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Sculpting MHC class II-restricted self and non-self peptidome by the class I antigen-processing machinery and its impact on CD4+ TCR repertoire and Th cell responses

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Abstract

It is generally assumed that the MHC class I antigen (Ag)-processing (CAP) machinery —which supplies peptides for presentation by class I molecules— plays no role in class II-restricted presentation of cytoplasmic Ags. In striking contrast to this assumption, we previously reported that proteasome inhibition or TAP- or ERAAP-deficiency led to dramatically altered T helper (Th) cell responses to allograft (HY) and microbial (*Listeria monocytogenes*) Ags. Herein, we tested whether altered Ag processing and presentation, altered $CD4^+$ T cell repertoire, or both underlay the above finding. We found that TAP- and ERAAP-deficiency dramatically altered the quality of class II-associated self peptides suggesting that the CAP machinery impacts class II-restricted Ag processing and presentation. Consistent with altered self peptidomes, the $CD4^+$ T cell receptor repertoire of mice deficient in the CAP machinery substantially differed from that of wildtype animals resulting in altered $CD4^+$ T cell Ag recognition patterns. These data suggest that TAP and ERAAP sculpt the class II-restricted peptidome, impacting the $CD4^+$ T cell repertoire, and ultimately altering Th cell responses. Together with our previous findings, these data suggest multiple CAP machinery components sequester or degrade MHC class II-restricted epitopes that would otherwise be capable of eliciting functional Th cell responses.

Keywords

antigen presentation; mass spectrometry; T helper cells; self peptidome; MHC

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Introduction

$CD4^+$ Th cells regulate multiple cellular and humoral responses to pathogenic microbes and parasites to protect against infectious diseases. These cells sense infections by recognizing short microbial peptides presented by MHC class II molecules on the cell surface of antigen (Ag)-presenting cells (APCs). Hence, alterations or deficiencies in factors that control class II-restricted Ag processing and presentation can alter the display of self and microbial peptides by APCs. Alterations in the presented self peptide repertoire (peptidome) can change the $CD4^+$ T cell repertoire that are activated in response to an infection, which in turn can affect the host's susceptibility to infectious disease.

Th cells recognize endogenous cytosolic as well as exogenous Ags. The mechanisms controlling exogenous class II-restricted Ag presentation are quite well established [1,2]. Nonetheless, endogenous cytosolic Ag presentation by class II molecules is less well understood. Endogenous cytosolic Ags existing within professional APCs are presented by class II molecules when they are delivered to the endo/lysosomes. These Ags are delivered to these compartments by various autophagic mechanisms —macro-autophagy [3–7] or chaperone-mediated autophagy [8–10]— and processed therein for presentation to $CD4^+$ T cells [11–17]. Alternatively, cytosolic Ags expressed by class II-negative cells —such as allograft, tumour and infected cells— are acquired by phagocytosis. Professional class II-positive APCs (e.g., dendritic cells (DCs) and macrophages (MΦs) phagocytose dying cells and process Ags into short peptides within the phago-lysosomes, assemble with class II molecules and are displayed at the cell surface [18–20]. This process, termed indirect presentation, was originally described to explain solid organ allograft rejection.

Newer data suggests that this dogmatic separation of class I and class II Ag processing and presentation is not so absolute. Interdependence between these two processing pathways has been observed either within the presenting APC or in damaged neighboring (donor) cells. As we reported previously, class II-restricted cytosolic Ags are exposed to modification by components of the MHC class I antigen processing (CAP) machinery in both the presenting and donor cells [21]. This modification is evident in animal models deficient in the CAP components TAP and ERAAP where an altered basal class I-restricted peptide repertoire is displayed [22–26]. However, the effect of their absence on the class II-restricted peptide repertoire has not been fully explored. Certain class II-restricted Ags, including several self peptides, that are dependent upon the actions of the CAP machinery have been identified [12–15,21,27–31]. Nonetheless, other investigators have not seen a dependence upon this processing machinery for class II-restricted Ag presentation [17,32–34]. Despite the identification of a few peptides that depend on CAP machinery for presentation, the global impact the CAP machinery has on the self and non-self peptidome remains unknown. Moreover, although previous studies have observed differences in Ag presentation, no notable alterations in the frequencies of TCR Vβ usage in TAP-deficient animals for either $CD4^+$ or $CD8^+$ T cells were observed [35]. It is therefore unclear whether the class II-restricted $CD4^+$ T cell repertoire is impacted by the CAP machinery.

We recently showed that $CD4^+$ T cell recognition of indirectly presented cytosolic, class II-restricted self (HY minor histocompatibility Ag) and non-self (*Listeria monocytogenes* (*Lm*)) peptides was enhanced in the absence of the CAP components TAP and ERAAP [21]. Curiously however, the donated HY alloantigen entered the cytosol of acceptor APCs and required LMP2- dependent immunoproteasomes for presentation [21]. Moreover, the effects of CAP components on HY alloantigen presentation were neither due to competition between class I and class II Ags nor due to competition between $CD4^+$ and $CD8^+$ T cells. They were also not caused by enhanced MHC class II, B7.1, B7.2, calreticulin or HSP90 expression nor enhanced macro-autophagy, or enhanced ER-associated degradation. Hence,

we concluded from that study that the CAP machinery must regulate the quantity and/or quality of peptides available for presentation by class II molecules. Hence, we hypothesized that by regulating the class II-restricted peptidome, CAP components could alter the robustness of the Th response to class II-restricted Ags [21].

We now report direct evidence that TAP and ERAAP influence the available class II-associated peptide pool. In their absence, a nearly unique self peptidome is displayed by $H2A^b$ molecules. These findings emerged from amino acid sequence analyses of the class II-associated self peptidomes isolated from wild type, $TAP^{-/-}$ or $ERAAP^{-/-}$ splenocytes. As previously described [35], we also found insubstantial alterations in the TCR Vβ usage. Nonetheless, we observed significant changes within the Ag-binding complementarity determining region 3 of TCR β-chains (CDR3β) expressed by $CD4^+$ T cells. Consistent with altered Ag processing and presentation and an altered TCR diversity, we found that functional Th responses to $H2A^b$-restricted vaccinia viral (VACV) epitopes were also altered. $TAP^{-/-}$ mice recognized novel epitopes not recognized by wild type mice and, conversely, had lost recognition of some epitopes recognized by wild type mice. Our in-depth analysis of the self peptidome, mature TCR repertoire and Th responses suggests that the CAP machinery meaningfully sculpts class II-restricted Ag presentation likely through sequestration or degradation of potential epitopes.

Results

TAP and ERAAP sculpt the self peptidome

Previous reports have documented an altered endogenous class I-associated self peptide repertoire (peptidome) in the absence of the CAP components TAP or ERAAP [22–26]. Recently, increasing interdependence of the class I- and class II-restricted Ag processing pathways and the identification of several class II-restricted peptides that require the activity of components of the CAP machinery have been reported [12–15,27–31]. This led us to query whether the basal class II-associated self peptidome might also have a similar dependence on TAP and/or ERAAP. To this end, class II-associated peptides were eluted from affinity purified $H2A^b$ molecules expressed by wild type, B6.129-$TAP^{-/-}$, B6.129-$ERAAP^{-/-}$ and B6.129-$H2Ab^{-/-}$ splenocytes. Importantly, deficiency in either TAP or ERAAP did not alter the frequency of APC within the spleen. Nor was the cell surface phenotype (e.g., class II and co-receptor CD80 and CD86 expression) different than wild type (data not shown; [24,25]). The recovered peptides were fractionated by reversed-phase chromatography (RPC) and their sequence deduced by LC-MS/MS tandem mass spectrometry (Fig 1; S1).

The mass/charge (*m/z*) pattern generated by MS/MS was compared against a dataset consisting of the *m/z* patterns of theoretical and known peptide sequences. The degree of concordance between these two patterns was assigned a cross correlation score X_{corr} (Cn). Higher Cn values are assigned to those peptides whose *m/z* pattern showed greater concordance between the observed and expected *m/z* patterns [36]. Only peptides with a Cn>1.5 were considered to be possible peptide sequences. However, the larger the Cn value the more confidence is placed in the peptide sequence identification. In addition, greater differences in the Cn values between the top two most likely peptide sequence identifications (ΔCn) provides greater confidence in the identification. Therefore, peptides with a highly confident identification were considered to have a Cn score >3.0 and ΔCn >0.2. Overall, this dataset had an average Cn = 3.536 and ΔCn = 0.324. In addition, 44% of the peptides had only a single possible sequence identification for which no ΔCn can be calculated.

To ascertain the specificity of the bound peptides, materials eluted from control $H2A^b$-deficient cells were isolated and analyzed by the same methods. We found that only ~7% of the peptide sequences (Cn >1.5) identified in wild type, $TAP^{-/-}$ and $ERAAP^{-/-}$ samples were also present in the control $H2Ab^{-/-}$ eluates (data not shown). These were largely derived from three sources; a, Ig —likely representing the antibody used for immunoaffinity purification or splenic Ig that bound to protein A Sepharose used to prepare the immunoaffinity column; b, complement —perhaps because they bind Ig; and c, fibronectin, fibrinogen and other secreted proteins — likely representing unspecific contaminants of the purification. Few peptides were derived from cytosolic/intracellular proteins as well. Hence, peptide sequences that matched those isolated from $H2Ab^{-/-}$ splenocytes were considered an artifact of the purification. Such peptide sequences with Cn>1.5 when present in wild type, $TAP^{-/-}$ and $ERAAP^{-/-}$ samples were removed from all downstream analyses.

Analysis of the peptides identified with high confidence in (Cn>3.0 and ΔCn>0.2) eluted from wild type, $TAP^{-/-}$ and $ERAAP^{-/-}$ splenocytes surprisingly revealed little overlap between the peptides displayed by wild type cells and either $TAP^{-/-}$ or $ERAAP^{-/-}$ cells (Fig 2; Table S1). Only 22.5% of the $H2A^b$-restricted self peptide sequences displayed by wild type cells were also presented by $TAP^{-/-}$ or $ERAAP^{-/-}$ cells (Fig 2*A*). In a different project, replicate MS samples that consisted of peptides with similar confidence levels eluted from MHC molecules, demonstrated a 63% concordance (SBC, CTS, AJL and SJ, unpublished data). Since class II-associated peptides expressed by wild type and CAP deficient cells have only 22.5% overlap, the differences in the wild type and CAP peptidomes are likely real and not caused by irreproducibility in the experiment. Conversely, 18.4% of self peptide sequences displayed by $TAP^{-/-}$ cells were presented by wild type cells, while 33% of self peptide sequences displayed by $ERAAP^{-/-}$ cells were presented by wild type cells. This lack of identity was not due to bias in selecting peptides with Cn>3.0 as datasets which included peptides identified with either moderate (Cn>2.5 and ΔCn>0.2; Fig 2*B*) or low (Cn >1.5 and ΔCn>0.2; Fig 2*C*) confidence also demonstrated little overlap in peptide sequence. However, to maintain focus on relevant naturally processed self peptides using this unbiased approach, all downstream analyses were performed on peptides with Cn >3.0 and ΔCn >0.2. Importantly, this peptide set was found to have a false discovery rate (FDR; described in Materials and Methods) of 0, i.e., no peptides were identified by random similarity.

Notably, the average length of $H2A^b$-associated peptides increased from 14—16 amino acid residues in wild type cells to 18—20 amino acids in $TAP^{-/-}$ and $ERAAP^{-/-}$ cells (Table S1 and Fig S2). This was consistent with peptide length changes previously observed for class I-associated peptides displayed by $ERAAP^{-/-}$ cells [22]. In addition, we observed numerous groups of nested peptides arising from the same protein (Table S2) as would be expected from class II-associated peptides expressed by wild type cells [37,38]. These nested peptides contained both N- and C-terminal extensions, consistent with previous reports on class II-associated peptides expressed by wild type cells [37,38]. Moreover, only two peptides identified in this study have been previously reported (Table S1) [37,38]. The lack of overlap in peptides identified in previous studies and this one may have resulted from the analysis of different cell populations. We used un-manipulated APC isolated directly *ex vivo* in this study compared with B cell lymphomas, LPS-induced B cell blasts, IFN-γ-induced BMC2.3 cell line and Flt3-induced cells used in the earlier reports [37,38]. In addition, although we found thousands of peptides by LC-MS/MS, we have focused solely on those with the highest Cn values. It is conceivable that the few hundred peptides previously reported were excluded based on the criteria used for sequence determination and validation and may be present in the larger dataset. Hence the differences observed in the different reports does not detract from the novel peptides reported herein as similar results were observed with the larger datasets as well (Fig 2*B*–*C*).

H2A^b-associated peptides were derived from both secreted/extracellular and cytosolic/intracellular proteins as defined in the LOCATE database [39]. However, the majority (~70%) were processed from cytosolic/intracellular proteins (Fig 2*D*), including proteins associated with endosomes. Comparing individual genotypes, the presentation of cytoplasmic/intracellular protein-derived peptides was increased in TAP$^{-/-}$ and ERAAP$^{-/-}$ splenocytes. Consistent with previous reports [40], ~63% of the H2A^b-associated self peptidome presented by wild type cells were generated from cytosolic/intracellular proteins. In contrast, 87.5% and 80.2% of the H2A^b-associated peptides displayed by TAP$^{-/-}$ and ERAAP$^{-/-}$ splenocytes, respectively, were derived from cytosolic/intracellular proteins (Fig 2*D*). These data demonstrate that numerous cytoplasmic/intracellular proteins, including endosomal proteins, are processed and presented by H2A^b in TAP$^{-/-}$ and ERAAP$^{-/-}$ mice. From these analyses, we conclude that CAP components can impact the H2A^b-associated self peptidome.

TAP and ERAAP deficiency alter the CD4$^+$ TCR repertoire

As the self peptidome instructs the developing TCR repertoire, we compared TCR Vβ usage by CD4$^+$ CD62L^{HI}CD44^{LO} naïve T (T$_n$) cells between wild type mice and for TAP$^{-/-}$ or ERAAP$^{-/-}$ animals using a panel of Vβ-specific antibodies. As previously reported [35], the frequencies of TCR Vβ usage between wild type-, TAP$^{-/-}$- or ERAAP$^{-/-}$-derived CD4$^+$ T$_n$ cells were quite similar, although not identical (Fig 3*A*). Likewise, TCR Vβ usage within *Lm*-reactive CD4$^+$ CD62L^{LO}CD44^{HI} effector T (T$_{eff}$) cells of wild type, TAP$^{-/-}$ or ERAAP$^{-/-}$ mice were similar as well (Fig 3*B*).

Since Ag recognition is mediated by the highly variable CDR3, we specifically examined this region of the TCR β-chains. CDR3β sequence diversity can be estimated by analyzing the number of amino acids spanning the V-D-J recombination site by spectratyping the nucleotides that encode them [41,42]. Although different sequences may have equivalent lengths, thereby underestimating the true diversity, differences in the number of amino acids, nonetheless, provide a high throughput estimate of Ag receptor diversity. The diversity of the TCR of flow sorted CD4$^+$ T$_n$ cells were analyzed by spectratyping 52 *V*β-*J*β pairings. This analysis revealed extensive alterations in some but not all CDR3β length profiles in the naïve TCR β-chain repertoire expressed by wild type, TAP$^{-/-}$ or ERAAP$^{-/-}$ mice (Fig 4, S3*A*). Similar analysis of flow sorted *Lm*-responsive CD4$^+$ T$_{eff}$ cells revealed extensive differences in the CDR3β length profiles between wild type and TAP- or ERAAP-deficient CD4$^+$ T$_{eff}$ cells (Fig 5, S3*B*). These data suggest that, despite similarities in Vβ usage, which was serologically determined, CD4$^+$ T cells utilize different CDR3β sequences in the absence of the CAP machinery. Since the CDR3β region of the TCR is predominantly involved in Ag recognition, sequence differences in this region could potentially lead to alterations in the CD4$^+$ T cell responses to microbial challenge.

TAP-deficiency alters class II-restricted microbial Ag recognition

Previously, we reported that the magnitude of the CD4$^+$ T cell response to minor histocompatibility Ag HY and *Lm*-derived LLO and p60 peptides were increased in animals deficient in TAP or ERAAP [21]. Here, we have shown that TAP and ERAAP impact the quality of the H2A^b-restricted self peptidome and alter the TCR repertoire. Therefore, we queried whether the CAP machinery could destroy and/or create class II-restricted microbial peptides recognized by CD4$^+$ T cells. To this end, wild type, H2Ab$^{-/-}$ and TAP$^{-/-}$ mice were inoculated with VACV and, 7 days later, the Th response tested against a panel of 448 15-mer peptides. This panel consisted of putative H2A^b-restricted peptides from VACV ORFs [43]. An initial screen of these peptides revealed few shared specificities and significant alterations in the magnitude of CD4$^+$ T cell responses to these shared peptides in TAP$^{-/-}$ mice when compared to wild type animals (data not shown). In addition, the loss of

response to some peptides and novel responses to others was suggested (data not shown). To confirm these results, wild type, $TAP^{-/-}$ and $H2Ab^{-/-}$ mice were inoculated with VACV. After 7 days, splenocytes were restimulated in vitro with increasing amounts of select peptides identified from the initial screen. This interrogation confirmed our previous observation [21] that $TAP^{-/-}$ Th cells responded to certain peptides with increased magnitude (Fig 6*A*). In addition, the reactivity against other peptides was lost when compared to the response elicited in wild type mice, suggesting they are dependent on the activity of TAP (Fig 6*B*). Still other peptides were uniquely recognized only by $TAP^{-/-}$ Th cells and not wild type Th cells (Fig 6*C*) suggesting that in wild type animals those epitopes are destroyed by the action of TAP. Importantly, VACV-immune spleen cells from $H2Ab^{-/-}$ mice recognized none of the peptides tested (Fig 6) indicating $H2A^b$-restricted recognition of these epitopes by Th cells and not $CD8^+$ T cells. Hence, these data demonstrate that the CAP machinery profoundly affected the Th response. The altered Th response is a reflection of both altered Ag processing and presentation as well as an altered $CD4^+$ T cell repertoire.

Discussion

$CD4^+$ T cells regulate the adaptive cellular- and antibody-mediated responses to numerous microbial pathogens as well as cancers and autoantigens. Hence, it is critical to understand the processes regulating $CD4^+$ T cell development and activation. The results presented herein provide direct evidence that components of the CAP machinery sculpt the self peptidome displayed by $H2A^b$ molecules. Alterations in the displayed peptidome subsequently impact both the $CD4^+$ T cell repertoire and Ag-specific Th responses. Though altered $CD4^+$ T cell repertoire and Ag-specific Th responses would be expected from an altered peptidome, these data imply that interference with the CAP machinery could profoundly affect anti-microbial Th responses. Many viruses and oncogenic mutations result in down regulation of TAP expression [44–49]. This down regulation is triggered to prevent class I-restricted peptide presentation. However, our data suggest that this down regulation would also alter class II-restricted self and viral peptide presentation and the subsequent Th response. Furthermore, the results presented herein enhance our understanding of $CD4^+$ T cell responses in those individuals who lack TAP expression or express natural genetic variants of TAP or ERAAP [50–59]. The altered $CD4^+$ T cell repertoire and the recognition of a different antigenic peptidome may help explain the recurrence of bacterial infections and tumors in individuals that lack TAP function [54,57,58].

With the discoveries of class I-restricted Ag cross-presentation and class II-restricted cytosolic Ag presentation, the division of the class I and class II Ag processing pathways is becoming blurred. It becomes important, therefore, to understand the effect(s) that components of the CAP machinery may have on cytosolic Ags presented by class II molecules. We have shown that activities of CAP components profoundly alter the class II-restricted self peptidome. Therefore, not only is class I-restricted Ag presentation affected by the CAP machinery [22- 26,59], but class II-restricted peptide presentation is altered as well [21]. By manipulating expression of CAP components, therefore, pathogenic microbes can both block class I- and skew class II-restricted peptide presentation. By skewing the Th response microbes could potentially evade sterilizing immunity or cause immunopathologic responses. Furthermore, these data have implications for next generation subunit vaccines and immunotherapies targeting Ag-specific T cells. Epitopes inducing protective immunity against microbes capable of manipulating the CAP machinery may only be presented in the absence of fully functional CAP components. In the absence of CAP suppression, e.g., peptide-pulsed APC, these protective epitopes may not be processed and presented rendering such vaccines ineffective. Therefore, our data suggests that studies utilizing the live pathogen capable of manipulating the CAP machinery would be most likely to identify protective epitopes processed and presented during a natural infection.

Selection of $CD4^+$ T cells with an altered self peptidome appeared to generate a distinct $CD4^+$ TCR repertoire in CAP-deficient mice compared with that of the wild type animals. Consistent with previous reports [35], this altered repertoire was not obvious when Vβ usage was queried. However, analysis of the CDR3β regions revealed clear differences between wild type and CAP-deficient repertoires. Functionally, TAP deficiency led to the enhanced recognition of certain peptides by $CD4^+$ T cells compared with recognition in wild type animals. In addition, the recognition of some epitopes in wild type mice was lost while at least two novel epitopes were recognized solely in the absence of TAP. This altered recognition pattern represents the combined effects of an altered T cell repertoire and alterations in viral Ag processing and presentation. This implies that, in wild type mice, the novel epitopes identified here were perhaps degraded by the CAP machinery within VACV infected cells and, hence, did not generate a $CD4^+$ T cell response. Alternatively, the TCRs specific for these epitopes normally may not be selected during development on the wild type self peptidome leaving a hole in the $CD4^+$ T cell repertoire.

By extension, humans deficient in TAP expression or those that express genetic variants of TAP or ERAAP might have similar alterations in their $CD4^+$ T cell repertoires [50–61]. This could result in altered recognition of microbial peptides leading to either limited immunogenicity or enhanced immunopathology. In this regard, it is noteworthy that herpetic stromal keratitis (HSK) —a leading cause of blindness that has an infectious etiology [62]— evolves as a consequence of chronic herpes simplex virus (HSV) infection. HSK is a chronic inflammatory disease that is mediated by $CD4^+$ T cells [63]. As ICP47 of HSV blocks TAP function [48], one might predict that the display of an altered peptidome by HSV-infected cells might lead to $CD4^+$ T cell-mediated inflammation resulting in HSK. Further investigations will be needed to understand the clinical outcome of CAP deficiencies in humans.

In sum, it is becoming clearer that many T cell epitopes are being processed by components of both cytosolic and endo/lysosomal Ag processing pathways [11–15,21,27–31,61]. Data obtained from tagged Ags have suggested that the subcellular localization of the Ag may be critical for its presentation [15,31,34,64–66]. Proteasomes and endo/lysosomal proteases may degrade proteins at the point of Ag entry, endogenous versus exogenous, respectively. Subsequently, peptides may then be shared between the two Ag presentation pathways depending on the efficiency of molecular components that transport processed Ags. While some peptides can be presented by both pathways [11–15,27–31], it is evident that other peptides are restricted to a single presentation pathway [32,34]. This is likely due to an as yet undefined biochemical mechanism(s) by which partially processed Ags are targeted from the cytosol to the endo/lysosome. Understanding the underlying mechanism will impact how T cell biology is harnessed for vaccinations and immunotherapies as well as in treating autoimmune disorders that have a microbial etiology (e.g., HSK).

Materials and Methods

Animals

C57BL/6J mice were purchased from The Jackson Laboratory. B6.129-$TAP^{-/-}$, B6.129-$ERAAP^{-/-}$ and B6.129-$Ab^{-/-}$ mice [21] were bred, maintained and used in experiments in compliance with Vanderbilt University's Institutional Animal Care and Use Committee regulations and approval. B6.129-$TAP^{-/-}$, B6.129-$ERAAP^{-/-}$ and B6.129-$Ab^{-/-}$ mice had been backcrossed to the C57BL/6 strain 8–10 generations before use.

Isolation of naturally processed H2A^b-associated self peptides

RBC-depleted single cell suspensions of splenocytes pooled from 68—70 mice per strain were solubilized, clarified and pre-cleared with normal mouse serum by previously described methods [67,68]. Pre-cleared lysates were passed twice over protein A Sepharose (Repligen)-bound W6/32 (anti-HLA class I, an irrelevant antibody; Cedarlane) columns followed by bead-bound H2A^b-specific antibody columns (NYRmI-A, Cedarlane) at 4°C. The eluates were adjusted to 2N acetic acid, incubated for 20 min in a boiling water bath and cooled on ice [68]. Eluted peptides were enriched by Centricon 10 ultrafiltration (Millipore), freeze dried, resuspended in ~0.1ml deionized distilled water (Sigma) and fractionated by reversed-phase chromatography (RPC; HP1090, Hewlett-Packard) as previously described [68]. Approximately 150 fractions were collected and lyophilized to dryness.

MS-ESI sequencing of naturally processed H2A^b-associated self peptides

Each lyophilized RPC fraction was resuspended in 0.1% formic acid and subjected to reversed-phase microcapillary LC-MS/MS analysis using an LTQ linear ion trap mass spectrometer (Thermofisher). A fritless, microcapillary column (100-μm inner diameter) was packed with 10 cm of 5-μm C_{18} reversed-phase material (Synergi 4u Hydro RP80a, Phenomenex). RPC fractionated peptides were loaded onto the column equilibrated in buffer A (0.1% formic acid, 5% acetonitrile) using the LCPacking autosampler. The column was placed in line with an LTQ mass spectrometer. Peptides were eluted using a 60-min linear gradient from 0 to 60% buffer B (0.1% formic acid, 80% acetonitrile) at a flow rate of 0.3μl/min. During the gradient, the eluted ions were analyzed by one full precursor MS scan (400–2000 *m*/*z*) followed by five MS/MS scans of the five most abundant ions detected in the precursor MS scan while operating under dynamic exclusion. Extractms2 program was used to generate the ASCII peak list and to identify +1 or multiply charged precursor ions from the native mass spectrometry data file [69]. Tandem spectra were searched with no protease specificity using SEQUEST-PVM against a RefSeq murine protein database [36]. For multiply charged precursor ions ($z \geq +2$), an independent search was performed on both the +2 and +3 mass of the parent ion. Data were processed and organized using the BIGCAT software analysis suite with a weighted scoring matrix used to select the most likely charge state of multiply charged precursor ions [70]. Fragmentation/ionization patterns were compared against a dataset consisting of the fragmentation/ionization patterns of theoretical and known peptide sequences. The degree of concordance between these two patterns was assigned a cross correlation score X_{corr} (Cn) with higher values representing greater concordance between the observed and expected fragmentation/ionization patterns [36]. Peptides with a Sequest Cn score >3.0 and ΔCn >0.2 compared with the second most likely assignment were considered highly concordant (see Fig S1).

The ion fragments were also searched against the reversed mouse proteome database to generate the false discovery rate (FDR) calculated as (2 × # reverse hits)/(# reverse hits + # forward hits). This generated an overall FDR of 7%. Whereas a search of only the highly concordant peptide spectra (Cn>3.0 and ΔCn>0.2) generated a FDR of 0, i.e., no peptides were identified in the reversed database. The parental ions representing peptides eluted from class II molecules of only 2 genotypes were manually searched against the database of parental ions of the third genotype. Of the 62 overlapping peptide sequences, only 2 (3.2%) were identified in the third genotype within 10 HPLC fractions and 10 minutes of LC elution of the same fraction number/retention time. Of these, 1 was inappropriately identified by the tandem MS and the other was not analyzed by tandem MS for identification. From this analysis, we conclude that 96.8% of peptides presented by class II molecules of only two genotypes were correctly identified and were not presented by that of the third genotype.

Immunisation, T cell purification and functional analysis

The indicated mouse strains were inoculated either retro-orbitally with ~5×10^4 cfu wild-type *Lm* or i.p. with 2×10^5 pfu vaccinia virus (VACV) WR strain. After 7d, splenocytes were harvested and either stained for flow cytometric characterisation or restimulated for functional analyses. *Lm*-immune splenocytes were stained with mAb against mouse CD62L and CD44 for flow sorting of naïve (T_n) and effector (T_{eff}) $CD4^+$ T cell populations (FACS Aria, BD Bioscience). Post-sort purity was ascertained by flow cytometry and found to be >98% (data not shown). A separate aliquot of $CD4^+$ T cells were analysed for Vβ usage with a panel of 15 anti-Vβ antibodies (BD Bioscience) within the naïve (T_n: $CD44^{lo}CD62L^{hi}$) or *Lm*-immune (T_{eff}: $CD44^{hi}CD62L^{lo}$) subsets.

IFN-γ ELISPOT co-culture of total VACV-immune splenocytes with $H2A^b$-restricted peptides derived from VACV [43] was performed as previously described [21].

TCR spectratyping

Total RNA was isolated from flow sorted non-immune $CD4^+$ T cells or flow sorted naïve $CD62L^{hi}CD44^{lo}CD4^+$ (T_n) cells and activated, effector $CD62L^{lo}CD44^{hi}CD4^+$ (T_{eff}) cells and converted to cDNA as described [71]. PCR amplification of individual *V*β-*C*β junctions and specific *J*β-specific run-off was performed using previously reported primer pairs [72] and Supermix (Invitrogen). The run-off *J*β primers were end-modified with WellRED D2, D3 or D4 fluorescent dyes (Sigma-Genosys) to detect products using capillary gel electrophoresis (CEQ8000; Beckman Coutler). CDR3β fragment sizes were determined by correlation against a size standard consisting of WellRED D1 fluorescent DNA strands of incremental 20nt residues (Beckman-Coulter) and the frequency within the population was determined by integration of the peak area. CDR3β length was calculated as the number of amino acids between the conserved last germline encoded Vβ Cys to the Jβ Gly-X-Gly motif.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAP MHC class I antigen processing

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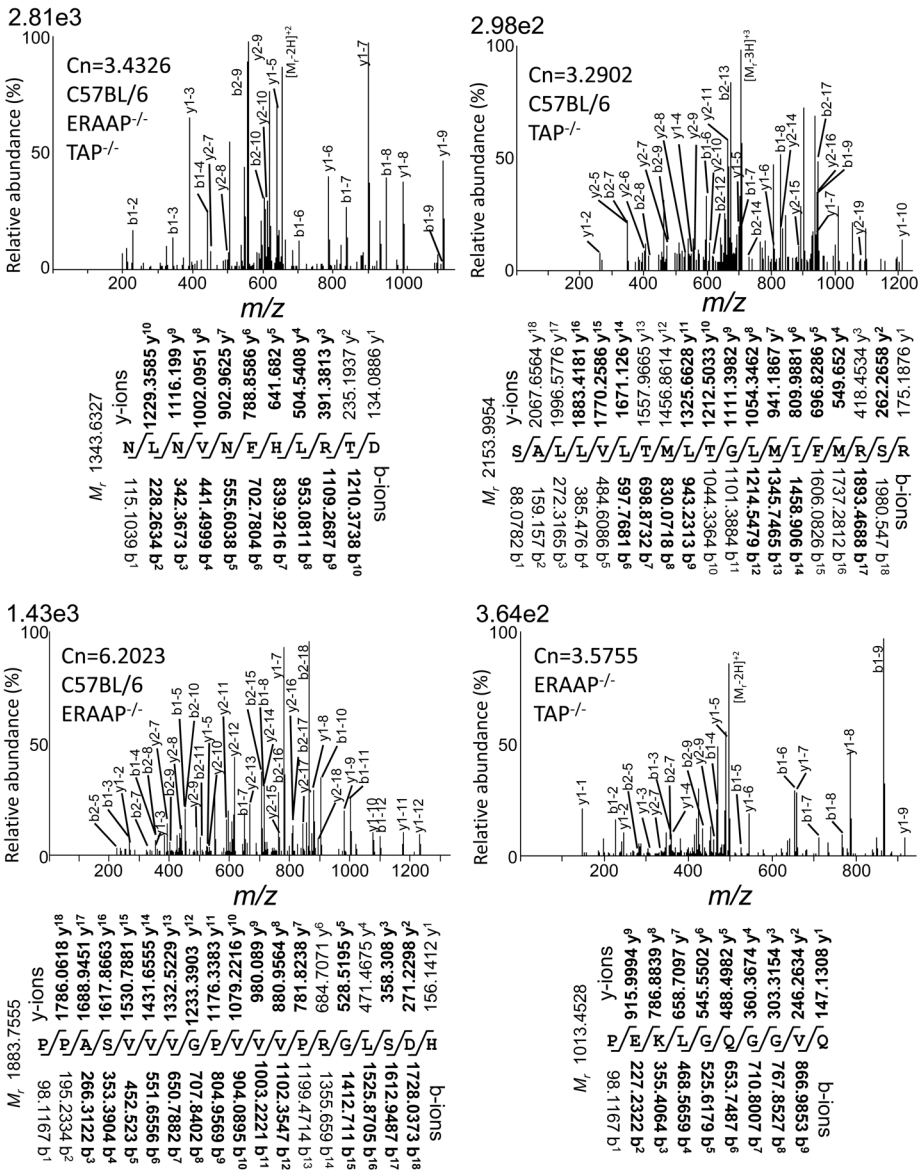

Figure 1. LC-MS/MS spectra of H2A^{b}-associated peptides commonly displayed by wild type, TAP$^{-/-}$ and ERAAP$^{-/-}$ splenocytes

Peptides eluted from immunoaffinity purified H2A^{b} molecules expressed by splenocytes from 68–70 wild type, TAP$^{-/-}$ and ERAAP$^{-/-}$ mice were separated by RPC and their amino acid sequence determined by LC-MS/MS. Representative mass spectra are presented. For each spectrum, the *b*- and *y*-ions are indicated along with the Sequest cross-correlation score (Cn) showing the degree of concordance between the observed and expected fragment ions. Within the spectrum, b1, b2, y1 and y2 refer to fragment ions that have mass/charge (*m/z*) +1 or +2. Below each spectrum are the +1 ion *m/z* values for each peptide (**bold**, observed ion masses). Note: the +2 ion mass/charge values are provided in Figure S1.

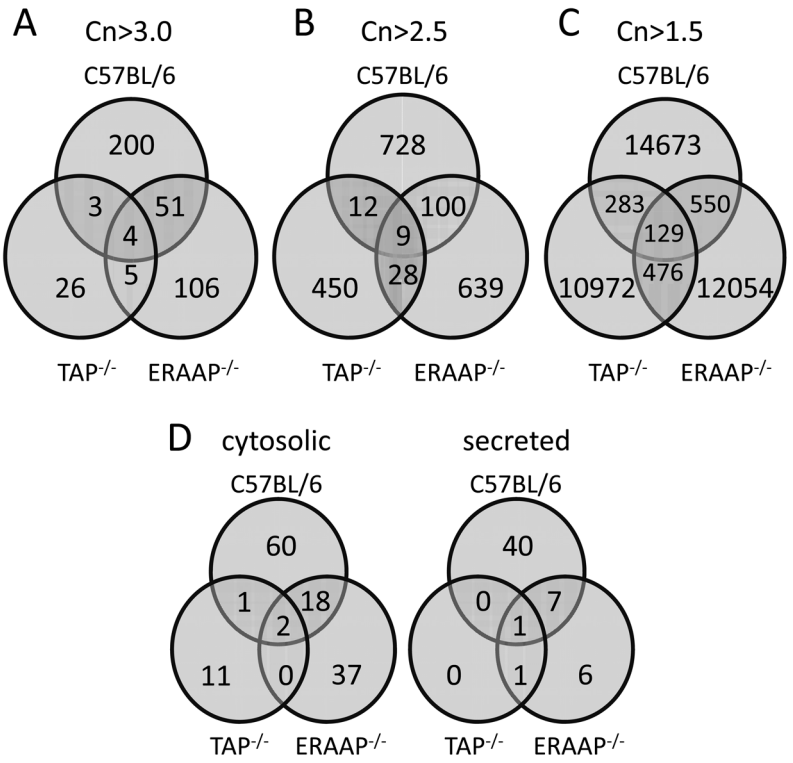

Figure 2. TAP and ERAAP deficiency alters the basal H2A^{b}-restricted self peptidome

The prevalence of H2A^{b}-restricted self peptide sequences was compared between wild type, TAP$^{-/-}$ and ERAAP$^{-/-}$ strains. Venn diagrams indicate the number of unique and common peptide sequences identified amongst the peptidomes displayed by the indicated strains. Cn>3.0 (**A**), Cn>2.5 (**B**) or Cn>1.5 (**C**) indicates increasing spectral confidence (see Materials and Methods). ΔCn≥0.2 distinguishes between the top two peptide sequences predicted from the spectrum; this criterion allows identification of the best peptide sequence that matches the observed spectrum. (**D**) Using the LOCATE database, the number of peptides derived from cytosolic and secreted proteins was compared amongst the peptidomes consisting of peptides with Cn>3.0.

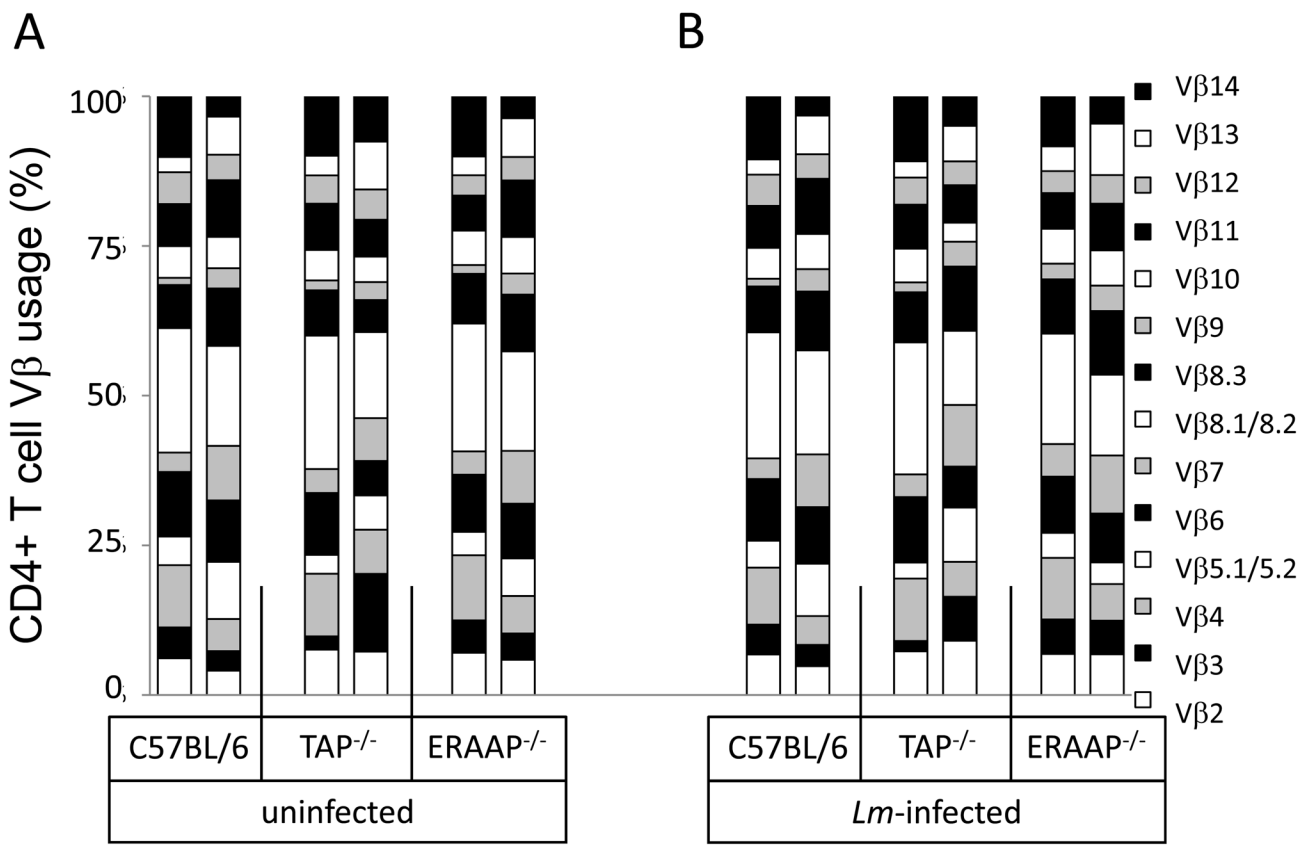

Figure 3. Differential self peptidome display has little impact on the TCR V β repertoire.

Wild type, $TAP^{-/-}$ and $ERAAP^{-/-}$ mice were inoculated with *Lm* or not and the TCR Vβ usage of the indicated $CD4^+$ T cell population was determined by flow cytometry after staining with a panel of Vβ-specific antibodies. The cumulative bar graphs indicate the proportion of each Vβ segment present within the (***A***) $CD4^+$ T_n ($CD44^{LO}CD62L^{HI}$) or (***B***) *Lm*-immune T_{eff} ($CD44^{HI}CD62L^{LO}$) population of duplicate experiments.

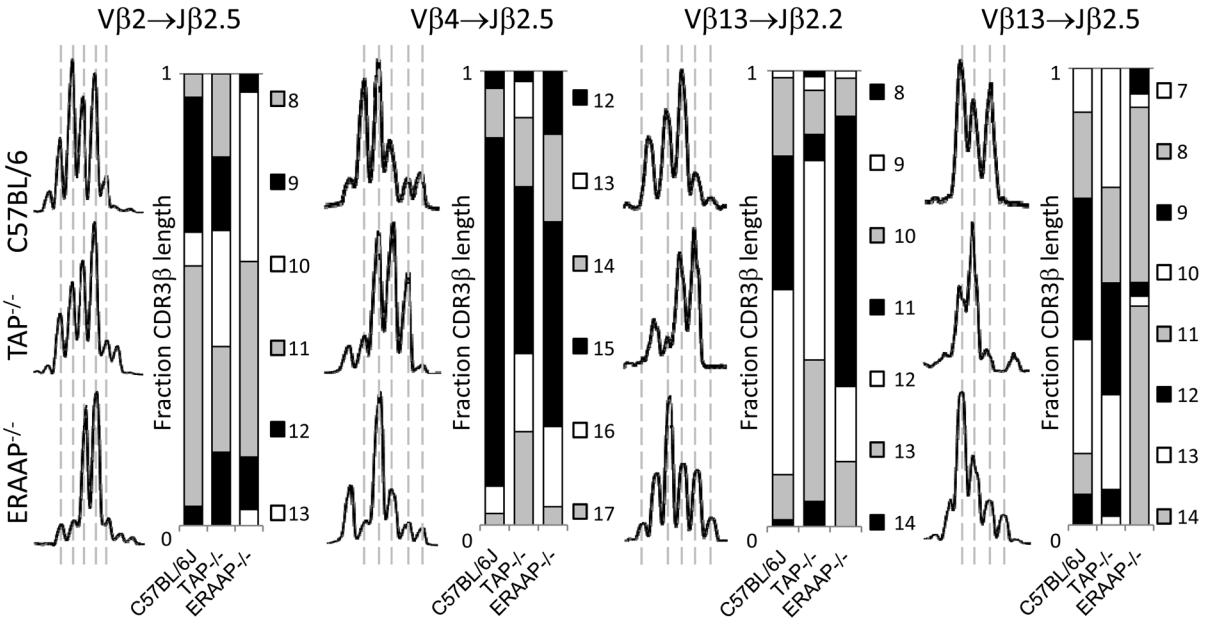

Figure 4. The TCR repertoire of naïve CD4$^+$ T cells in both TAP$^{-/-}$ and ERAAP$^{-/-}$ mice is substantially different from that of wild type mice

Total RNA was isolated from purified CD4$^+$ T_n cells from naïve, uninfected mice and amplified by RT-PCR using primers specific for the indicated *V*β→*J*β rearrangements. CDR3β length diversity was detected by capillary-gel electrophoresis and quantified by calculating the area under each peak. Representative spectrograms of four TCR *V*β→*J*β CDR3 length distributions are shown from wild type, TAP$^{-/-}$ and ERAAP$^{-/-}$ derived CD4$^+$ T_n cells. Bar graphs depict the fraction of specific CDR3β lengths present in the total population. Representative data from duplicate experiments (*n*=3–5 mice per experiment). Replicates sometimes displayed minor alterations in the absolute frequencies of CDR3 lengths but no alteration in their presence or relative frequencies were observed within a sample.

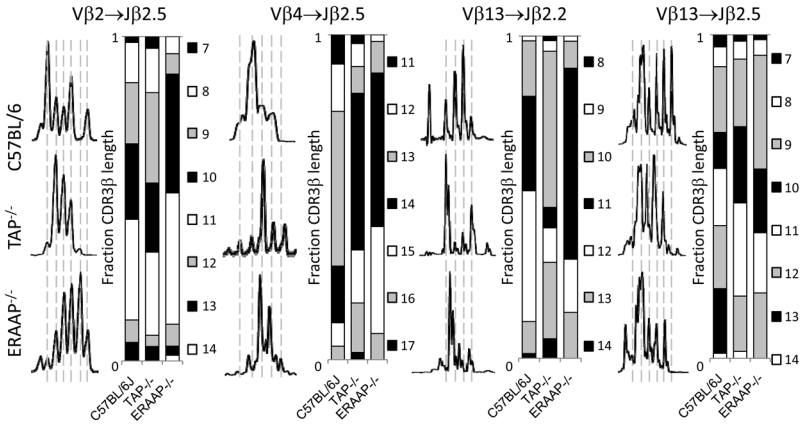

Figure 5. The *Listeria*-immune TCR repertoire is altered in TAP$^{-/-}$ and ERAAP$^{-/-}$ CD4^{+} T$_{eff}$ cells compared with wild type T$_{eff}$ cells

Wild type, TAP$^{-/-}$ and ERAAP$^{-/-}$ mice were inoculated with *Lm*. After 7 days, CD4^{+} T$_{eff}$ cells were flow sorted to ≥95% purity and total RNA was isolated, processed and analyzed as described in Figure 4. Representative spectrograms of four TCR *V*β→*J*β CDR3 length distributions are shown for wild type, ERAAP$^{-/-}$ and TAP$^{-/-}$ CD4^{+} T$_{eff}$ cells. Bar graphs depict the fraction of specific CDR3β lengths present in the total population. Representative data from duplicate experiments (*n*=3–5 mice per experiment). Replicates sometimes displayed minor alterations in the absolute frequencies of CDR3 lengths but no alteration in their presence or relative frequencies were observed within a sample.

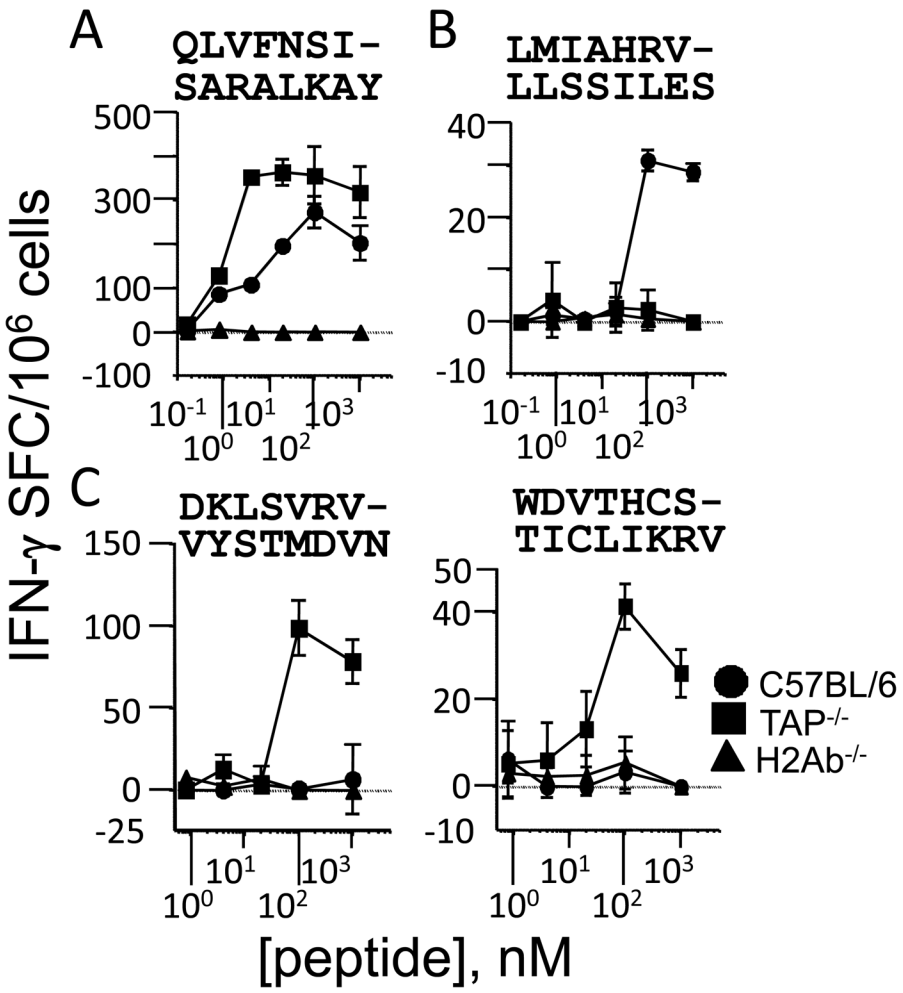

Figure 6. Wild type and TAP$^{-/-}$ CD4^{+} T cells recognize a different subset of vaccinia viral epitopes

Wild type, TAP$^{-/-}$ or H2A$^{b-/-}$ mice were inoculated with 5×10^5 pfu VACV. After 7 days, 10^6 splenocytes were restimulated *in vitro* with individual class II-restricted VACV peptides and the number of IFN-γ producing cells determined by ELISPOT. Peptide recognition was either enhanced (**A**), lost (**B**) or uniquely generated (**C**) in TAP$^{-/-}$ animals compared with wild type responses. Representative data from duplicate experiments (*n*=3–5 mice per experiment); mean ± sd is shown.