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Short Communication:

Tapasin shapes immunodominance hierarchies according to the kinetic stability of peptide – MHC class I complexes

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Peptide loading of MHC class I molecules involves multiple cofactors including tapasin. We showed previously *in vitro* that tapasin edits the peptide repertoire by favoring the binding of peptides with slow dissociation rates. Here, using tapasin-deficient mice and a DNA vaccine that primes directly, we confirm that tapasin establishes hierarchical responses *in vivo* according to peptide-MHC stability. In contrast, this hierarchy is lost when the peptides are cross-presented *via* an alternative DNA vaccine. By regulating transgene expression, we found that the dominant response modifier was antigen persistence. Our findings reveal strategies for activating T cells against low-affinity peptides, of potential importance for patients with repertoires narrowed by deletional tolerance.

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Introduction

Effector $CD8^+$ T cell responses to complex immunogens often focus on a few epitopes compared to the total number of potential epitopes available. One of the most important determinants of this phenomenon, termed immunodominance, is the abundance of peptide – MHC (pMHC) complexes at the surface of the initial priming APC [1]. Several factors will contribute to this, including the efficiency of antigen processing, the kinetics and thermodynamics of pMHC interaction, and the efficiency of peptide loading inside APC. Although the molecular mechanism by which peptides are selected and loaded onto MHC class I molecules is becoming clearer, the immunological consequences of these events remain largely uninvestigated.

MHC class I molecules are assembled with peptide cargo in the lumen of the ER while they are part of a peptide-loading complex comprising TAP, tapasin, calreticulin, ERp57, and protein disulfide isomerase [2–4]. Loss of any of these components gives rise to a quantitative loss of MHC class I peptide complexes that are exported to the cell surface with the approximate order TAP > tapasin (depending on the allele) > calreticulin ≥ ERp57 [5–9]. The cofactor tapasin additionally edits the peptide repertoire that is loaded onto class I molecules in favor of those with slow dissociation kinetics [10]. This is a similar function to that described for DM in MHC class II loading [11].

We have shown previously in cell lines that the hierarchy of MHC class I-restricted presentation of peptides generated endogenously (from translation of a minigene) is controlled by the kinetics of peptide binding to class I in a tapasin-dependent way [10]. Thus, the peptide variants of the ovalbumin-derived peptide SIINFEKL, SIINFEK**M** and SIIN**Y**EKL, represent a hierarchy with decreasing biological half-lives. Trans-

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Abbreviations: N.FrC: N-terminal domain of fragment C from tetanus toxin · **pMHC:** peptide-MHC

fectants stably expressing minigenes encoding each of these peptides display a level of surface presentation of H2-K^b complexes that corresponds exactly with this half-life but only in the presence of the peptide editor tapasin. When tapasin is absent, the hierarchy distorts in such a way as to lead to preferential presentation of an intermediate half-life peptide. Thus tapasin edits the repertoire of peptides supplied endogenously in a way that is sensitive to half-life [10].

There is evidence from studies investigating MHC class II-restricted antigen presentation that the DM-edited repertoire dictates the intensity of $CD4^+$ T cell responses in mice [12–14]. However, it is not known whether the tapasin editing we have observed in cell culture is physiologically relevant for the generation of immunodominant $CD8^+$ T cell responses *in vivo*. To this end we have measured $CD8^+$ T cell priming to the same epitopes we have studied previously *in vitro* by measuring a single $CD8^+$ T cell reactivity (anti-K^b/SIINFEKL), following the immunization of mice with DNA vectors containing class I- and class II-restricted determinants in two different configurations [15, 16].

In the first design, the $CD8^+$ T cell epitope is expressed as a minimal peptide specifically targeted to the ER by an N-terminal leader sequence. To provide critical "help" for the minigene product, a separate expression cassette is incorporated within the plasmid backbone encoding a hybrid invariant chain molecule, with the CLIP sequence replaced by a Th determinant from tetanus toxin (p30) [15]. This vaccine fails to cross-present antigen because of the short half-life of minigene products [17] and induces Th-dependent $CD8^+$ T cell responses *via* directly transfected APC [15]. In the second design, the $CD8^+$ T cell epitope is fused immediately 3′ of the N-terminal domain of fragment C (N.FrC) from tetanus toxin [16]. Expression of this fusion protein allows both direct presentation and cross-presentation of the class I-restricted epitope. However, we have shown that priming of $CD8^+$ T cell responses to the C-terminal peptide of this fusion is dependent upon cross presentation, with responses to directly presented determinants aborting due to the absence of help [15].

Thus, the two different DNA vaccine designs allowed us to explore whether tapasin impacts on direct presentation and cross-presentation pathways. We find that tapasin establishes a hierarchical $CD8^+$ T cell response when peptides of decreasing half-life are presented directly. However, this hierarchy is lost if peptides are cross-presented over prolonged periods. These observations underline the importance of the nature and persistence of antigen in the priming and breadth of $CD8^+$ T cell responses, and have relevance for vaccine design.

Results and discussion

Direct priming elicits hierarchical $CD8^+$ T cell responses that mirror pMHC stability

To study the relationship between peptide-off rates and immunogenicity, we used a DNA immunogen designed to specifically target nominal MHC class I- and class II-restricted epitopes into the ER of transfected APC. This vaccine, designated *pDUO*, minimizes the requirement for antigen processing and induces Th-dependent $CD8^+$ T cell responses by direct presentation [15]. Naive C57BL/6 mice were immunized intramuscularly with *pDUO* encoding the peptide variants we have studied previously *in vitro* [10]: SIINFEKL (FL), SIINFEK**M** (FM), and SIIN**Y**EKL (YL). After 12 days, the number of SIINFEKL-specific T cells induced was measured directly *ex vivo* by IFN-γ ELISPOT.

As shown in Fig. 1A, the magnitude of the response to each peptide mirrored the hierarchy of antigen presentation levels measured in cell transfectants [10], which in turn is dictated by the kinetic stability of the pMHC complex. Responses were also measured to the immunizing peptide and we observed no change in the relative number of IFN-γ-producing cells (data not shown). Therefore, as described earlier [17], $CD8^+$ T cells elicited to these variant peptides are fully cross-reactive with the wild-type peptide. This is also consistent with our observation that the T cell hybridoma B3Z, which was raised to the wild-type K^b/SIINFEKL peptide complex, recognized each of the peptide variants equally well when present at saturation (T. Elliott, unpublished data). These data, combined with previous finding [18, 10], demonstrate that kinetic stability is a key parameter in determining the $CD8^+$ T cell response hierarchy to these variant peptides. A similar direct relationship between pMHC stability and immunogenicity was reported by Melief and colleagues [19].

Hierarchical $CD8^+$ T cell priming is lost in tapasin-deficient mice

The hierarchy of SIINFEKL peptide variant presentation, though underpinned by kinetic stability of the pMHC complex, is nevertheless dependent on the peptide-editing function of tapasin [10]. Thus, in tapasin-negative cells transfected with the same variant peptide minigenes, the hierarchy of presentation is different with a peptide of intermediate kinetic stability (SIINFEK**M**) being presented to higher levels than wild-type peptide. Furthermore, the difference in level of presentation between peptide variants with the slowest half-life (SIINFEK**L**) and fastest half-life (SIIN**Y**EKL) is drastically reduced in tapasin-negative cells [10].

In order to determine the extent to which the response hierarchy that we observed in Fig. 1A is dependent on tapasin-mediated editing, we repeated the *pDUO* immunizations in tapasin-deficient (tapasin$^{-/-}$) mice [6]. The hierarchy between SIINFEKL and SIINFEK**M** response intensities seen in wild-type mice was lost (Fig. 1B), with an inversion in the response magnitudes to SIINFEKL and SIINFEK**M**. Thus, in the absence of tapasin, CD8$^+$ T cell responses to intermediate- rather than high-stability pMHC complexes dominate. Failure to respond to SIIN**Y**EKL is consistent with the low levels of this peptide that can be directly presented in the absence of tapasin [10]. The correlation observed between the preferential formation of intermediate stability complexes in transfected cells [10], and the level of CD8$^+$ T cell response observed after DNA immunization (Fig. 1B), results in an alteration in the immune-response hierarchy.

Taken together, these data indicate that *in vivo*, peptide stability is a necessary but insufficient parameter in determining the hierarchy of primary T cell responses and that the additional influence of tapasin editing is essential to establish immunodominance: the mechanism being its ability to select peptide cargo for loading onto MHC class I molecules according to the kinetic stability of the resulting pMHC complex.

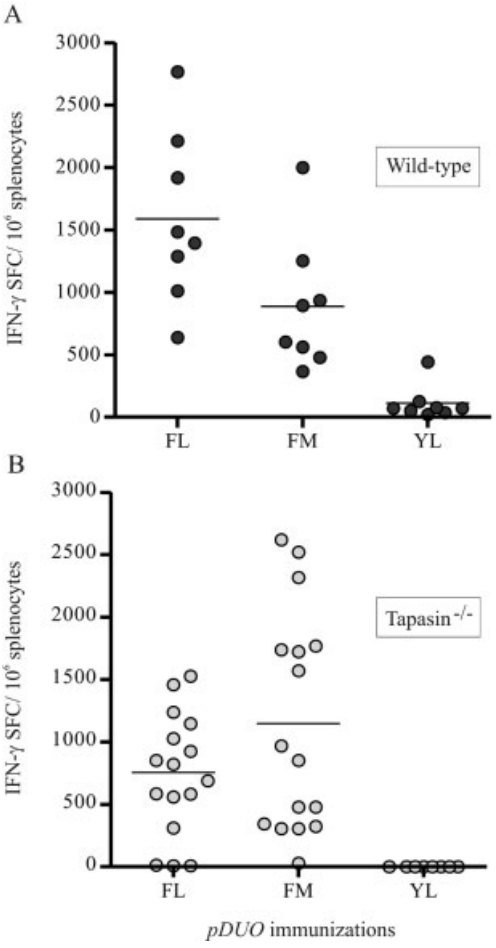

Figure 1. Immunization with *pDUO* elicits a hierarchical CD8$^+$ T cell response, mirroring kinetic stability of pMHC complexes that is controlled by tapasin. C57BL/6 mice (A) and tapasin$^{-/-}$ mice on a C57BL/6 background (B) were immunized by intramuscular injection with *pDUO* encoding the peptide variants indicated. After 12 days, the number of SIINFEKL-specific T cells induced was measured directly *ex vivo* by IFN-γ ELISPOT. The results shown are combined from at least two separate experiments, with each data point representing an individual mouse. Horizontal bars depict group means.

A DNA-encoded fusion protein breaks the immunodominance response hierarchy

We investigated a second DNA-based immunogen in which the SIINFEKL variant epitope was appended immediately 3′ of N.FrC from tetanus toxin. This DNA vaccine, designated *pDOM-peptide*, induces Th-dependent CD8$^+$ T cell responses by cross-presentation [15].

We initially established whether tapasin-sufficient cells transfected with *pDOM-peptide* maintained the hierarchy observed in minigene transfectants [10]. H-2^b RMA cells were transiently transfected with DNA expression vectors encoding each of the SIINFEKL peptide variants fused to N.FrC. After 6 h in culture, peptide presentation was detected by flow cytometry with the monoclonal antibody 25-D1.16 [20], which is able recognize all of the analogues bound to H-2K^b [10]. The fusion proteins were co-expressed with enhanced GFP from a single bicistronic transcript, enabling normalization for transfection efficiency. As shown in Fig. 2A, their relative presentation matched that of nominal minigene products, giving rise to the hierarchy SIINFEKL > SIINFEK**M** > SIIN**Y**EKL.

In contrast, when we immunized C57BL/6 mice with SIINFEKL variant-encoding *pDOM-peptide* constructs, we found that the immune response hierarchy we observed with the RMA transfectants and *pDUO* was absent (Fig. 2B). Instead, responses to all three peptides were roughly equivalent. Most notably, the response to SIINFEKL was significantly lower compared to *pDUO* immunization (mean 481 SFC/10^6 splenocytes compared to 1590, $p=0.0006$) and the relative response to SIIN**Y**EKL was significantly higher (mean 491 SFC/10^6 splenocytes compared to 113, $p=0.0027$). This flattening of the hierarchy appeared therefore to be specific for DNA delivery (*via pDOM-peptide*) of cross-presented antigen.

Regulated antigen expression restores hierarchical CD8$^+$ T cell responses induced by cross-priming

We have shown recently that uncontrolled, CMV promoter-driven *DOM-FL* transgene expression results in prolonged cross-presentation of the SIINFEKL peptide (>20 days) that limits the magnitude of a primary CD8$^+$ T cell response [21], possibly due to supra-optimal stimulation by the high-stability K^b/SIINFEKL complex leading to physical deletion [22]. Conversely, using a mifepristone-responsive gene regulation system (GeneSwitch^{TM} [23]) to control antigen presentation, we have shown that reducing antigen expression to a short burst (<5 days) results in a significant amplification of the SIINFEKL-specific response.

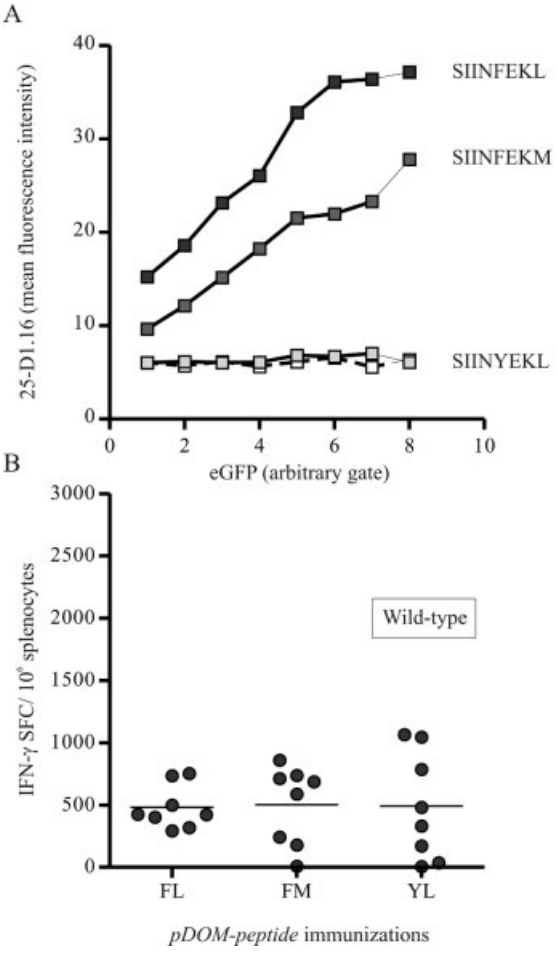

Figure 2. The relationship between kinetic stability and immunodominance *in vivo* is lost with *pDOM-peptide* DNA immunization. (A) RMA cells were transfected with *pIRES-DOM-peptide* constructs, encoding each of the SIINFEKL variants indicated, or *pIRES* alone (dashed line) and stained 8 h later with H-2K^b/SIINFEKL-specific antibody 25-D1.16 [20]. Arbitrary gates were introduced into dot plots according to enhanced GFP expression levels, and the 25-D1.16 MFI of cells within the gates calculated using WinMDI 2.8 software. (B) The immunogenicity of *pDOM-peptide*-encoded SIINFEKL variants in wild-type mice was assessed by IFN-γ ELISPOT analysis 12 days after intramuscular DNA injection. The results shown are combined from two separate experiments, with each data point representing an individual mouse.

To examine whether it might be possible to re-establish the response hierarchy by shortening the duration of antigen expression, wild-type C57BL/6 mice were co-injected with the GeneSwitch^{TM} plasmids, encoding each of the peptide variants fused to N.FrC, and antigen expression induced with a single dose of 0.5 mg/kg mifepristone. This dose of the inducer leads to transient antigen expression (<5 days). As shown in Fig. 3A, when expression of *DOM-peptide* is temporally regulated, a hierarchy is established that is comparable to *pDUO* immunization. To confirm that this hierarchy is dependent upon the editing function of tapasin, we repeated these experiments in tapasin-deficient mice. Akin to the results obtained with *pDUO*, shuffling of the response hierarchy was observed, with the peptide of intermediate kinetic stability (SIINFEK**M**) inducing more robust responses than the wild-type peptide (Fig. 3B). These data suggest that tapasin establishes hierarchical CD8$^+$ T cell responses to cross-presented antigen but such hierarchies are shaped by persistence of high-affinity peptide ligands.

It is not clear why a response was seen to the low-stability peptide SIIN**Y**EKL only when it was cross presented – either as a bolus or by sustained delivery. One possibility is that its level of presentation simply breeches a threshold required for T cell activation that is not reached following immunization with *pDUO*. Consistent with this idea is the frequent observation that antigen processing by cross-presentation pathways is more efficient than *via* the endogenous pathway [24], and our own observation that responses to all peptides in the hierarchy, including SIIN**Y**EKL, are elevated following delivery of a bolus of cross-presented antigen (compare Fig. 1A and 3A), resulting in a detectable anti-SIIN**Y**EKL response.

Concluding remarks

We have shown that tapasin establishes a hierarchical CD8$^+$ T cell response that reflects peptide half-life when peptides are targeted directly to the ER of the priming APC. This is consistent with our previous findings that the relative level of presentation of each of these peptides in transfected cells is predicted by their half-life when tapasin is present, this relationship breaking down in its absence [10], and with other studies that correlate immunogenicity with kinetic stability of the pMHC complex [19, 25]. Taken together, therefore, these data indicate that tapasin controls the hierarchical response to diverse peptides by regulating their level of expression at the surface of APC. This was true for both direct

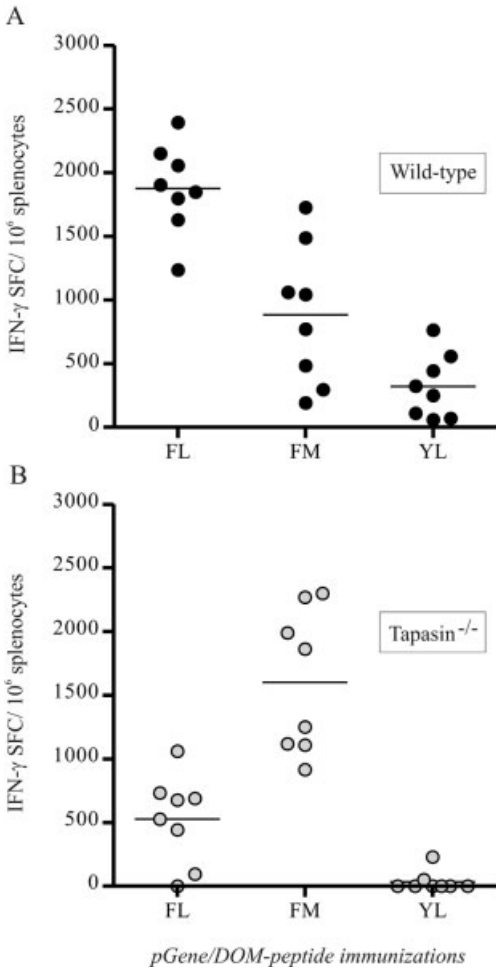

Figure 3. Transient antigen expression restores a hierarchical $CD8^+$ T cell response that is controlled by tapasin. Wild-type (A) and tapasin$^{-/-}$ mice (B) were immunized with *pSwitch* and *pGene/DOM-peptide*, and transgene expression induced with 0.5 mg/kg mifepristone. After 12 days, splenocytes were prepared and the number of SIINFEKL-specific T cells measured by IFN-γ ELISPOT. The results shown are combined from two separate experiments.

presentation and cross-presentation pathways, although for the latter, hierarchical responses to the tapasin-groomed peptide repertoire could be altered by antigen persistence.

Materials and methods

Plasmids

pDUO, *pDOM-peptide* and *pIRES-DOM-peptide,* encoding each of the SIINFEKL peptide variants, were constructed as previously described [15]. To construct the GeneSwitch™ vectors, open reading frames flanked by *Hind III* and *Not I* restriction sites were amplified by PCR using *pDOM-peptide* as template and subcloned into *pGene/V5* (Invitrogen, Carlsbad, CA). Plasmid DNA was purified for immunization using a QIAfilter Giga kit (Qiagen, Hilden, Germany). All constructs were sequenced and checked for expression *in vitro* using the TNT T7 Coupled Reticulocyte Lysate System (Promega, Southampton, UK).

In vitro transfection

RMA cells (ATCC) were transfected by electroporation (320 V, 750 μF in 400 μL serum-free RPMI) using a Gene Pulser II (BioRad, Richmond, CA).

Mice and *in vivo* experiments

Tapasin$^{-/-}$ mice [6] used in these studies were backcrossed for at least seven generations onto the C57BL/6 background. Wild-type C57BL/6 and tapasin$^{-/-}$ mice, both bred in-house, were vaccinated at 8-10 wk of age with a total of 50 μg of plasmid DNA in normal saline injected into two sites in the quadriceps. For the GeneSwitch(tm) experiments, mice were injected intramuscularly with 50 μg *pGene/DOM-peptide* and 25 μg *pSwitch* (Invitrogen). Four hours after plasmid DNA injection, mifepristone (Sigma, Poole, UK) was given to the mice intraperitoneally at the indicated dosage. Animal experiments were conducted according to the UK Home Office license guidelines and approved by the University of Southampton's ethical committee.

Evaluation of peptide-specific T cell responses

ELISPOT analysis was performed using the BD™ ELISPOT Set for murine IFN-γ. Spots were developed using 5-bromo-4-chloro-3-indolyl phosphate (Zymed Laboratories, San Francisco, CA) and counted with a Transtec 1300 ELISPOT reader (AID Diagnostika GmbH, Strassberg, Germany).

Statistical analysis

Statistical significance between vaccination groups was determined by the nonparametric Mann–Whitney test (two-tailed) using Prism 4 (GraphPad) software.

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