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Multiple synergizing factors contribute to the strength of the CD8$^+$ T cell response against listeriolysin O

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Abstract

Immunodominance in CD8$^+$ T cell responses against *Listeria monocytogenes* is a well recognized but still not fully understood phenomenon. From listeriolysin, the major virulence factor of *Listeria monocytogenes* only a single epitope, pLLO91-99, is presented by MHC class I molecules in BALB/c mice which dominates the cytotoxic T cell response against this bacterial pathogen. To obtain more insight into the molecular and cellular mechanisms underlying immunodominance of this particular epitope, we compared the various steps involved in presentation and recognition of pLLO91-99 derived from wild type toxin with an equivalent epitope from a mutated toxin. This fully functional variant contains within the pLLO91-99 epitope a conservative Isoleucin to Alanin replacement at the C-terminal anchor residue which results in loss of antigenicity. The binding properties of the variant peptide to soluble K^d remained unaffected and cytotoxic T cells capable of recognizing the pLLO99A/K^d complex were detectable in BALB/c mice. However such T cells required higher concentrations of antigen to be optimally activated *in vitro*.

A comparison between TAP translocation efficiency of wild type and mutant peptide demonstrated that the mutation at the C-terminus leads to a reduced transportation rate. Furthermore, the amino acid substitution changes the *in vitro* proteasomal cleavage pattern resulting in a reduced liberation of the correct peptide from a polypeptide precursor. Thus, in all assays employed the immunodominant epitope performs optimal while the variant was found to be inferior. The synergy of all these steps most likely is the decisive factor in immunodominance of pLLO91-99.

Introduction

The immune response against microbial pathogens comprises T cells that are reactive against a broad range of various antigenic peptides. Nevertheless, the strength of the T cell response towards particular epitopes of such pathogens exhibits a remarkable constant hierarchy i.e. the majority of the T cells are directed against a few immunodominant epitopes only (1-4). This hierarchy can be accomplished at various levels (5, and extensively reviewed in 6). For instance, peptides with high affinity towards the MHC molecule could compete more successfully for the binding site of the MHC molecule than low affinity peptides. Alternatively, the T cell response could be influenced by previous encounter with cross-reactive epitopes from other pathogens. This could favor reactivity against one particular determinant. On the other hand, T cells could be tolerant against potential epitopes or the T cell repertoire might lack high affinity receptors against subdominant or unrecognized epitopes. However, the most decisive factor imparting immunodominance is most likely how the antigen presenting cell (APC) is handling the antigen during the multiple steps of processing and presentation.

Cytolytic $CD8^+$ T cells (CTL) play an important role in the immune defense against *Listeria monocytogenes*, a Gram-positive bacterium that enters the cytosol of host cells (7, 8). When BALB/c mice are infected with *L. monocytogenes*, $CD8^+$ T cells specific for only a limited number of pathogen-derived peptides are induced (4, 9-11). The most dominant of these epitopes is derived from the secreted bacterial toxin, listeriolysin O (LLO), the major virulence factor of the pathogen. Although a few additional potential epitopes are found within this protein, only one, consisting of amino acid (aa) 91-99 (pLLO91-99), is activating cytotoxic T cells (12).

Interestingly, a quantitative investigation of APC infected by *L. monocytogenes* revealed that pLLO91-99 is found at very low abundance when compared with two epitopes of another listerial secretory protein p60. Only 700 pLLO91-99/K^d complexes could be recovered (13). This is in accordance with the finding that listeriolysin secretion is drastically down regulated intracellularly. In addition, the presence of a PEST motif subjects listeriolysin to rapid degradation by proteasomes (14). Nevertheless, even though the toxin is practically undetectable in infected cells, already the primary $CD8^+$ T cell response is mainly directed towards pLLO91-99 (13).

We have shown previously that purified listeriolysin, due to its pore-forming activity enters the cytosol of APC and thereby is accessible to the MHC class I processing and presentation pathway (15, 16). Thus, a simple experimental way exists to test physiological parameters of presentation of pLLO91-99 that might be responsible for its immunodominace. In this context, we first replaced the H-$2K^d$ anchor residue isoleucin at position 99 in the peptide pLLO91-99 by alanin. The replacement did not change the affinity of the peptide towards isolated K^d molecules. We subsequently introduced this mutation into the listeriolysin gene. The variant toxin LLO99A maintained pore forming property required for targeting the cytosol of APC. However, although binding of the epitope to the MHC class I molecule remained unaffected, the variant toxin lost its ability to stimulate listeriolysin specific $CD8^+$ T cells. This prompted us to dissect and analyse the molecular and cellular basis of the immunodominance of wild type pLLO91-99.

Methods

Mice

Female BALB/c mice 6 to 8 week of age were obtained from Harlan Winkelman (Borchen, Germany). All experiments performed with mice have been reviewed and approved by an official review committee.

Cell lines, bacterial strains, and antigens

The mastocytoma cell line P815 (H-2^d) and the macrophage-like cell line J774 (H-2^d) were used as target cells in cytotoxic T cell assays. To test for TAP dependency of LLO derived peptide presentation T2K^d and T2K^{d}TAP1/2 cells were used (17, kindly provided by Frank Momburg, German Cancer Research Center, Heidelberg) as targets. T2K^d and T2K^{d}TAP1/2 are derivatives of T2 transfected with TAP1/2 and/or H-2K^d cDNA. RMA cells originated from a Rauscher virus-induced C57BL/6 T-cell lymphoma. RMA-S cells, a subline of RMA, are deficient for TAP and were used for TAP translocation assays.

LLOwt and LLO99A were hyperexpressed in the nonpathogenic bacterium *Listeria innocua* and purified as described before (18). Nucleoprotein (NP) from influenza virus A/Loi/4/57 was expressed in baculovirus and purified from infected Sf9 cells by chromatography over Sephacryl S-100 HR (16).

Peptide synthesis and fluorescence peptide binding assay

Peptides were synthesized using the standard F-moc chemistry and purified by reversed-phase HPLC prior to use. Peptide affinities to soluble H-2K^d was measured based on the nonradioactive energy transfer to dansylated (DNS) peptides as

described before (18). Peptide equilibrium dissociation constants were calculated as the ratio k_{off} to k_{on} constants of peptide dissociation/binding from/to H-2K^d heavy chain/β_2m heterodimer. As was described earlier the rate constant of peptide binding to the heavy chain/β_2m heterodimer does not depend on the particular peptide sequence (19-21) and was taken as to be the same as for TYQRC(Dansyl)RALV (19). Peptide dissociation kinetics from the ternary complex was initiated by the addition of 100 fold excess of the dansylated peptide TYQRC(Dansyl)RALV to the heavy chain/β_2m heterodimer preincubated for two hours at room temperature with a peptide of the LLO series. Experiments were carried out in a 4 x 4 mm magnetically stirred, quartz optical cuvette. The fluorescence energy transfer from the heavy chain tryptophans to the peptide dansyl group was monitored at 530 nm upon excitation at 290 nm.

PCR mutagenesis and plasmid construction

The introduction of the point mutation leading to the aa substitution Ile99 to Ala within the LLO encoding gene *hly* and the subsequent cloning of the mutagenesis PCR products was performed as previously described (18). Point mutation was introduced by the internal mutagenesis primers 5´$_{285}$AATGAATAT*GC*TGTTGTGGAG$_{305}$3´ and 5´$_{305}$CTCCACAACA*GC*ATATTCATT$_{285}$3´. The final *Listeria* expression plasmid harboring the mutated *hly* gene and the positive regulator factor prfA were used to transform *L. innocua* to hyperexpress and purify the LLO99A as described before (18). Alternatively a ∆hly strain of *Listeria monocytogenes* EGD was transformed with the plasmid. The resulting strain *L. monocytogenes∆hlyLLO99A* was used for *in vitro* infection and presentation assays.

Hemolysin titer test

To assess hemolytic activity purified LLOwt and LLO99A were incubated with 10mM DTT prior to use. Sheep erythrocytes were washed 3 times in PBS adjusted to pH 5,7 and seeded in V-bottom microtiter plates. Graded amounts of LLOwt and LLO99A were added and after 1 hour incubation at 37°C supernatants were collected and OD_{414} was measured. Hemolytic Units (HU) were determined as protein concentration required to release 50 % hemoglobin from sheep erythrocytes. 100% lysis was obtained by incubation with de-ionized water.

Generation of Dendritic Cells

Bone marrow macrophages were isolated from BALB/c mice and incubated overnight in the presence of IL-4 and GM-CSF. Non adherent cells were removed and adherent cells were cultured for further six to eight days in IL-4 and GM-CSF containing medium to stimulate maturation into dendritic cells.

CTL induction and CTL assay

Cytotoxic T cells were generated either by immunization of BALB/c mice with protein in iFA as described before (18) or with peptide loaded syngeneic splenocytes or DC. Splenocytes or DC were pulsed for 2hrs to overnight with 10 µM peptide, washed carefully, resuspended in PBS and injected into mice (10^6 DC or 10^7 splenocytes / mouse, i.v.). Nine days after immunization, spleen cells were harvested and restimulated with the respective peptide for five to six days before use as effector cells in a cytotoxic assay. Cytotoxicity was measured using the JAM-assay as described before (18). When activated J774 cells were used as target cells stimulation with INF-γ was carried out 48hrs before the assay. To analyze TAP-

dependency of LLOwt presentation, T2.K^d or T2.K^dTAP cells were radioactively labeled overnight with 5 µg/ml ^{3}[H]-thymidine, then cells were pulsed with LLOwt for 30 min at room temperature, washed and seeded into microtiter plates. As positive control pLLO91-99 peptide was added to the target cells. Cytotoxic T cells were added after 2 hrs to allow sufficient surface presentation to take place. The assay was harvested after additional 4 hrs of incubation.

TAP-Translocation Assay

The peptide translocation assay was performed as described (22). Briefly: RMA and RMA-s cells were permeabilized with 2 U/ml streptolysin O (dissolved in 130mM KCl, 10mM NaCl, 1mM $CaCl_2$, 2mM EGTA, 2mM $MgCl_2$, 5mM HEPES, pH7.3). 200 ng of radioiodinated peptide (TNKTRIDGQY) containing an N-glycosylation motif were added to the cells including 20mM ATP and graded amounts of competitor peptide. After incubation for 20 min cells were lysed with 1% NP-40 in PBS containing protease inhibitors. The translocated and glycosylated peptide fraction was isolated on Con A-Sepharose and quantified by counting the radioactivity. Translocation efficiency was calculated as: cpm bound/total cpm.

Purification of 20S proteasomes, peptide digests and analysis

Proteasomes were purified from outdated blood as described previously (23). For the digestion of synthetic peptides typically 1 µg of human 20S proteasomes was incubated with peptide substrates (final concentration of 30-40 µM) at 37°C in a total volume of 300 µl assay buffer (20 mM HEPES/KOH [pH 7.8], 2 mM $MgAc_2$,). Aliquots of 100 µl were removed at different time points between 0 and 30 hrs. The reaction was stopped by the addition of 10 µl 10% acetic acid. Aliquots were desalted in two

alternative ways: peptides were adsorbed to C18 resin (ODS Hypersil (5 µm), Hewlett-Packard) in pipette tips, washed with water and eluted with 50% methanol/1% acetic acid. Alternatively, the aliquot was adsorbed to µRP SC 2.1/10-columns (Pharmacia), washed with buffer A (0,1% TFA) for 10 min and eluted with buffer B (0,081% TFA, 80% acetonitrile). In both cases the eluate was concentrated under vacuum, resuspended in 10-30 µl of eluent and then used for Edman sequencing or mass spectrometry (MS) analysis. HPLC-separation of peptide digests: for the separation of degradation products, unfractionated peptide digests were subjected to µRP SC 2.1/10-columns (Pharmacia) on a Microbore HPLC-system (SMART-system, Pharmacia). Gradients : 0% B for 5 min, in 40 min to 40% B, in 15 min to 75% B and up to 85% in another 5 min. The flow rate for all separations was 150 µl/min.

Matrix-associated laser desorption ionization (MALDI) analysis

Routinely, 1 µl of DHAP-matrix (20 mg 2,5-dihydroxy-acetophenone, 5 mg ammoniumcitrate in 1 ml 80% isopropanol) was mixed with 1 µl of concentrated desalted eluate on a gold target. Measurements were performed using a LD-TOF (laser desorption-time of flight)-system (Hewlett-Packard G2025A) at a vacuum of 10^{-6} Torr. For signal generation 50 to 100 laser shots were added up in the single shot mode. Fragmentation of degradation products, unfractionated peptide digests were subjected to µRP SC 2.1/10-colums (Pharmacia) on a Microbore HPLC system (SMART-system, Pharmacia). When dominant masses in MALDI spectra could not be identified by a fragment search program, MS/MS experiments were performed on a hybrid quadrupole orthogonal acceleration tandem mass spectrometer (Q-TOF, Micromass). Fragmentation of the parent ions was achieved by collision with argon

atoms. Q1 was set to the mass of interest +/- 0.5 Da and the collision energy optimized for each fragment. The integration time for the TOF analyzer was 1s with an inter scan delay of 0.1s.

N-terminal sequencing (Edman degradation)

For the identification of degradation products and for the relative quantitation of peptide products, 5-15 µl of unfractionated digests were applied to a glass fiber disc coated with 0.75 mg of polybrene and sequenced in a pulsed-liquid sequencer Procise model 494A (Applied Biosystems) following standard protocols.

Results

Replacement of Ile99 by Ala abolishes recognition by LLOwt specific CD8$^+$ T cells without affecting peptide binding to H-2K^d

To assess the influence of amino acid substitutions in the C-terminal anchor residue of the immunodominant listeriolysin epitope pLLO91-99 on peptide binding and T cell stimulatory potential, Ile99 was replaced by selected amino acids. None of the peptides shown in Table I was able to sensitize target cells for cytotoxicity when tested with T cells specific for pLLO91-99. However, binding of these peptides to K^d was not affected as strongly as might have been expected from the cytotoxic assays. In particular the variant peptide pLLO99A, harboring an Ile99 to Ala99 exchange, was found to bind to H-2K^d molecules with nearly the same affinity as the wild type peptide, although it was unable to sensitize the target cells.

LLO99A efficiently enters the MHC class I processing compartment without stimulating specific cytotoxic T cells

Using site-directed mutagenesis we introduced the Ile99 to Ala99 replacement into the listeriolysin encoding gene and purified the protein from culture supernatants of recombinant *L. innocua* using a previously published procedure (18). As displayed in Fig. 1A specific hemolytic activity of purified LLO99A (2500 HU/mg) was comparable to that of the wild type toxin (2632 HU/mg).

We have shown previously that purified LLOwt by its pore-forming activity enters the cytosol and thereby the MHC class I presentation pathway of APC (15, 16). Immunization with LLOwt therefore induces pLLO91-99 specific CD8$^+$ T cells. The

variant LLO99A however was no longer able to induce CTL responses to either the wild type peptide pLLO91-99 or to the mutant peptide pLLO99A (Fig. 1B).

To ascertain that LLO99A still has the capacity to target the cytosol of APC we made use of the capability of LLO to introduce, in addition to it self, also soluble passenger proteins into the MHC class I processing compartment (15, 16). Therefore, BALB/c mice were immunized with mixtures of LLO99A and soluble nucleoprotein (NP) from influenza virus. A $CD8^+$ T cell response against the passenger protein NP would indicate successful access of both proteins to the cytosol of the target cells. Indeed, cytotoxic T cells specific for NP were induced under these conditions but not against the LLO99A mutant (Fig 1B). Even restimulation of splenocytes from mice immunized with LLO99A with the corresponding peptide pLLO99A did not result in expansion of pLLO99A specific cytotoxic T cells as indicated by absence of specific cytolytic activity (data not shown). Thus, the conservative replacement of Ile by Ala completely abolished $CD8^+$ T cell responses against LLO99A without altering its capacity to enter the cytosol of APCs. Stimulation of $CD8^+$ T cells against subdominant determinants of LLO was not observed under these conditions as shown before using similar mutants (18, 24).

Cytotoxic T cells specific for the peptide pLLO99A can be induced in BALB/c mice but require higher antigen doses to be optimally triggered

The lack of a $CD8^+$ T cell response against LLO99A could be a consequence of the absence of specific T cells in the repertoire of BALB/c mice (5, 6, 25). To test this hypothesis, BALB/c mice were immunized with syngeneic bone marrow derived DC pulsed with pLLO99A, or pLLO91-99 as positive control. As shown in Figure 2, immunization with both the pLLO91-99 (Fig. 2A) as well as pLLO99A (Fig. 2B)

induced a specific cytotoxic T cell response. In contrast, when mice were immunized with LLOwt or LLO99A protein, only LLOwt induced a specific CTL response (Fig. 2C, D). Interestingly, T cells specific for epitope pLLO91-99 showed no crossreactivity with the epitope pLLO99A and *vice versa* although the peptides differ solely in the C-terminal anchor residue that is not expected to interact with the TCR. Thus, the full length mutant protein fails to induce CTL response *in vivo* although cells specific for pLLO99A are present in the T cell repertoire.

To explain the non-responsiveness to pLLO99A, we examined whether the affinity of specific $CD8^+$ T cells might be lower and would therefore require higher concentrations to be optimally triggered. To analyze this possibility, $CD8^+$ T cells specific for either the pLLO91-99 or the pLLO99A peptide were tested with graded amounts of the respective peptide in cytotoxicity assays. Care was taken to use identical concentrations of peptides during the priming and restimulation steps to avoid differential selection of T cells with particular affinities. As shown in Fig 3, cytotoxic T cells specific for pLLO99A required about ten times more antigenic peptide to be optimally triggered compared to pLLO91-99 specific CD8 T cells. Confirmatory results were obtained when restimulation of T cells from mice immunized with pLLO99A was carried out with graded amount of the particular peptide. No “high affinity” T cells could be obtained when low concentrations of pLLO99A were used. In contrast, low concentrations of pLLO91-99 were able to activate specific T cells (data not shown).

Taken together, BALB/c mice have the capacity to initiate a cytotoxic T cell response towards pLLO99A. However, the TCR repertoire appears to be comprised of “low affinity” receptors only. Moreover, when intracellular processing of the LLO99A is required for peptide presentation, no cytotoxic T cell response is induced.

Increase in antigen dose does not result in presentation of LLO99A

If the observed lack of a pLLO99A specific $CD8^+$ T cell recognition is exclusively due to a low TCR affinity it should be possible to compensate this by increasing the numbers of stimulatory peptide/class I complexes at the surface of APC. An increase of the amount of purified LLO99A was not possible due to the toxic effects of the hemolysin. However, cells infected with recombinant *L. monocytogenes* producing elevated amounts of LLO remain viable and functional. We infected the macrophage-like cell line J774 with recombinant *L. monocytogenes∆hlyLLO99A.* This variant lacks production of endogenous wild type LLO but overproduces the recombinant LLO99A because of the presence of the virulence transcriptional regulator PrfA in these bacteria. Since the antigen is continuously overproduced by these bacteria within infected APC, the number of peptide/class I complexes at the cell surface should be sufficient for triggering even $CD8^+$ T cells with low affinity TCRs.

While LLOwt secreted by *L. monocytogenes* EGD was readily presented and recognized by specific $CD8^+$ T cells under these conditions (Fig. 4A), J774 cells infected with *L. monocytogenes∆hlyLLO99A* were not recognized by pLLO99A specific $CD8^+$ T cells (Fig. 4B). Only target cells pulsed with the peptide pLLO99A were efficiently lysed. Thus, even high intracellular concentration of LLO99A fail to stimulate pLLO99A specific T cells when processing of the protein and peptide transport within the APC is required.

IFN-γ increases the class I presentation capacity of APC by influencing the efficiency of TAP transport and the composition of proteasomes (26-33). Therefore, we treated J774 cells with this cytokine 48 hrs before infection with *L. monocytogenes∆hlyLLO99A.* Infected cells were then tested for their capacity to

trigger pLLO99A specific T cell killing. Again, no activity was observed. Only activated J774 target cells pulsed with the pLLO99A were efficiently lysed by pLLO99A specific cytotoxic T cells (Fig. 4B).

To determine that infection of the J774 cells with *L. monocytogenes∆hlyLLO99A* was indeed efficient and that the infected cells retained their capacity to present antigen, we tested presentation of the listerial membrane protein ActA as an internal control. Since in short term assays ActA only becomes available for MHC class I presentation when intracellular bacteria are killed (34) ampicillin was added during these experiments. As shown in Fig. 4C, target cells infected with *L. monocytogenes∆hlyLLO99A* are lysed to the same extend by ActA specific cytotoxic T cells as cells infected with wild type *L. monocytogenes EGD*. This indicates that both strains infect J774 cells equally well.

In addition, viability of infected J774 cells was demonstrated by these controls since processing and presentation of the ActA protein was unaffected in cells infected with the recombinant strain hyperexpressing LLO99A. Presentation of LLO was not influenced by this treatment (data not shown). Therefore, the observed lack of activation of pLLO99A specific $CD8^+$ T cells is most probably due to an absence of presentation of the epitope by the APC as well as the low affinity of the specific TCR.

Reduced efficiency of TAP translocation due to the Ile to Ala replacement at the C-terminus of the epitope

Since the TAP transporter displays preferences regarding peptide length (35) and amino acid composition (21, 36), the aa replacement at the C-terminus of pLLO99A might influence the translocation efficiency of the peptide. In previous experiments we have shown, that presentation of passenger proteins that are introduced into the

MHC class I pathway of APC via the pore-forming activity of LLO is dependent on TAP (15). However, this has not yet been demonstrated for LLO itself. To this end, we incubated TAP-deficient T2.K^d cells with native LLOwt or pLLO91-99 and used these cells as targets in cytotoxicity assays. As indicated in Figure 5A only T2.K^d cells pulsed with the pLLO91-99 were efficiently lysed by LLOwt specific cytotoxic T cells while T2.K^d cells pulsed with the native protein LLOwt were not capable of stimulating cytotoxic T cells. On the other hand, T2.K^d cells that were reconstituted with a functional TAP, presented peptide and protein equally well (Fig. 5B). Thus, the presentation of pLLO91-99 by MHC class I requires TAP-dependent transport from the cytosol to the ER.

Murine TAP preferentially transports peptides with an aliphatic C-terminus. Hence, the Ile to Ala exchange might result in a decreased translocation rate. This indeed turned out to be the case. In a TAP translocation assay, pLLO99A displayed a 10-fold reduced competitive capacity compared to pLLO91-99 (Fig. 6). This is consistent with a 10-fold lower translocation efficiency of the mutant peptide.

Since TAP has been shown to transport N-terminally extended epitope precursors (37, 38) it might also differentially translocate N-terminally extended precursor of pLLO91-99 *in vivo*. Therefore we included pLLO91-99 or pLLO99A peptides N-terminally elongated by 1, 2 or 3 aa of the original sequence in our transport competition assay. In every case, we found wild type peptides competing more efficiently than the mutant peptides (data not shown).

The Ile to Ala exchange alters the proteasomal cleavage pattern

Although the 10-fold higher concentration requirement of pLLO99A specific T cells together with the tenfold lower efficiency of TAP transport of pLLO99A should have a

severe effect on the induction of $CD8^+$ T cells against the epitope pLLO99A it can not explain the complete absence of this reaction. Since proteasomes are involved in the degradation of listeriolysin (39) it was possible that changing the Ile99 to Ala also altered the proteasomal cleavage pattern. As has been shown previously, changing aa`s either within the epitope or its flanks can strongly influence the cleavage preference of the proteasome (40-44). We therefore performed *in vitro* proteasomal digestion of 24meric petides that contained the pLLO91-99 or pLLO99A epitopes and were extended at the N- and C-termini by aa`s that naturally flank them. The resulting proteolytic fragments were analyzed by mass spectrometry and Edman degradation.

Reproducibly and as indicated by arrow sizes, the most dominant cleavage sites are located after Gly9, Tyr10, and Asp12 (corresponding to Gly91, Tyr92 and Asp94) in both the pLLO91-99 and pLLO99A peptide (Fig. 7). The only significant difference in the cleavage and fragment pattern generated from the two synthetic peptides is the cleavage after Y16 and the aa at position 17 where the two peptides differ by Ile or Ala. Both cleavage sites are used in both peptides, but to a different degree. In the extended pLLO91-99 peptide the cleavage after Ile17 dominates over the cleavage after Tyr16. This could be shown by the detection of a strong MALDI-signal for fragment 1-17 present already early in the digest (data not shown). For the extended pLLO99A peptide however, the situation is exactly the opposite. In MALDI-spectra, the fragment 1-16 dominates clearly over the fragment 1-17 as quantified by nanospray MS. This is indicated by the thickness of the horizontal bars in Fig. 7. MALDI spectra at 25 hrs revealed that the fragment 1-17 dominates the digestion pattern of the extended pLLO91-99 peptide, while the fragments 1-16 and 1-17 are found in equal amounts in the digest of the extended pLLO99A peptide (data not shown).

The most convincing indication, however, is obtained by Edman sequence analysis of unfractionated digests at 25 hrs. The fragment 17-24 starting with Ala was detectable in large amounts in the digest of the variant peptide while in the digests of the wild type peptide, no fragment starting with Ile17 could be detected. Only minute amounts of fragment 17-24 were found in the digest of wild type peptide by sensitive MS analysis (data not shown).

Thus, our data strongly suggest that during proteasomal processing of pLLO91-99, generation of the correct C-terminus of the T cell epitope is favored over its proteolysis. The opposite is found for the processing of pLLO99A. This was confirmed by analyzing the cleavage pattern of both peptides *in silico* (www.mhc-pathway.net). Scores obtained using this software can be interpreted as logarithms of the probability of cleavage site usage, thus, the difference between two scores can be directly translated into a difference of amounts. Consistent with our *in vitro* data, the constitutive proteasome is predicted to cleave LLOwt between Tyr16 and Ile17 less efficient than LLO99A between Tyr16 and Ala17 (estimated score 1,4 for LLOwt compared to 1,6 for LLO99A), thus destroying the epitope to a certain extend. Importantly, the immunoproteasome as will be found in activated J774 cells is predicted to even more efficiently cleave LLO99A between Tyr16 and Ala17 (estimated score 1,95 compared to 1,5 for LLOwt), i.e. the epitope pLLO99A is predicted to be destroyed to even a larger degree by the immunoproteasome than by the constitutive proteasome. Immunoproteasomes are probably the type of proteasomes present in our J774 subline, since untreated cells already constitutively express low amounts of MHC class II, a marker for activation. In conclusion, the proteasome-dependent part of LLO processing favors the generation of the pLLO91-

99 epitope whereas the pLLO99A is destroyed to a large extent by proteasomal processing.

Discussion

Originally it was postulated that the affinity between peptide and MHC class I molecule is the major factor in determining epitope immunogenicity (45, 46). However, now it is clear that many additional factors contribute to the decision as to whether or not an epitope becomes available for MHC class I presentation (47-49) and especially becomes the dominant target of an immune response. In this study, we systematically analyzed the different steps of antigen recognition, processing and presentation that under normal conditions renders the listeriolysin O extremely immunogenic. We followed the impact of a single amino acid exchange within the dominant epitope of listeriolysin on MHC class I antigen presentation and found that changing the C-terminal anchor residue of pLLO91-99 from Il99 to Ala99 does not alter the peptide binding-affinity to isolated H-2K^d. Nevertheless, MHC class I presentation of the mutanted protein was abrogated. This indicates that the selection of the dominant epitope is mainly determined by appropriate proteasomal processing and TAP transport rather then binding capacity to MHC class I molecules.

Studies by Hahn et al. have shown that a mutation within the influenza hemagglutinin T cell epitope HA210-219 from Leu219 to Ala resulted in an epitope that only weekly bound K^d (42). In case of pLLO99A the Ala in position 9 of the nonameric peptide obviously can substitute for the anchor amino acid Ile. The binding capacity of pLLO99A to K^d was comparable to that of the wild type epitope pLLO91-99. Nevertheless, CD8$^+$ T cells raised against the pLLO91-99 determinant did not recognize pLLO99A. Similar results were obtained with other peptides in which exchanges at aa99 had been carried out. Since anchor residues and especially the C-terminus of a peptide should face inwardly into the binding groove of

class I molecules they should not be in contact with the TCR. Thus, even conservative replacements at this aa position of LLO91-99 must have significant influence on the conformation of the peptide or the class I molecule in order to affect interactions of contact residues with the TCR. This issue is presently investigated in more detail. Similar observations have been made with a conservative exchange at the second anchor residue of this epitope (17, 23). Along this line, exchange of non-TCR contact residue within an epitope has been shown before to result in antagonism of T cell recognition (50, 51).

Selective forces on the presentation of particular peptides have impressively been demonstrated by Davenport et al. previously (52). Comparing naturally presented peptides and peptides bound from mixtures to recombinant MHC class I molecules *in vitro,* they found similar amino acid distribution within the core of the epitopes. However, the C-terminus of peptides eluted from the *in vitro* loaded HLA-A2 and HLA-B8 molecules was clearly different from peptides eluted from the same molecules loaded under physiological conditions. Natural peptides showed a strong preference for hydrophobic C-termini which was not observed in the experimental system. In case of peptides eluted from *in vitro* loaded HLA-B8 the yield of Ala at the C-terminus was found to be even higher than Ile, Val, and Leu that were observed normally. This is in line with our results. We demonstrate that Ala at the C-terminus of the LLO91-99 epitope results in binding to MHC class I H-2K^d with almost the same affinity as the wild type peptide does. Thus, the observed preference for hydrophobic aa at the C-terminus of peptides binding to K^d is most likely an effect of epitope processing and transport rather than peptide binding. This hypothesis is supported by extensive studies on immunodominance and subdominance of lymphocytic choriomeningitis virus (LCMV) in a mouse model. Although the gp276 determinant

exhibits a higher affinity to isolated H-2D^b then the dominant epitope gp33 and was highly efficient in stimulating lysis of LCMV infected target cells in adoptive transfer experiments (53, 54), it remains a subdominant epitope during viral infections.

The Ile to Ala substitution in pLLO99A negatively influences the TAP translocation efficiency. This was not unexpected as the correct C-terminal residue is known to play a critical role for TAP transport (55). Murine TAP preferentially translocates peptides with hydrophobic C-termini. Indeed exchanging this residue in LLO99A led to a 10-fold decrease in translocation efficiency compared to the wild type epitope. In line with these findings is the significant effect of the affinity of TAP for a particular epitope and its presentation as described by Fruci et al. (56).

The combined effects of a) a TCR that needs around 10 times more antigen to be optimally triggered and b) the inefficient TAP-dependent transport of the epitope could explain a reduction of pLLO99A presentation to a level ineffective for triggering cytotoxic T cells. However, if antigen presentation capacity would only be reduced at these steps it should be possible to overcome this defect by using higher amounts of antigen. Studies performed by Chen and Niedermann (48, 57) on the presentation of a subdominant ovalbumin epitope indeed demonstrated that the lower binding capacity of the subdominant epitope Ova55-62 and its inefficient proteolytic processing can be compensated by the use of high ovalbumin concentrations. In our case, even loading APC with excessive amounts of intracellularly produced LLO99A did not result in T cell stimulation. This suggests that the observed absence of T cell stimulation by LLO99A is not simply a consequence of a reduced TAP transport and reduced TCR affinity but due to a severe defect in processing of the pLLO99A epitope.

In vitro proteasome digestion profiles of mutant and wild type 24meric peptides clearly indicated that the exchange of the C-terminal anchor residue of the LLO T cell epitope changes the cleavage preference of the proteasome. This effect is caused by the aa substitution itself as under our experimental conditions positional or flanking effects on proteasomal cleavage preference could be excluded due to the fact that the mutation was introduced in the original aa sequence without alterations within the flanking region. These results are in line with proteasomal cleavage motifs described by Nussbaum et al. (58) where Tyr is a preferred C-terminal residue at the P1 position of an epitope after cleavage by the chymotryptic activity of yeast and human 20S proteasomes (59 and unpublished observations). According to these cleavage motifs a small amino acid is preferred at the position C-terminal of the cleavage site (P1´-position). Thus, the mutation from Ile to the smaller amino acid Ala at the C-terminus of the T cell epitope could explain the shifting of the dominant proteasomal cut from Ala17 to Tyr16.

Several studies have been performed by now showing that mutations within an epitope or its flanking region can influence the proteasomal cleavage pattern (40-44, 59-61). Some of them indicate a strong effect of Ala-spacing on the processing frequency of an epitope (59, 60). Single as well as penta Ala-spacers were found to improve the antigen presentation capacity of a nonameric epitope by enhancing the proteasomal cut between the correct C-terminus Leu and the flanking Ala. This is in line with our results since we also observed a strong preference of the proteasome to cleave before Ala17 rather then before the natural flanking aa Val18. The introduction of an Ala in the C-terminal position of the LLO T cell epitope apparently functions as a cleavage signal leading to the destruction of most of the T cell epitope by the proteasome.

The hydrolysis of proteins by proteasomes obviously represents the key step in the generation of most antigenic peptides (reviewed in 62). Although proteasomes are needed for the presentation of most antigens, including LLO, they in fact destroy more epitopes than they generate (63, 64). Consistent with this, we observed during *in vitro* proteasomal digestion of 24meric precursors major cleavage events after Gly9, Tyr10, and Asp12, which all result in destruction of the pLLO91-99 and pLLO99A eptitope. The only significant difference in the proteasomal cleavage pattern of wild type versus variant 24meric peptide, however, was the cleavage at position 16 and 17, respectively. For various epitopes it has been demonstrated that N-terminally extended precursors are the main proteasomal products generated (65, 66). In contrast, proteasomal activity is required to process the correct C-terminus needed for proper binding to MHC class I molecules. This is due to the lack of carboxypeptidases in the cytosol and the endoplasmic reticulum (67). Indeed, by cleaving the wild type peptide precursor at position Ile17 the proteasome generates an N-terminally extended 17mer that exhibits the correct C-terminus. In case of the variant precursor, a major cleavage takes place at position Tyr16 deleting the C-terminal anchor position. Only minute amounts of the 17mer precursor with the correct C-terminus were detectable in proteasomal digests.

The proteasomal degradation of a protein is the initial step in generating an antigenic epitope, and most precursors extended N-teminally are processed by aminopeptidases in the cytosol and the endoplasmic reticulum (68). These down stream processes may also be involved in the establishment of dominance of the pLLO91-99 epitope. Clearly, *in vitro* proteasomal digestion of extended pLLO91-99 as well as pLLO99A peptide did not result in the generation of the correct nonameric peptide but only to N-terminally extended epitope precursors (Fig. 7). Therefore, it is

very likely that aminopeptidases have a major impact on the processing of the correct epitope.

Little is known about the specificities of aminopeptidases residing in the cytosol and ER. Although some of them appear to destroy many antigenic peptides (69, 70) they exhibit the potential to generate epitopes that match the requirements for MHC class I binding (71, 72). Overexpression of the IFN-γ inducible ER aminopeptidase 1 (ERAP1) has been shown to result in enhanced processing of the SIINFEKL epitope from an N-terminally extended precursor peptide (73) while inhibition of ERAP1 leads to downregulation of MHC class I presentation (73, 74). Moreover, ERAP1 exhibits peptide selectivity and differential effects on antigen presentation by various MHC class I molecules (75). In addition, ERAP1 strongly prefers peptide substrates with hydrophobic C-terminal residues as described (67). Such impact of post proteasomal trimming on the generation of the immunodominant epitope pLLO91-99 has not been studied so far. However, the sequence preference of some of the down stream proteases might affect the correct trimming of the epitope.

A similar discrepancy between the ability of CTL to lyse peptide pulsed APC and their lack of lysing cells that express the full length protein has been described by Valmori et al. (76). As in our study, inappropriate processing of the C-terminus of the antigenic peptide was identified as the factor limiting presentation. Interestingly, peptide presentation was restored by addition of the proteasome inhibitor lactacystin. Inhibition of the proteasomal trypsin- and chymotrypsin-like activity prevented destruction of the epitope in that case and allowed presentation by MHC class I. In our hands, treatment of APC with lactacystin did not result in restoration of processing of pLLO99A from LLO99A (data not shown).

Basler et al. have demonstrated that immunoproteasomes, but not constitutive proteasomes lead to the preferential destruction of the gp276 epitope in LCMV infected cells. This indicates the participation of the IFN-γ inducible proteasomal subunits on shaping the epitope hierarchy of CTL responses *in vivo* (47). No effect on the presentation of the wild type or the lack of presentation of the variant listeriolysin epitope was observed by the addition of IFN-γ to APCs. Thus, the composition of the proteasome is most likely excluded as decisive factor for the dominance of pLLO91-99. This is in agreement with predictions of cleavage patterns of immunoproteasomes as revealed by the mhc-pathway software (www.mhc-pathway.net).

The fact that intracellular LLO secretion by *Listeria monocytogenes* is drastically down regulated shortly after infection but nevertheless still suffices to accumulate enough pLLO91-99/H-2K^d complexes to stimulate a primary protective CTL response towards the pathogen suggests an extremely rapid and efficient processing and presentation of the toxin. A conservative single aa exchange at the C-terminal anchor position of the immunodominant epitope pLLO91-99 had, with the exception of peptide / MHC binding, a dramatic effect on all major steps involved in generation and presentation of an antigenic determinant. This includes interference with proteasomal digestion, TAP transport and T cell recognition, resulting in the complete loss of immunogenicity of the formerly highly immunogenic listeriolysin. Thus, our data underline a highly optimized coordination of processing and presentation of pLLO91-99 that suggests a role if not a major role in the establishment of immunodominance towards this particular epitope. It also demonstrates the complexity of molecular mechanisms that lead to the dominance of

a single MHC class I presented epitope from an organism that in principal could provide thousands of epitopes for T cell recognition.

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Legends to the figures

FIGURE 1: LLO99A exhibits full pore forming activity and enters the cytosol of cells. (A) Hemolytic activity of DTT-activated LLOwt and LLO99A were compared in a standard hemolysis assay using sheep erythrocytes as target and declining listeriolysin concentrations as indicated. (B) Splenocytes from BALB/c mice immunized with mixtures of 1µg LLOwt + 2,5µg NP, 1µg LLO99A + 2,5µg NP or 2,5µg NP alone in incompletes Freund´s adjuvant (iFA) were used *ex vivo* as effector cells at an E/T ratio of 50:1 on P815 cells sensitized with 10µM of the indicated peptides. All assays were performed in triplicates and SEM never exceeded 10%. One representative result from three independent experiments is shown.

FIGURE 2: Cytotoxicity of T cells against the pLLO99A. Restimulated spleen cells from BALB/c mice that were immunized with syngeneic DC pulsed either with 10 µM pLLO91-99 (**A**) or pLLO99A (**B**) or alternatively with purified 1 µg LLOwt (**C**) or LLO99A (**D**) in iFA were tested at an E/T ratio of 25:1 on P815 cells sensitized with 10µM pLLO91-99, pLLO99A or pNP147-155. All assays were performed in triplicates and SEM never exceeded 10%. One representative result from three independent experiments is shown.

FIGURE 3. Concentration dependency of cytotoxic T cell specific for pLLO91-99 and pLLO99A. BALB/c mice were immunized with DC pulsed with either 10 µM pLLO91-99 or pLLO99A. Restimulated splenocytes were analyzed for cytotoxicity in a standard JAM-assay on P815 target cells loaded with increasing amounts of peptide as indicated in the figure. Squares indicate splenocytes from pLLO91-99

sensitized mice, triangles indicate splenocytes derived from mice immunized with pLLO99A. The E/T-ratio was 20:1. All assays were performed in triplicates and SEM never exceeded 10%. One representative result from three independent experiments is shown.

FIGURE 4. Overexpression of LLO99A in infected cells does not restore antigen presentation capacity. (**A and B)** J774 target cells, either non-activated or activated with INF-γ 48 hrs before the assay, were infected with *L. monocytogenes* EGD or *L. monocytogenes∆hlyLLO99A* overexpressing LLO99A. Alternatively, APC were pulsed with either 10 µM peptidepLLO91-99 or pLLO99A. Restimulated splenocytes from BALB/c mice that were immunized with LLOwt (A) or pLLO99A loaded syngeneic spleen cells (**B**) were added at an E/T ratio of 25:1. (**C)** Infection and functional integrity of APC was controlled by infection with *L. monocytogenes* EGD or *L. monocytogenes∆hlyLLO99A* and testing for presentation of the listerial membrane protein ActA. ActA-specific cytotoxic T cells were added at an E:T-ratio of 15:1 in the presence of ampicillin to kill intracellular bacteria necessary for ActA presentation. The efficient presentation of the ActA epitope indicates the effective infection with *L. monocytogenes∆hlyLLO99A* and integrity of APC. All assays were performed in triplicates and SEM never exceeded 10%. One representative experiment out of three is shown.

FIGURE 5. Presentation of pLLO91-99 requires functional TAP transport. TAP deficient T2.K^dcells (**A)** or TAP reconstituted T2.K^dTAP cells (**B)** were incubated with 1 µg/ml LLOwt, 10 µM pLLO91-99 or left untreated. LLOwt specific cytotoxic T cells were added 2 hours later at an E:T ratio of 25:1. All assays were performed in

triplicates and SEM never exceeded 10%. One representative experiment out of three is shown.

FIGURE 6. Reduced TAP translocation efficiency due to the Ile to Ala substitution. Permeabilized RMA cells were treated with radioiodinated reference peptide and unlabeled pLLO91-99 or pLLO99A as competitors. Ratio indicates 10, 100, or 1000 fold excess of the competitor over the reference peptide. TAP deficient RMA-S cells were included as negative control, RMA cells treated with the reference peptide alone served as positive control. 10-fold higher concentrations of pLLO99A are required to obtain competition similar to pLLO91-99 indicating a 10-fold lower transport efficiency of the variant petide. One out of two independent experiments with similar results is shown.

FIGURE 7. Qualitative and quantitative comparison of fragments in digests of extended pLLO91-99 and pLLO99A peptide. (**A**)The figure shows the analysis of a 25h proteasomal digest. At this time, approximately 50-70% of the synthetic peptide was still undigested. Horizontal bars indicate the major products obtained in these digestions revealed by MALDI-TOF analysis. Thickness of the bars represents the semi-quantitative estimation of the digestion products. Earlier time points showed that the dominant fragments are produced early on in the digest and accumulate over time. Internal fragments (e.g. 5-17 in the WT, 4-16 in the mutant) are only produced later during digestion. Some minor fragments and cleavage sites were left out for clarity of presentation. Arrow sizes reflect relative quantities as determined by Edman sequencing and mass spectrometry. Controls without proteasomes showed that there was no spontaneous auto-hydrolysis of the synthetic peptide under digestion

conditions. **Diamont**: This fragment was not detected in the digest of the pLLO99A 24meric peptide. **Circle**: This fragment was not detectable in the pLLO91-99 24meric peptide. One out of two experiments with similar results is shown.

Table I. Binding and stimulatory capacity of variant LLO peptides

peptide code	sequence	k_{off}/k_{on} (nm)	ratio	stimulation[2)]
pLLO91-99	GYKDGNEY**I**	0.58	1.0 [1)]	**+**
pLLO99A	GYKDGNEY**A**	0.65	1.1	**-**
pLLO99Y	GYKDGNEY**Y**	4.5	7.8	**-**
pLLO99Q	GYKDGNEY**Q**	3.3	5.7	**-**
pLLO99S	GYKDGNEY**S**	1.5	2.6	**-**
pLLO99F	GYKDGNEY**F**	1.3	2.2	**-**
pLLO99G	GYKDGNEY**G**	3.0	5.2	**-**
pLLO99N	GYKDGNEY**N**	2.7	4.7	**-**
pLLO99T	GYKDGNEY**T**	1.2	2.1	**-**
pNP147-155	TYQRTRALV	0.45	0.8	**-**

1) Value arbitrarily set to 1.
2) Capacity of the peptide to stimulate pLLO91-99 specific cytotoxic T cell response at an E:T ratio of 20:1; + corresponds to specific lysis > 60%, - corresponds to lysis at background level.

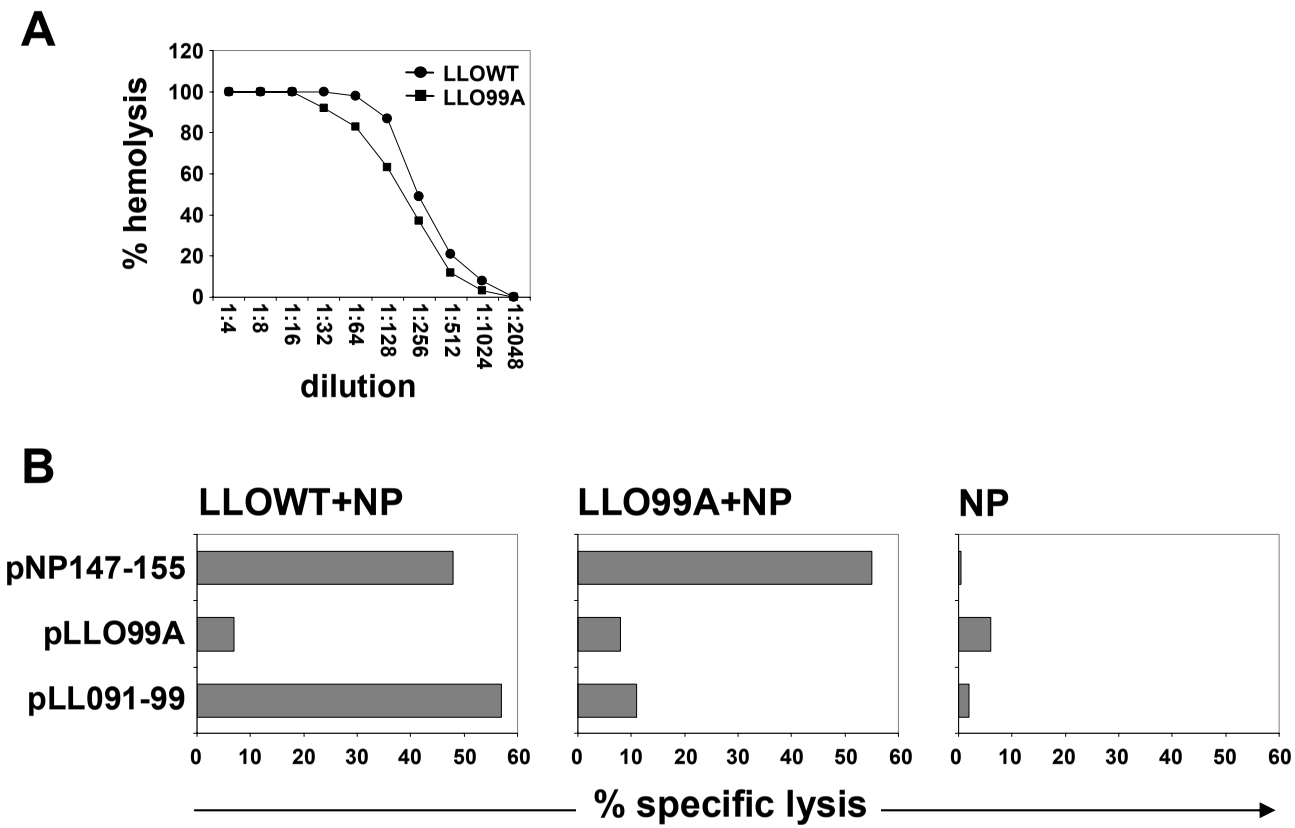

A
% hemolysis
120
100
80
60
40
20
0
LLOWT
LLO99A
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
1:2048
dilution
B
LLOWT+NP
LLO99A+NP
NP
pNP147-155
pLLO99A
pLL091-99
0
10
20
30
40
50
60
% specific lysis

Figure 1

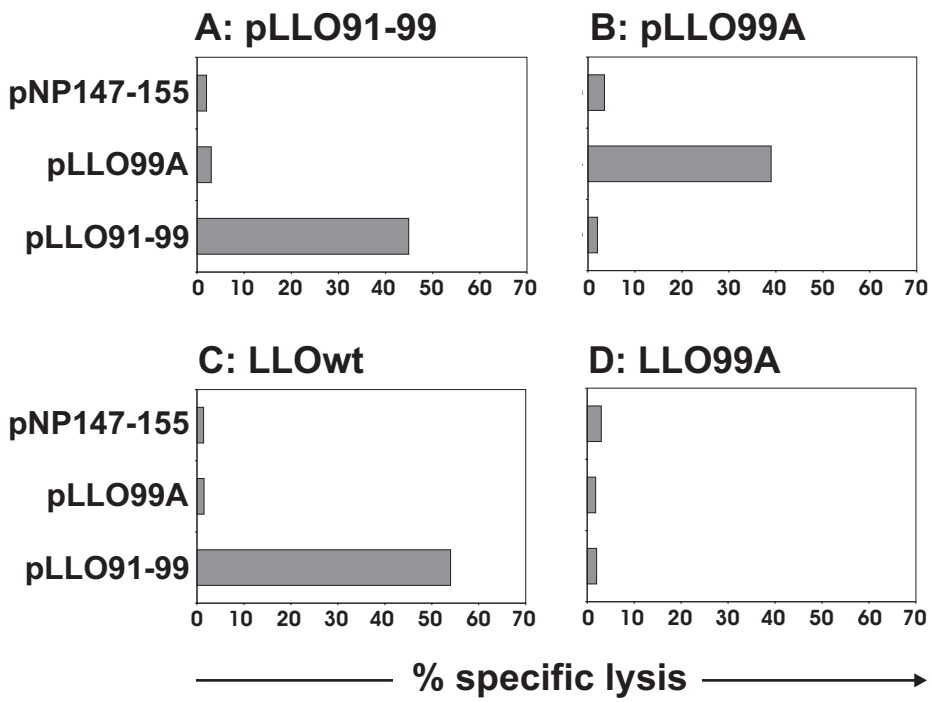

A: pLLO91-99
B: pLLO99A
C: LLOwt
D: LLO99A
pNP147-155
pLLO99A
pLLO91-99
0 10 20 30 40 50 60 70
% specific lysis

Figure 2

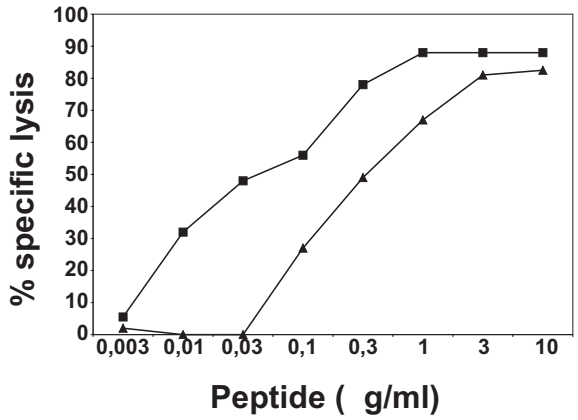

100
90
80
70
60
50
40
30
20
10
0
% specific lysis
0,003
0,01
0,03
0,1
0,3
1
3
10
Peptide (g/ml)

Figure 3

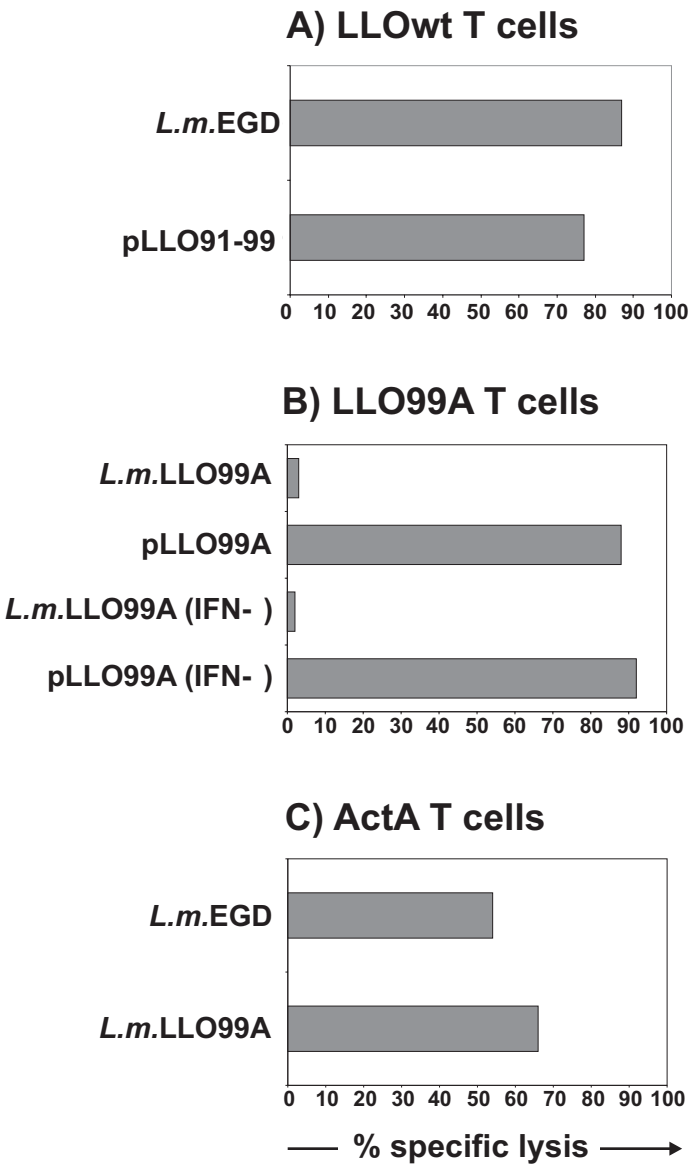

A) LLOwt T cells
L.m.EGD
pLLO91-99
0 10 20 30 40 50 60 70 80 90 100
B) LLO99A T cells
L.m.LLO99A
pLLO99A
L.m.LLO99A (IFN-)
pLLO99A (IFN-)
0 10 20 30 40 50 60 70 80 90 100
C) ActA T cells
L.m.EGD
L.m.LLO99A
0 10 20 30 40 50 60 70 80 90 100
% specific lysis

Figure 4

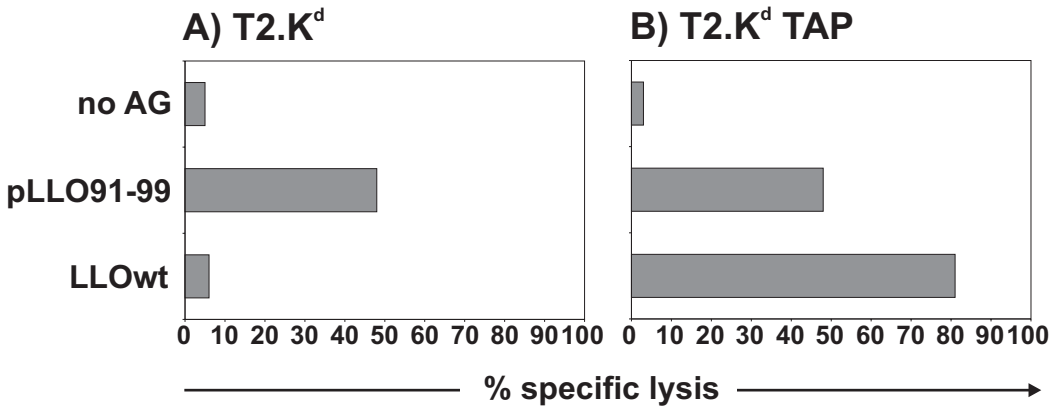
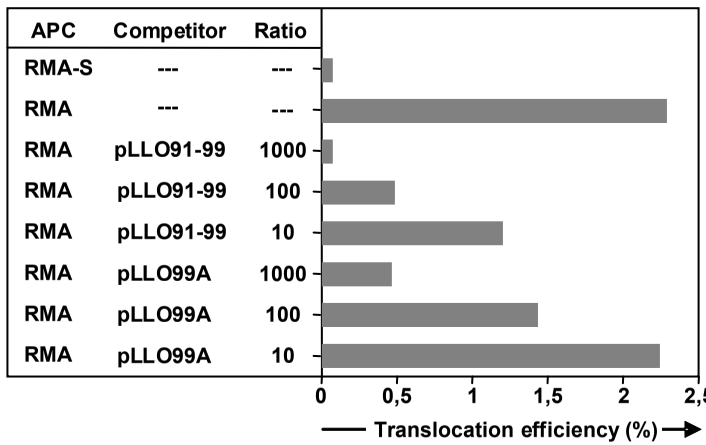

A) T2.K^d
B) T2.K^d TAP
no AG
pLLO91-99
LLOwt
0 10 20 30 40 50 60 70 80 90 100
0 10 20 30 40 50 60 70 80 90 100
% specific lysis

Figure 5


APC
Competitor
Ratio
RMA-S

RMA

RMA
pLLO91-99
1000
RMA
pLLO91-99
100
RMA
pLLO91-99
10
RMA
pLLO99A
1000
RMA
pLLO99A
100
RMA
pLLO99A
10
0
0,5
1
1,5
2
2,5
Translocation efficiency (%)

Figure 6

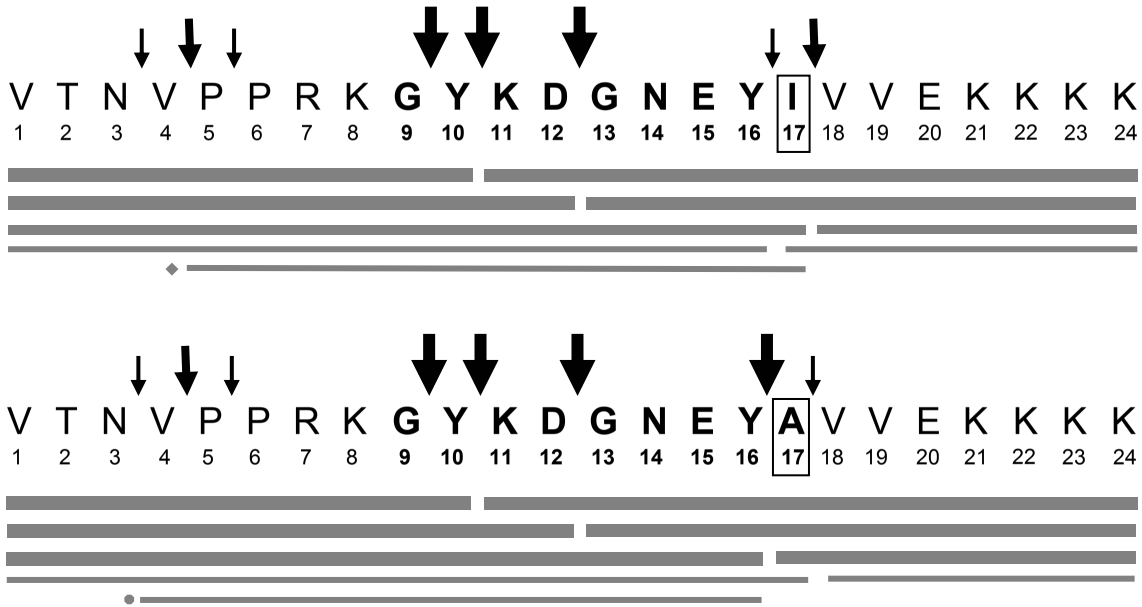

V T N V P P R K G Y K D G N E Y I V V E K K K K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
V T N V P P R K G Y K D G N E Y A V V E K K K K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 7