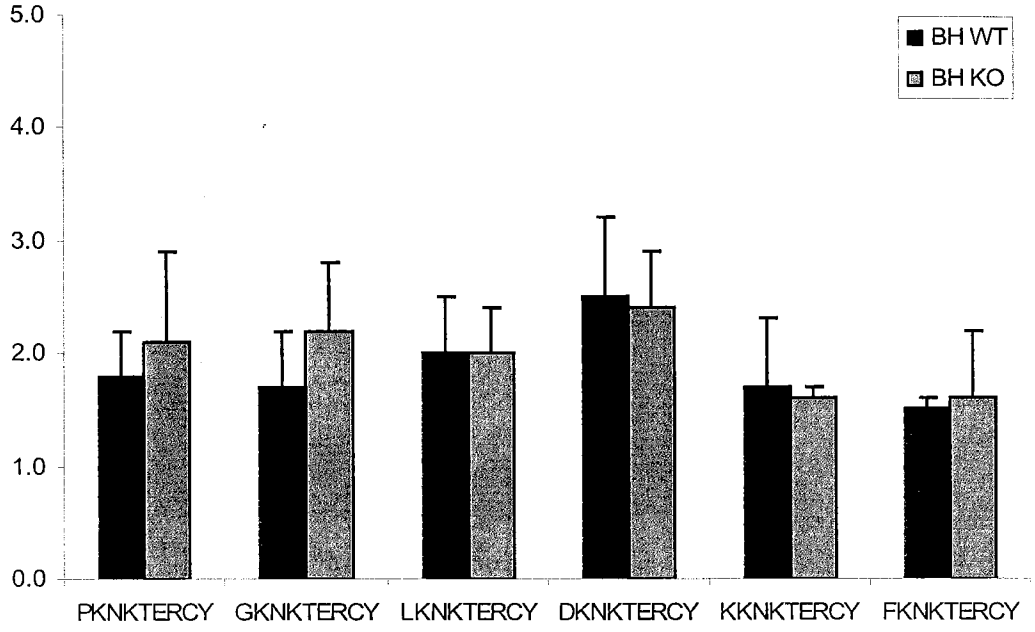


Figure 25 - Intracellular IFNγ staining of peptide-specific T-cells. Spleen cells from BH -/- and wild-type mice infected with virus were harvested and stimulated on, A) day 9 for LCMV, B) day 7 for VSV, or C) day 7 for recombinant vaccinia. After isolation, splenocytes were stimulated for five hours CTL responses to viral epitopes in infected mice. BH and wild type mice were infected with A) LCMV for 9 days, B) VSV for 7 days, or C) recombinant Vac-OVA virus for 7 days. At which point, spleens were harvested from these animals and the splenocytes were incubated with one of the indicated peptides for 5 hours with anti-CD3ε, as a control for CTL viability, or A) LCMV peptides gp33, np205, gp276, and gp396, B) VSV peptide NP52-59, or C) vaccinia peptides p10, B8R, or SIINFEKL peptides as described in the materials and methods. They were then surface stained with anti-CD8 and anti-CD44, and intracellularly stained with anti-IFNγ. Graphs represent the average percentages of CD8+, CD44hi T-cells that were IFNγ-positive (n=5 mice). Blue bars = wild type, and red bars = BH-/-. Error bars represent the standard deviation within each group. There was no significant difference between BH-/- and wild type mice in their response to any of the epitopes tested.

A)



B)

	KO		WT		
	avg	stdev	avg	stdev	p-value
PKNKTERCY	2.1	0.8	1.8	0.4	0.68
GKNKTERCY	2.2	0.6	1.7	0.5	0.22
LKNKTERCY	2.0	0.4	2	0.5	1.00
DKNKTERCY	2.4	0.5	2.5	0.7	0.83
KKNKTERCY	1.6	0.1	1.7	0.6	0.71
FKNKTERCY	1.6	0.6	1.5	0.1	0.57

Figure 26 - Half-life of microinjected peptides in BH-/- and wild type MEFs. Peptides with different residues in the P1 position were microinjected into BH-/- or wild type control MEFs. Half-life of peptide was determined by following the generation of fluorescence signal. The data is expressed as A) a graph, or as B) a chart with p-values. Statistical significance was determined by students t-test (two-tailed distribution assuming equal variances). No difference in half-life was detected in any of the tested peptides between the two cell lines.

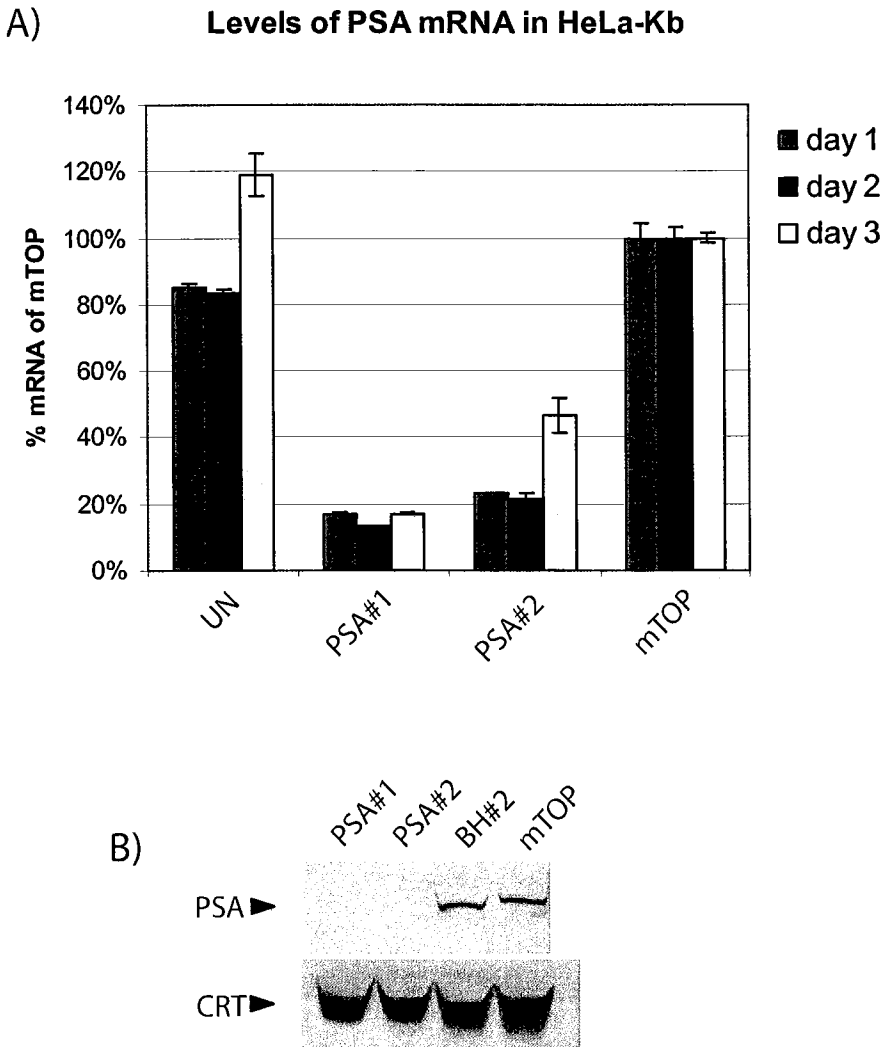


Figure 27 - PSA expression in HeLa-K^b cells after siRNA knock-down. HeLa-K^b cells were transiently transfected with one of two siRNAs targeting PSA mRNA, with control siRNA (mTOP), or untransfected (UN). A) Results from real-time PCR for BH mRNA on day 1 (blue), day 2 (red), or day 3 (yellow) after transfection. Data is normalized to β-actin, and is presented as a percent of total PSA mRNA in cells transfected with control siRNA (mTOP). Error bars represent variation between duplicate wells. Data is representative of at least two independent experiments. B) A western blot for PSA overexpressed in COS-K^b cells. COS-K^b cells were transfected with the indicated siRNA. 24 hours later, the same cells were transfected with PSA plasmid. 3 days post-siRNA transfection, cytosolic extracts were taken. Western blot for PSA indicates specific reduction of PSA protein by PSA siRNAs. Calreticulin (CRT) was used as a loading control.

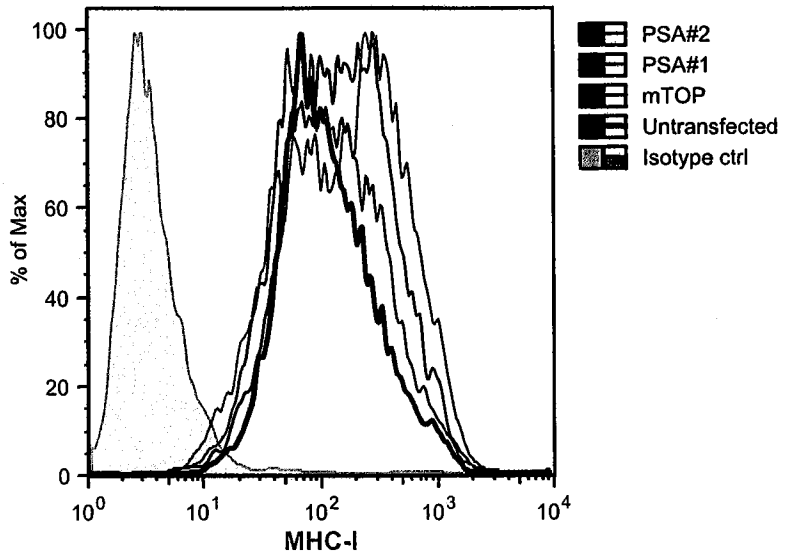


Figure 28 - MHC class I expression on HeLa-Kb cells after transfection with various siRNAs. 2 days after transfection with the indicated siRNA, HeLa-Kb cells were stained with PA2.6 and analyzed by flow cytometry. Live cells were gated and compared. Red line = ctrl siRNA (mTOP), blue line = PSA#1, green = PSA#2, and orange thick line = no siRNA. Data is representative of at least three independent experiments.

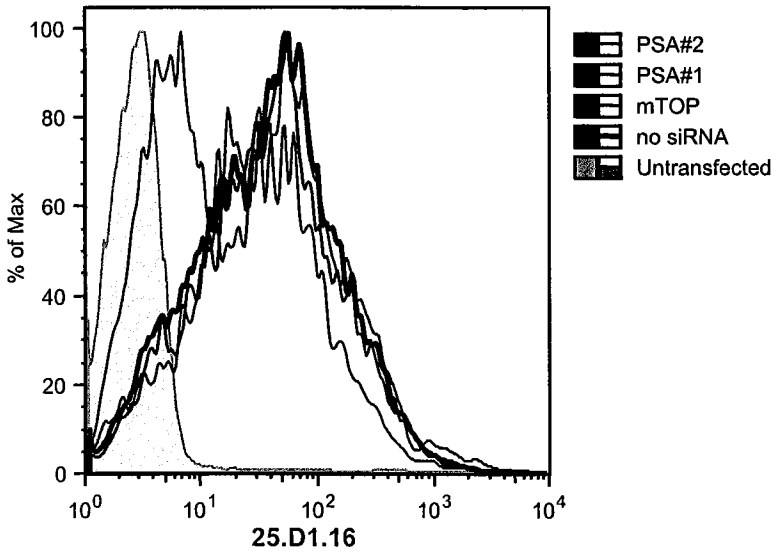


Figure 29 -SIINFEKL presentation from a peptide precursor on COS-K^b cells after transfection with various siRNAs. 1 day after transfection with the indicated siRNA, COS-K^b cells were transfected with a plasmid expressing a N25+SIINFEKL peptide precursor and GFP. On day 3 (post siRNA transfection), the cells were stained with 25.D1.16 and analyzed by flow cytometry. Cells were gated based on comparable GFP expression. Red line = ctrl siRNA (mTOP), blue = PSA#1, green = PSA#2, orange = no siRNA, and gray are cells that did not get a SIINFEKL plasmid. Data is representative of at least 2 independent experiments.

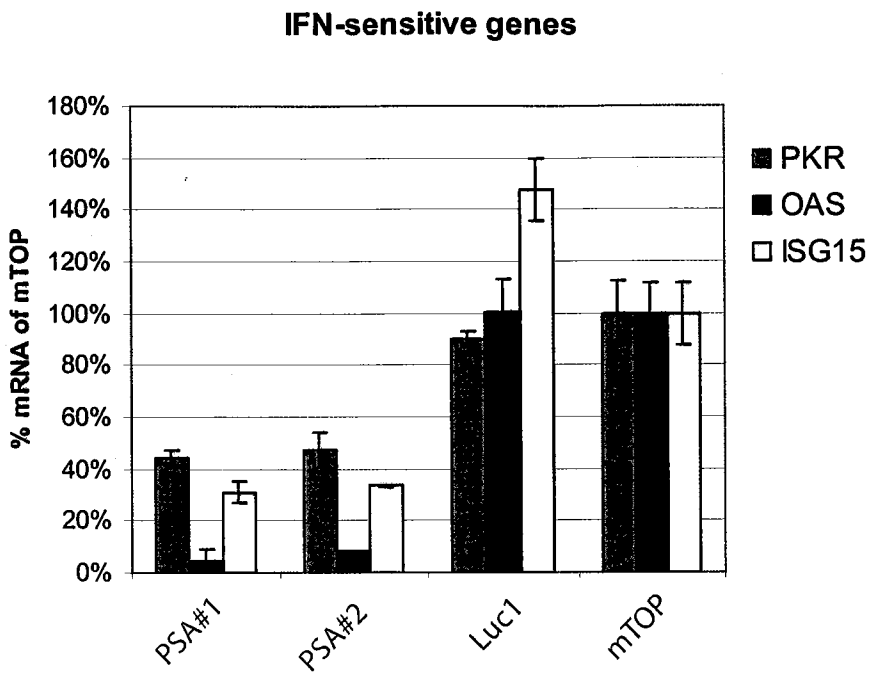


Figure 30 - Real-time PCR analysis of IFN-sensitive genes in HeLa-K^b cells treated with siRNA. Three days after transfection with the indicated siRNA, mRNA was isolated from HeLa-K^b cells and used for real-time analysis as previously described. Luc1 and mTOP siRNAs are compared to PSA#1 and PSA#2. Blue = PKR, red = OAS, and yellow = ISG15. All data is normalized to levels of β-actin. Error bars represent standard deviation between duplicate wells. Data is representative of at least two independent experiments.

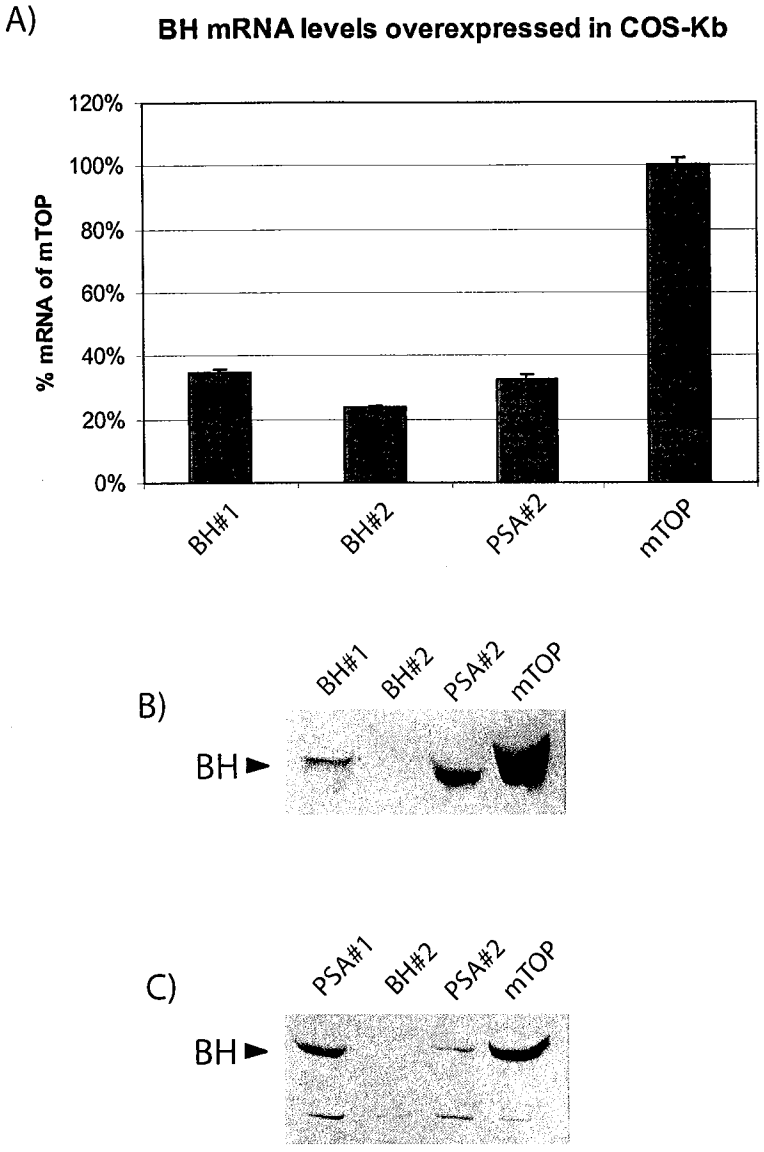


Figure 31 - Silencing ability of non-homologous siRNA sequences. COS-K^b cells were transfected with the one of two siRNAs targeting BH mRNA, with mTOP (ctrl), or with the non-homologous PSA#2 siRNA. 24 hrs later, the same cells were transfected with a BH plasmid. On day three post-siRNA transfection, the cells were harvested and analyzed by A) real-time PCR for BH, or B) western blot for BH. A) and B) represent separate experiments. C) Experiment was performed as in A) and B), except PSA#1 was used instead of BH#1. Unlike PSA#1, PSA#2 appears to silence non-homologous mRNA sequences, which leads to a reduction of untargeted proteins.

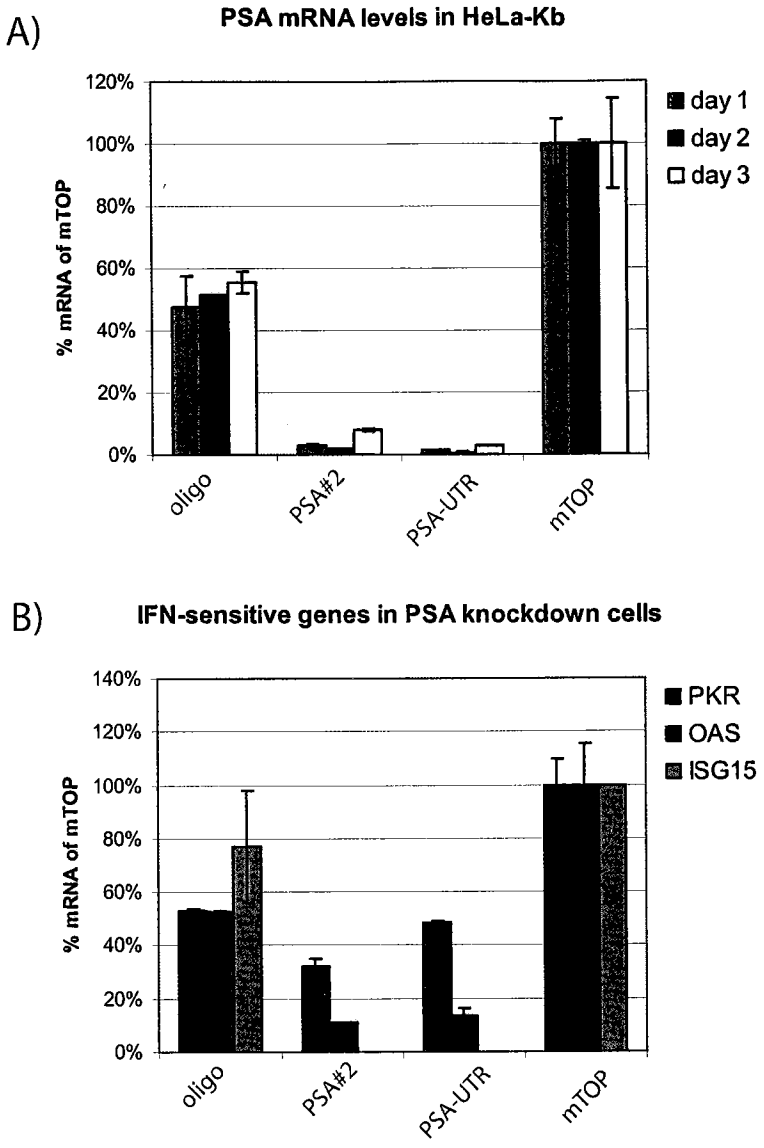


Figure 32 - mRNA expression in HeLa-K^b cells after PSA siRNA knock-down. HeLa-K^b cells were transiently transfected with one of two siRNA's targeting PSA mRNA, with control siRNA (mTOP), or mock transfected (oligofectamine alone). A) Results from real-time PCR for PSA mRNA on day 1 (blue), day 2(red), or day 3 (yellow) after transfection. Data is normalized to β-actin, and is presented as a percent of total PSA mRNA in cells transfected with control siRNA (mTOP). Error bars represent variation between duplicate wells. Data is representative of at least two independent experiments. B) mRNA from day 2 of the experiment in A) was used to determine induction of IFN-sensitive genes by the indicated siRNAs as previously described. green = PKR, orange = OAS, purple = ISG15. Induction of ISG15 by PSA#2 and PSA-UTR siRNAs was undetectable. Error bars and normalization were done as in A).

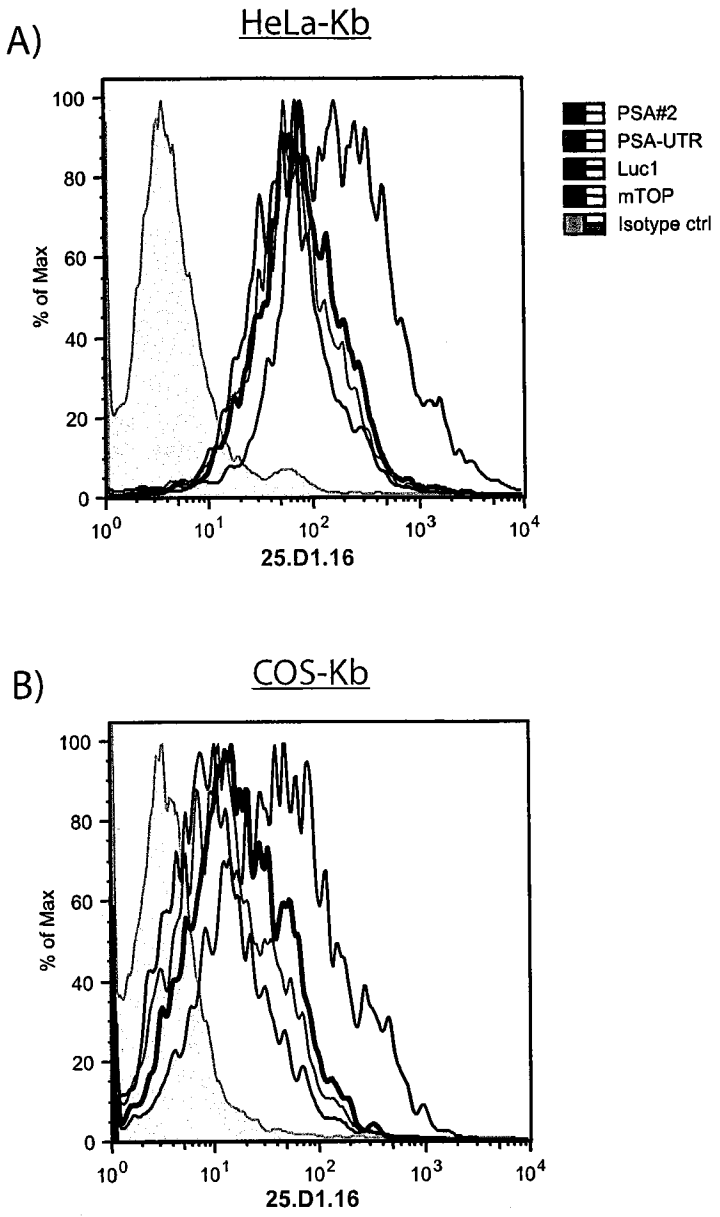


Figure 33 - Presentation of SIINFEKL on cells treated with siRNAs. A) HeLa-K^b cells were transfected with the indicated siRNA as previously described. 24 hrs later, all cells were transfected with a GFP construct expressing an N25+SIINFEKL peptide precursor. Three days post-siRNA transfection, the cells were stained for SIINFEKL+K^b presentation with 25.D1.16. Cells were gated based on comparable GFP expression. B) The same experiment was performed in COS-K^b cells and analyzed as in A) with similar results. PSA-UTR reduces SIINFEKL presentation as well as (or more than) Luc1 and PSA#2 siRNAs.

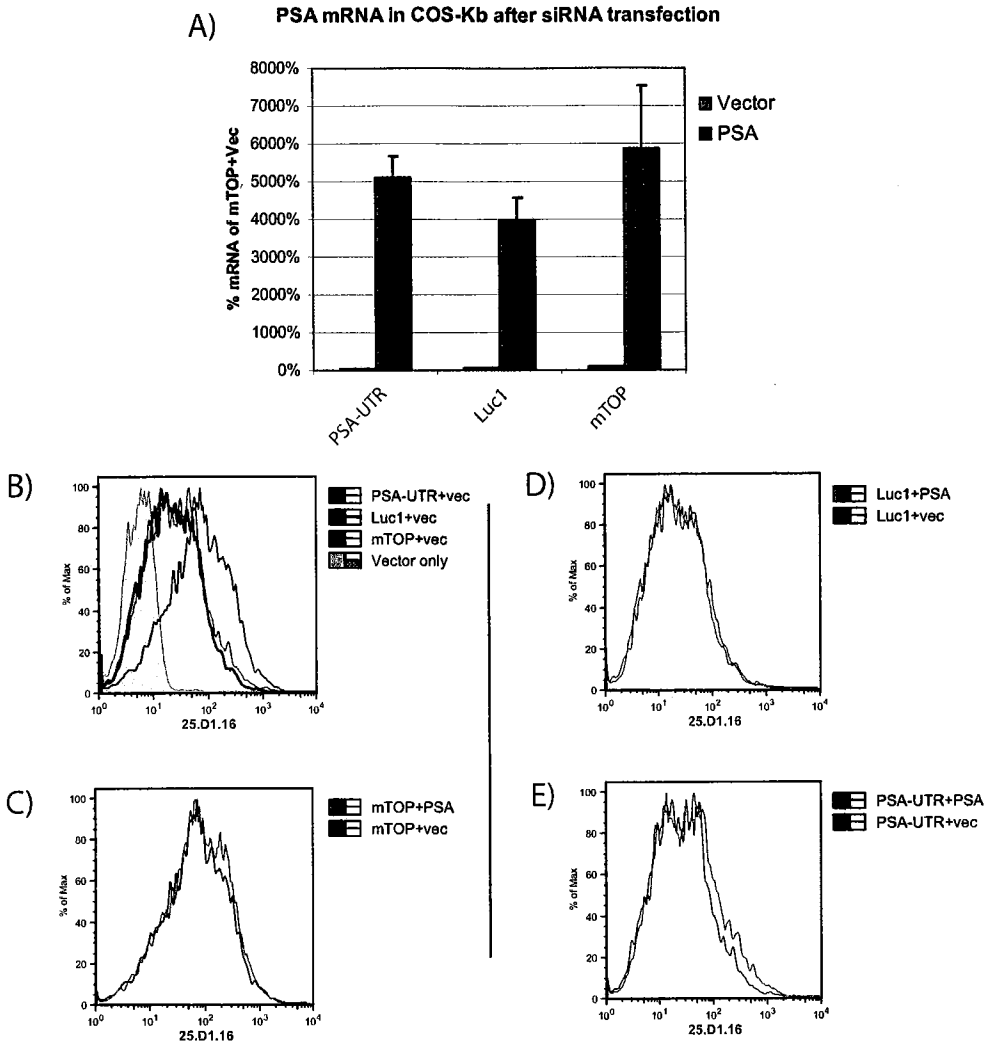


Figure 34 - PSA siRNA effects on SIINFEKL presentation is not due silencing PSA. COS-K^b cells were tranfected with the indicated siRNA. Then 24 hrs later, all cells were transfected with pcDNA3.1-N5+SIINFEKL plasmid (N-extended precursor), and a GFP-expressing plasmid encoding PSA-ORF or GFP-vector. Three days after siRNA transfection the cells were analyzed for A) mRNA content by real-time PCR, and B) SIINFEKL presentation. A) Real-time PCR for PSA of cells that were transfected with PSA-ORF (green), or vector (blue). Data is normalized to β-actin, and is presented as a percent of total PSA mRNA in cells transfected with mTOP siRNA and vector. B) The same cells (transfected with the SIINFEKL precursor and GFP-vector) were stained with 25.D1.16 and analyzed for SIINFEKL presentation by FACS. Cells were gated based on comparable GFP expression. C) Cells received PSA-UTR siRNA, N5+SIINFEKL, and either PSA plasmid or vector were analyzed as in B). C) Cells received Luc1 siRNA and the same plasmids as in C) and were analyzed as in B). D) Cells received mTOP ctrl siRNA and the same plasmids as in C) and were analyzed as in B).

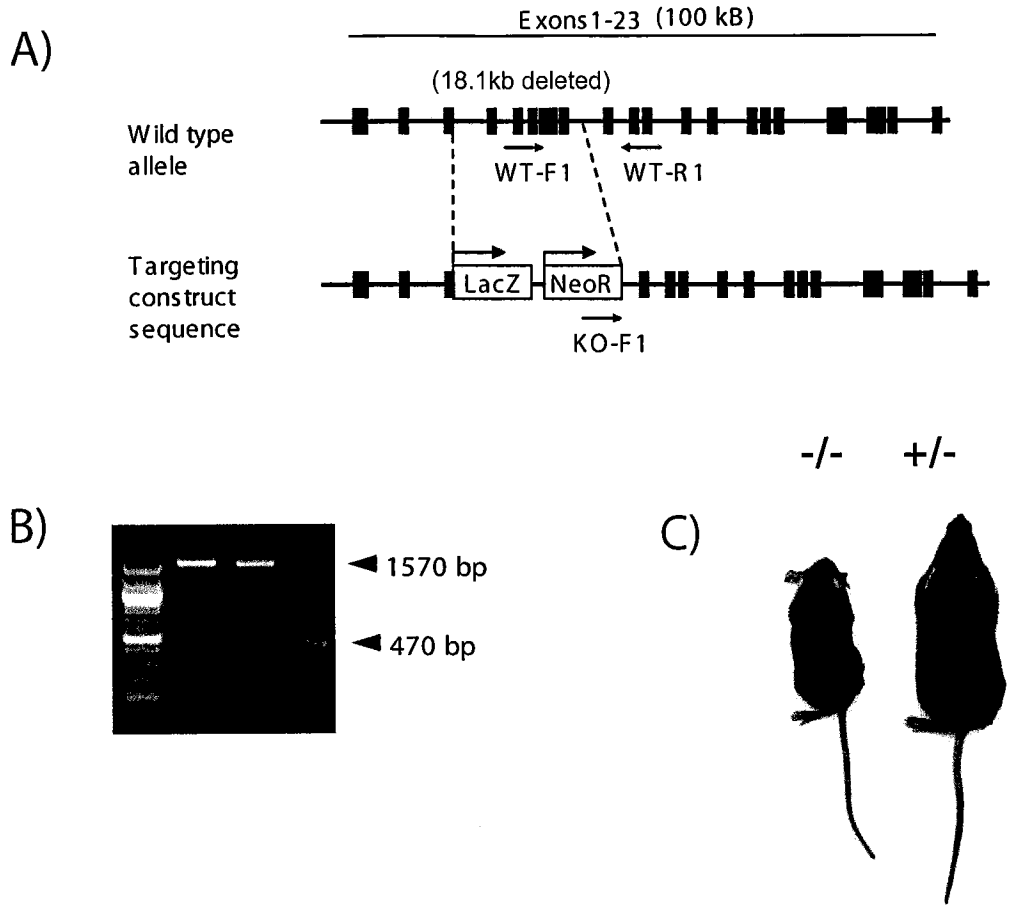


Figure 35 - Generation of PSA-deficient mice. A) Genomic organization of the mouse PSA gene (upper) and structure of the targeting vector (lower). Exons 1-23 are shown as boxes with the coding regions in black. Exons 3-9 (a total of 18.1 kb) were deleted by homologous recombination. The active site of PSA is located in exons 9 and 10. The locations of the primer sequences used for PCR genotype analysis are shown with arrows. B) PCR genotype analysis of wild-type and PSA-deficient mice. Amplification of the wild-type allele results in a 1570bp fragment, whereas the disrupted allele produced a 470bp fragment. C) Comparison of homozygote PSA-/- and +/- littermates at 4 wks of age.

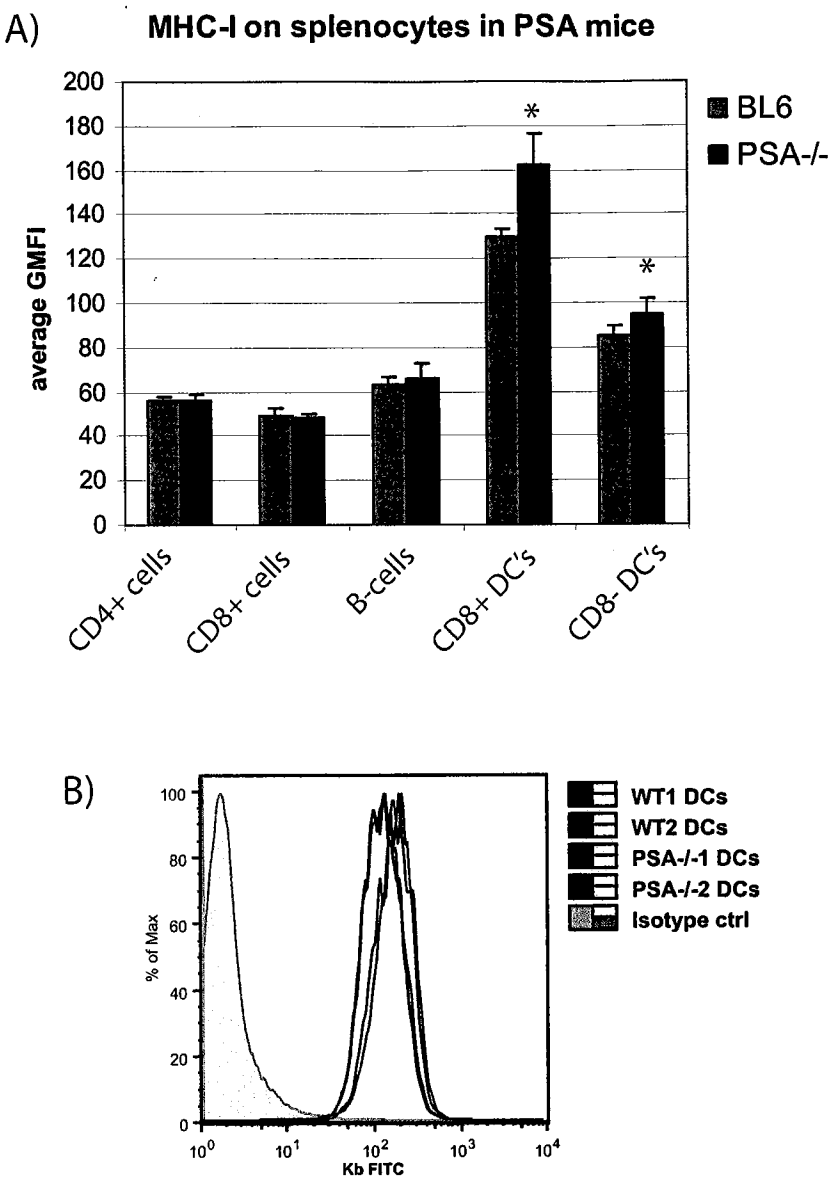


Figure 36 - MHC class I expression on splenocytes from PSA-/- and wild type mice. A) Splenocytes were stained with appropriate antibodies and analyzed by FACS. CD4+ cells were gated based on B220-, CD8-, CD4+. CD8+ cells are B220-, CD4-. B-cells are B220+, CD4-, CD8-. Dendritic cells (DC's) are CD11c+, CD86hi, and CD8+ or CD8-. B) Representative FACS traces of H-2Kb levels on CD8+ DC's from C57BL/6 (red & orange), or PSA-/- (green & blue) mice. Students t-test was used to determine statistical significance (* = $p<0.05$). Data represent 5 wild-type mice and 3 PSA-/- mice. Data is representative of three independent experiments. Error bars represent standard deviation within each genotype.

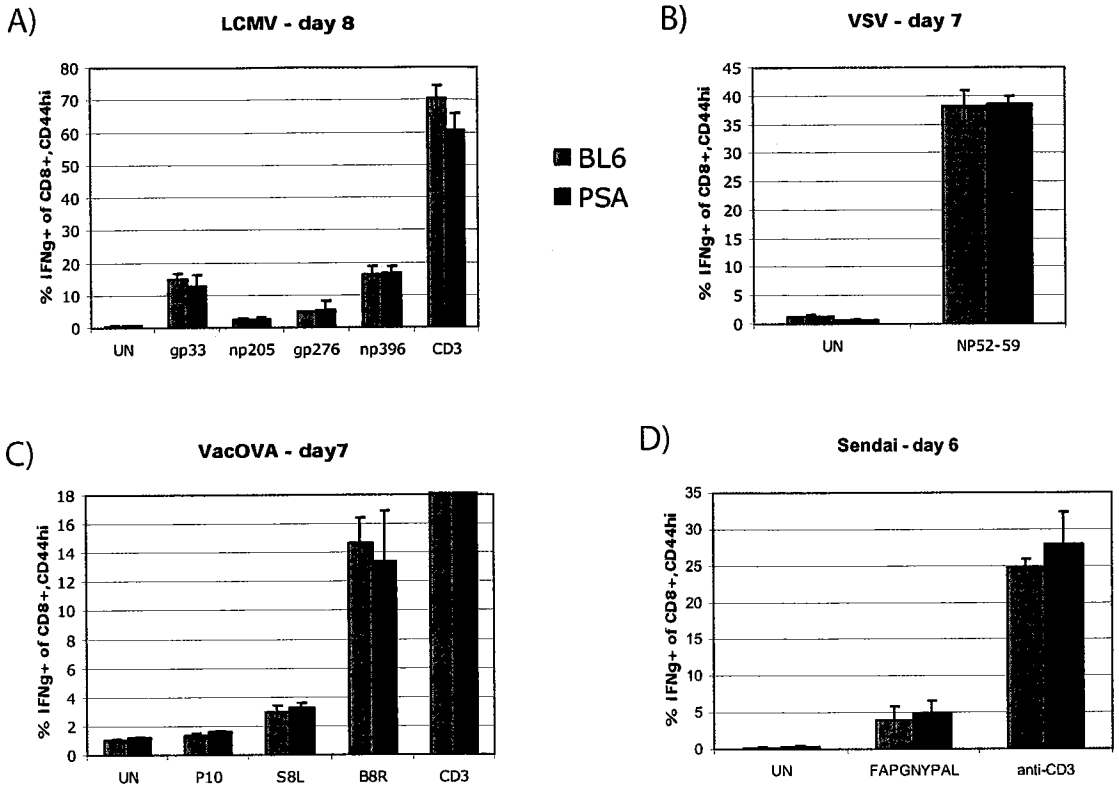
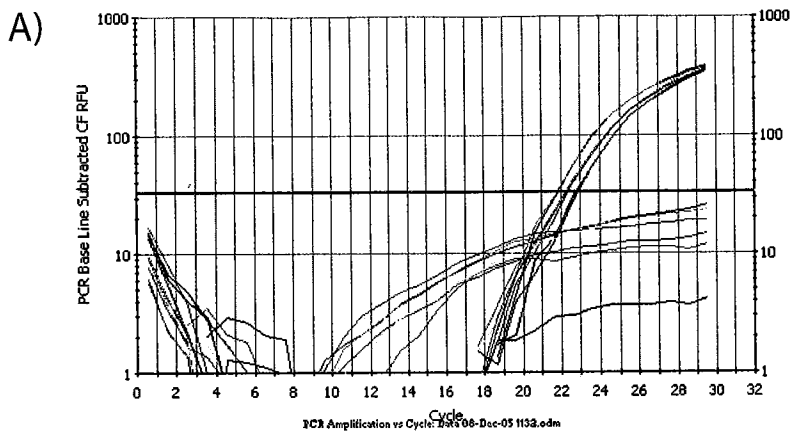


Figure 37 - Intracellular IFNγ staining of peptide-specific T-cells. Spleen cells from PSA-/- and wild-type mice infected with virus were harvested and stimulated on, A) day 9 for LCMV, B) day 7 for VSV, or C) day 7 for recombinant vaccinia. After isolation, splenocytes were stimulated for five hoursCTL responses to viral epitopes in infected mice. BH and wild type mice were infected with A) LCMV for 8 days, B) VSV for 7 days, C) recombinant Vac-OVA virus for 7 days, or D) Sendai virus for 6 days. At which point, spleens were harvested from these animals and the splenocytes were incubated with one of the indicated peptides for 5 hours with anti-CD3ε, as a control for CTL viability, or A) LCMV peptides gp33, np205, gp276, and gp396, B) VSV peptide NP52-59, C) vaccinia peptides p10, B8R, or SIINFEKL peptide, or D) NP52-59 from Sendai NP as described in the materials and methods. They were then surface stained with anti-CD8 and anti-CD44, and intracellularly stained with anti-IFNγ. Graphs represent the average percentages of CD8+, CD44hi T-cells that were IFNγ-positive (n=5 mice). Blue bars = wild type, and red bars = PSA-/-. Error bars represent the standard deviation within each group. Each graph represents at least two independent experiments. There was no significant difference between PSA-/- and wild type mice in their response to any of the nine epitopes tested.



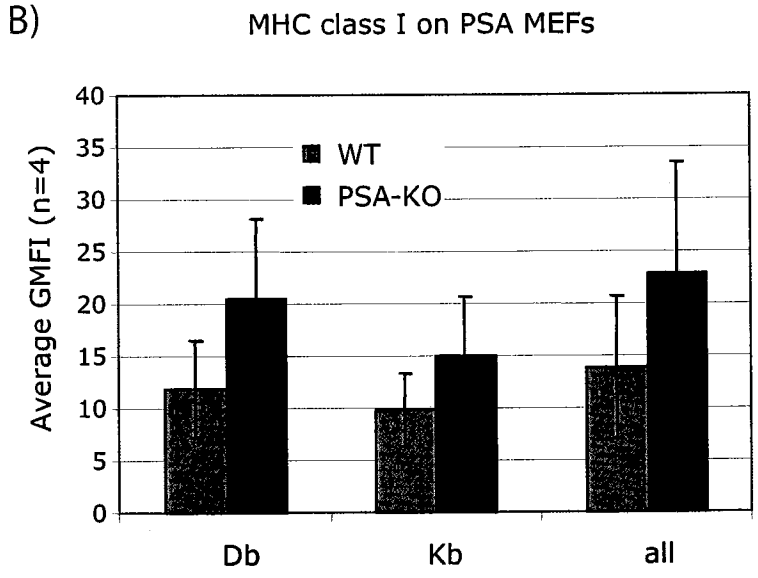


Figure 38 - Generation and characterization of PSA MEFs. A) MEFs were generated from embryos as previously described and tested by real-time PCR for expression of truncated forms of PSA mRNA. Traces above the orange line (cycle threshold) represent PSA expression in wild-type MEFs. Traces below the line represent PSA-/- MEFs. Thus, no truncated forms of PSA mRNA were detected. B) Four independent lines from each genotype were stained for MHC class I with B8.14.8S (D^b), Y3 (K^b), or M142 (all) and analyzed by FACS. Bars represent the average Geometric Mean Fluorescence of the independent MEF lines. Error bars represent the standard deviation within each group. Data is representative of at least three independent experiments.

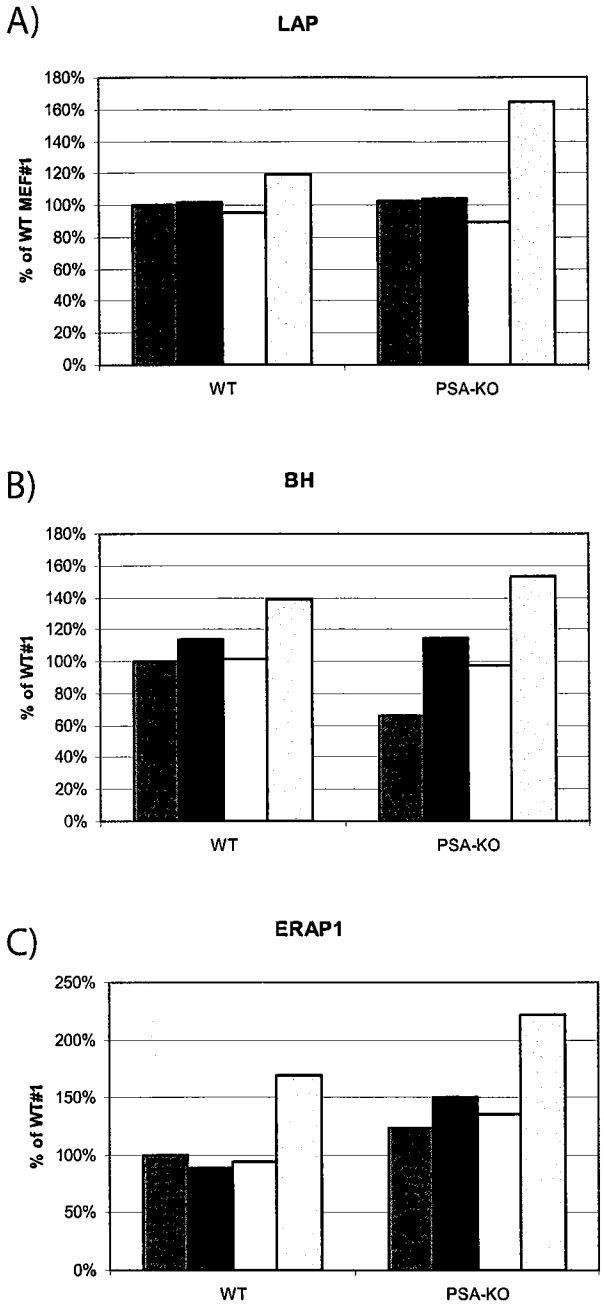


Figure 39 - Aminopeptidase expression in PSA MEFs. Real-time PCR for A) LAP, B) BH, and C) ERAP1 in each of the four MEF lines from each genotype. On the right of each graph are the four wild type MEFs. On the right are the four PSA-/- MEFs. MEFs are presented in the same order in each graph. All data are normalized to β-actin, and are represented as the percent of the mRNA in wild-type MEF#1. All data are representative of two independent experiments.

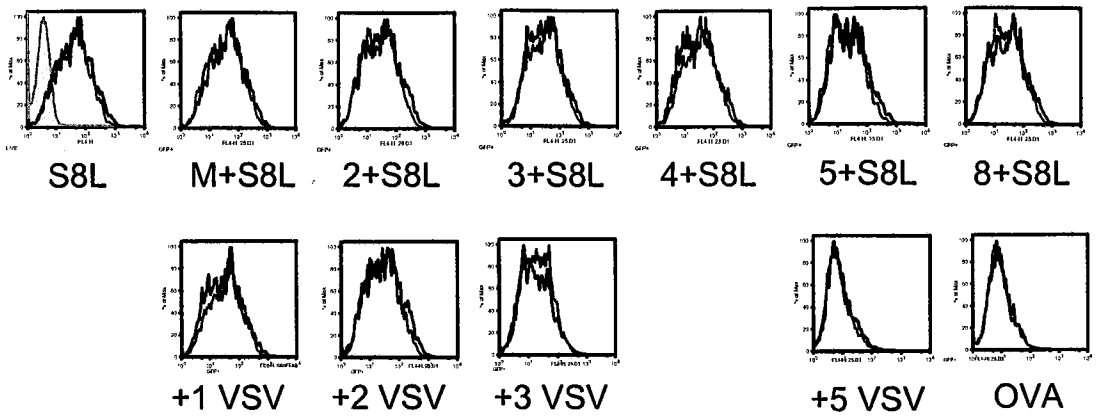
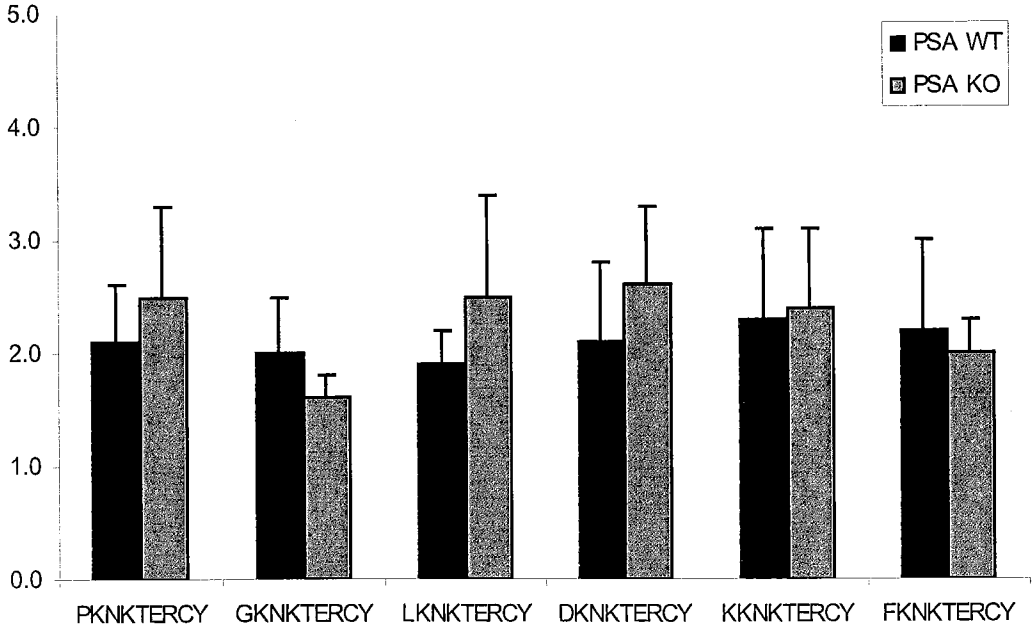


Figure 40 - SIINFEKL presentation in PSA MEFs from peptide precursors. MEFs were transfected with the indicated GFP-expressing construct and analyzed 48 hrs later. Cells were stained with 25.D1.16 and analyzed by FACS. Red = wild type, blue = PSA-/- MEFs. Cells were gated based on GFP expression, and SIINFEKL+K^b presentation was compared. Data is representative of at least three independent experiments.

A)



B)

	KO		WT		
	avg	stdev	avg	stdev	p-value
PKNKTERCY	2.5	0.8	2.1	0.5	0.31
GKNKTERCY	1.6	0.2	2	0.5	0.19
LKNKTERCY	2.5	0.9	1.9	0.3	0.35
DKNKTERCY	2.6	0.7	2.1	0.7	0.14
KKNKTERCY	2.4	0.7	2.3	0.8	0.71
FKNKTERCY	2.0	0.3	2.7	0.8	0.69

Figure 41- Half-life of microinjected peptides in PSA-/- and wild type MEFs. Peptides with different residues in the P1 position were microinjected into PSA-/- or wild type control MEFs. Half-life of peptide was determined by following the generation of fluorescence signal. The data is expressed as A) a graph, or as B) a chart with p-values. Statistical significance was determined by students t-test (two-tailed distribution assuming equal variances). No difference in half-life was detected in any of the tested peptides between the two cell lines.

Table 1 – shRNA and siRNA oligomers

Name	Sequences
LAP shRNA	GAATGATCCCATTGCCTGTTCCTCAATCgaagcttgGGTTGAGGAATAGGCA GTGGGATCATTCTTCttttttt
	gatcaaaaaaGAAGAATGATCCCACTGCCTATTCCTCAACCcaagcttcGATTGA GGAACAGGCAATGGGATCATTCcg
mERAP shRNA (ctrl cell line)	TTCTGACACCAAGAAGGAGATGACACAGgaagcttgCTGTGTCGTCTTCTTC TTGGTGTCAGGATAGttttttt
	gatcaaaaaaCTATCCTGACACCAAGAAGAAGACGACACAGcaagcttcCTGTG TCATCTCCTTCTTGGTGTCAGAAcg
BH#1 (1058)	GAUGGAGAGGCUGUGUGGUdTdT
	ACCACACAGCCUCUCCAUCdTdT
BH#2 (1252)	GGAUGAUCAGGAUGGUGCUdTdT
	AGCACCAUCCUGAUCAUCCdTdT
mTOP1	CCUCAACGAGGACACCACCdTdT
	GGUGGUGUCCUCGUUGAGGdTdT
BH-UTR (2002)	GGUUGAUCAGAUCUGACAUdTdT
	AUGUCAGAUCUGAUCAACCdTdT
Luc1	GAAACGAUAUGGGCUGAAUACdTdT
	GUAUUCAGCCCAUAUCGUUUCdTdT
PSA#1 (2320)	GCAUGGUGAUGGCACUACUdTdT
	AGUAGUGCCAUCACCAUGCdTdT
PSA#2 (2403)	GAGUCCUUGGCGCUACUCUdTdT
	AGAGUAGCGCCAAGGACUCdTdT
PSA-UTR (2883)	CCGAACAGCUGAUUCAUAUdTdT
	AUAUGAAUCAGCUGUUCGGdTdT

Table 2 – Primer Sequences

Gene name	Primer Name	Sequence (5'→3')	Notes
Human LAP	LAP 781F	gcacgccaattgatggag	For real time PCR in HeLa-K^b
	LAP 876R	ggtctgatatggacctcg	
	hLAP(F)-3 (KpnI)	gaggtacccgagccgacgagatgttc	For cloning LAP-ORF
	LAP 1826R (cloning)	gctctagataccatcctttgtctccgtta	
Mouse LAP	LAP BAC (F1)	AGACCCTAGAAAGGACGACGG	To generate LAP targeting construct (upstream)
	LAP BAC (R1)	GGCCCTGTGACTGGCTACTC	
	LAP BAC (F2)	TGGTGCCATCTTTCTCAGGAC	To generate LAP targeting construct (downstream)
	LAP BAC (R2)	GTGGTCACCTTGGTCTGCAAG	
	TaqMan F1	aggattgtcccaaagcctgctacgct	Taqman – screening ES clones
	TaqMan R1	TGGTGTTCAGTGATGGAGGTCTAG CATGCA	
	mLAP-KO (F5)	cctctgttccacatacacttc	For genotyping LAP mice
	mLAP-WT (F1)	gcacacttagacatagcag	
	mLAP-WT (R1)	cagatatggctgattctagc	
	mLAP-RT(F3)	CTTAACAGGTGCCATGGATGTAG	For real-time PCR in MEFs
	mLAP-RT(R3)	ACATCAGCAAGCTGGCAATC	
Human PSA	hPSA 2049F (PSA-F3)	ctcaactctcttgtcccac	For real-time PCR in HeLa-K^b
	hPSA 2158R (PSA-R3)	ggagtgcatcgagatgacc	
	hPSA-NotI	ataagaattgcggccgcacactgtgggtggtgaggccttcc gctg	For cloning PSA-ORF
	hPSA-KpnI 2	gggggtaccatgccggagaagaggcccttcgagcgg	
Mouse PSA	mPSA-WT(F3)	gttgcataagttctgaagctgagtc	For genotyping PSA mice
	mPSA-KO (F1)	TCATTCTCAGTATTGTTTTGCC	
	mPSA-(R2)	ggagcattgaataccttccagag	
	mPSA-1205F	cttgttactatggaatggtgg	For real-time PCR in MEFs
	mPSA-1386R	taggatggctgttatctaagg	
Human BH	hBH-Bgl II2	ggaagatctgatgagcagctcgggactgaattcg	For cloning BH-ORF
	hBH-XhoI2	ccgctcgagtctcagccaaagctcccatggggtcc	
	BH 686F	ggaacctggtacacagtg	For real-time PCR in HeLa-K^b
	BH 787R	ggtgaatgtctctggtgg	

Mouse BH	mBH (F)	cactgtagctgtactcacac	For genotyping BH mice (161)
	mBH (R-Vector)	atttgtcacgtcctgcacgacg	
	mBH (R-WT)	gcgacagagtaccatgtagg	
	mBH-702F	ggaacctggtacacagtgg	For real-time in MEFs
	mBH-820R	caGGTGAAGGTCTCTGGTGG	
Human ISG15	hISG15-KpnI	ggggtaccttagctccgcccgccaggctc	For cloning ISG15-ORF
	hISG15-XbaI	tgctctagaatgggctgggacctgacggtga	
	ISG-175F	cagaagattggcgtgc	For real-time PCR in HeLa- K^b
	ISG-319R	gaggttcgtcgcatttgtc	
Human PKR	hPKR-KpnI	ggggtaccctaacatgtgtgtcgttcatt	For cloning PKR-ORF
	hPKR-XbaI	tgctctagaatggctggtgatctttcagca	
	PKR-733F	gctactacgtgtgagtcc	For real-time PCR in HeLa- K^b
	PKR-882R	gagaccattcataagcaacg	
Human OAS	OAS2-XbaI	tgctctagatcagaggatggtgcaggtccagt	For cloning OAS2-ORF
	OAS2-BamHI	ccggatccatgatggatctcagaaatacccca	
	OAS2-540F	ctatgtcaagctcatcgag	For real-time PCR in HeLa- K^b
	OAS2-636R	gcgctgcttcaggaagtc	
Mouse ERAP1	mERAP1-2215F	gaggatgctcaggagccagctc	For real-time PCR in MEFs
	mERAP1-2358R	aacacagccaaggtcacatca	
β-actin	B-actin F1	cgaggcccagagcaagagag	For real-time PCR (162)
	B-actin R1	cggttggccttagggttcag	

SIINFEKL-containing pUG plasmids

Plasmid Name	Peptide Sequence
pUG1 vector	GFP only
0+S8L	**SIINFEKL**
1+S8L	E**SIINFEKL**
2+S8L	LE**SIINFEKL**
3+S8L	QLE**SIINFEKL**
4+S8L	EQLE**SIINFEKL**
5+S8L	LEQLE**SIINFEKL**
8+S8L	VSGLEQLE**SIINFEKL**
NPN1+S8L	L**SIINFEKL**
NPN2+S8L	DL**SIINFEKL**
NPN3+S8L	SDL**SIINFEKL**
NPN4+S8L	LSDL**SIINFEKL**
NPN5+S8L	SLSDL**SIINFEKL**
pIG+OVA	GFP+OVA under control of IRES

Table 3 - Plasmids used to transfect BH MEFs and PSA MEFs. 0+S8L thru 8+S8L contain the natural OVA sequence of upstream of SIINFEKL peptide. NPN1 thru NPN5 contain the upstream sequence of the VSV NP peptide. pIG+OVA encodes full-length OVA followed by an IRES and a GFP gene.

Table 4 - PSA-/- Mouse Viability and Fertility

	Total litters	Total weaned progeny	Avg. litter size	Avg. PSA-/- progeny
PSA+/- breeders	18	113	6.28	14.1%
PSA -/- breeders	11	36	3.27	N/A

Table 4 - PSA-/- mice are less viable as embryos and subfertile as adults. PSA+/- males were bred with PSA+/- females, and PSA-/- males were bred with -/- females. Data represent litter sizes at weaning (at least three weeks of age), and genotyping results fromvweaned mice over a period of at least 7 months. Breeding pairs were housed in the same facility, and maintained in accordance with facility protocols.