Published in final edited form as:
J Immunol. 2011 June 15; 186(12): 6683–6692. doi:10.4049/jimmunol.1100525.

Proteasomes, TAP and ERAAP control CD4+ T_H cell responses by regulating indirect presentation of MHC class II-restricted cytoplasmic antigens[1]

Srdjan M. Dragovic[2], **Timothy Hill**[2], **Gregory J. Christianson**[3], **Sungjune Kim**[2], **Tim Elliott**[4], **Diane Scott**[5], **Derry C. Roopenian**[3], **Luc Van Kaer**[2], and **Sebastian Joyce**[2,6]

[2]Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232;

[3]The Jackson Laboratory, Bar Harbor, ME 04609, USA

[4]Cancer Sciences Division, University of Southampton School of Medicine, Southampton General Hospital, Southampton SO16 6YD

[5]Transplantation Biology Group, Department of Immunology, Imperial College London, Hammersmith Hospital, London W12 0NN, UK

SUMMARY

Cytoplasmic Ags derived from viruses, cytosolic bacteria, tumours and allografts are presented to T cells by MHC class I or class II molecules. In the case of class II-restricted Ags, professional Ag-presenting cells acquire them during uptake of dead, class II-negative cells and present them via a process called indirect presentation. It is generally assumed that the cytosolic Ag-processing machinery—which supplies peptides for presentation by class I molecules—plays very little role in indirect presentation of class II-restricted, cytoplasmic Ags. Remarkably, upon testing this assumption, we found that proteasomes, TAP and ERAAP, but not tapasin, partially destroyed or removed cytoplasmic, class II-restricted Ags such that their inhibition or deficiency led to dramatically increased T_H cell responses to allograft (HY) and microbial (*Listeria monocytogenes*) Ags, both of which are indirectly presented. This effect was neither due to enhanced ER-associated degradation nor competition for Ag between class I and class II molecules. From these findings a novel model emerges in which the cytosolic Ag-processing machinery regulates the quantity of cytoplasmic peptides available for presentation by class II molecules, and hence modulates T_H cell responses.

INTRODUCTION

T cell responses are primed when the innate immune response culminates in the display of processed Ags by Ag-presenting cells (APC) in the context of *Major histocompatibility complex*-encoded class I and class II molecules (1, 2). Class I molecules, which regulate cytotoxic $CD8^+$ T lymphocyte (CTL) functions, present endogenous/cytoplasmic Ags, whilst class II molecules, which control $CD4^+$ T helper (T_H) cell functions, present exogenous/extracellular Ags (3, 4). Notwithstanding, CTL responses can also be primed by class I-restricted extracellular Ags by a well-defined process termed cross presentation (4, 5). Likewise, studies of T_H cell responses to allografts, cancers, viruses and cytosolic

[1]Supported by NIH grants HL54977 and AI40079 to SJ; AI70305 and HL89667 to LVK; and AI28802 to DCR. The US Army supported TH.

[6]Corresponding author: Sebastian Joyce, Telephone: 615-322-1472/Facsimile: 615-343-7392, Electronic: sebastian.joyce@vanderbilt.edu.

bacteria have suggested that class II molecules can also present cytoplasmic Ags (6–18). Furthermore, biochemical analyses have revealed that a substantial proportion of naturally processed self peptides associated with class II molecules are derived from nuclear and cytoplasmic proteins (19–21). Because such proteins are exposed to the cytosolic Ag-processing (CAP) pathway, one would predict that this pathway might impact the repertoire of cytoplasmic Ags presented by class II molecules.

Class II-restricted presentation of exogenous Ags requires the endolysosomal processing pathway (22). Thus, extracellular pathogens or derived proteins and toxins are delivered to the endolysosomes, wherein the action of the disulphide reductase–γ-interferon induced lysosomal thiol reductase (GILT)—and cysteine and asparate proteases generate peptides for assembly with peptide-receptive class II-CLIP complexes in a process catalysed by the class II-like molecule DM. Thence, stably assembled class II-peptide complexes egress to the cell surface for an appraisal by T_H cells (3, 5, 22).

In contrast to extracellular Ags, class II-restricted cytoplasmic Ags (8–13, 15–17) can take two routes towards the class II-containing endolysosomes. If Ags are presented by the same cell, then macroautophagy, microautophagy and chaperone (HSP70-LAMP2a)-mediated autophagy (12–14, 23, 24) delivers them from the cytosol into the endolysosomes. If Ags are synthesised by class II-negative cells, then they need to be presented by a professional APC. In such cases, the Ag is delivered to the endolysosomes by endocytic or phagocytic uptake by APCs. For alloantigens this process is called indirect presentation (6, 25, 26), a term used herein for all class II-restricted Ags donated from infected cells to APCs. In both cases, prior to reaching the lysosomes, cytoplasmic Ags are exposed to the CAP machinery—which is predominantly thought to assist in Ag processing and presentation by class I molecules. This machinery consists of the proteasomes, transporter associated with Ag-processing (TAP), tapasin and ER-associated aminopeptidase associated with Ag processing (ERAAP). They function to proteolytically process nucleo-cytoplasmic proteins, transport the emerging peptides into the ER and trim such peptides to a length conducive for binding to class I molecules or to degrade those that are incompatible with class I binding (27–30). Therefore, one would predict that all cytoplasmic proteins—including those from which class II-restricted Ags are generated—could become substrates for degradation by the CAP machinery. If such cytoplasmic degradation occurs, it would regulate the quantity of cytoplasmic proteins reaching the endolysosomes for presentation by class II molecules.

Notwithstanding the above regulatory mechanism, none of the numerous studies focused on determining whether the proteasomes and TAP regulate class II Ag presentation have thus far indicated a dramatic role for the CAP machinery in this process. For example, a few reports have demonstrated a positive role for the proteasome and TAP in generating class II-restricted Ags (14, 31–35), whilst the majority of such studies have indicated that the CAP machinery exerts no influence on class II-restricted Ag presentation (11, 36–39). It should be noted however, that all of these studies were focused on direct presentation of cytoplasmic Ags by class II molecules. Additionally, many such studies were performed using in vitro models. However, most infected, tumour and allogeneic cells do not express class II molecules themselves, and hence indirect class II-restricted presentation is a major pathway for their recognition by T_H cells. Hence, how the CAP machinery impacts indirect presentation of class II-restricted, cytoplasmic Ags in vivo remains a critical unanswered question of fundamental import.

To acquire Ags for indirect presentation, APC are thought to phagocytose dying cells from which derived Ags are presented by class II molecules (3, 5, 40); It is thus generally assumed that indirect presentation of class II-restricted Ags—including those of donor cytoplasmic origin—follows the same principles as direct presentation because it involves

phagocytosis of exogenous Ags from apoptotic cells and direct delivery of cargo to the endolysosomal processing pathway (3).

We sought to gain insights into the role of the CAP machinery in sculpting the repertoire of cytoplasmic Ags indirectly presented by class II molecules and its impact on T_H cell responses. Toward this goal, we defined the cellular and molecular bases for class II-restricted indirect presentation of cytosolic Ags derived from the HY alloantigen and *Listeria monocytogenes*. Remarkably, our findings revealed that proteasomes, TAP and ERAAP played destructive roles, thereby regulating the quantity of cytoplasmic Ags indirectly presented by class II molecules. Such alteration in Ag presentation modulated the magnitude of T_H cell responses to cytoplasmic Ags in vivo.

MATERIALS AND METHODS

Mice

All mouse strains, their histocompatibility genotype and sources are described in Table S1. All mice were bred, maintained and used in experiments in compliance with Vanderbilt University Institutional Animal Care and Use Committee regulations and approval.

Cell lines

Wild type K41 and calreticulin-null K42 MEF (41) as well as Hsf1-null MEF (42) were maintained in RPMI-1640 (Invitrogen) supplemented with 5% foetal calf serum (FCS; Hyclone), L-glutamine, HEPES and antibiotics (Invitrogen). These MEFs were transfected with *Dby* cDNA (43) and selected with 0.5 mg/ml G418 for ~4 weeks to express the HY alloantigen. *Dby* expression was verified by RT-PCR using forward (GGTCTGGAAAAACTGCTGC) and reverse (TTGGTGGCATTGTGTCCTGC) primers (43).

Preparation of donor cells

In some experiments, donor splenocytes were treated with PBS or the irreversible proteasome inhibitor epoxomicin or protein glycosylation inhibitor/ER stress inducer (Sigma) for 2 or 3hrs, respectively, at 37°C. In other experiments, donor splenocytes were starved for 2hrs in Hanks balanced-salt solution (Cellgro) or maintained in DMEM containing 10% foetal calf serum, penicillin, streptomycin, L-glutamine, sodium bicarbonate and HEPES buffer. Cells were washed thoroughly, resuspended at ~2×10^8 cells/ml and used for immunisation.

Peptides

All peptides used in this study (Table S2) were synthesized using Fmoc chemistry and determined to be >90% pure by MALDI-MS analysis (The Pennsylvania State University College of Medicine, Hershey, PA). Peptide stocks and working dilutions were prepared as described (44).

Immunisation and ELISpot assay

Recipient mice were immunised i.p. with 2×10^7 donor splenocytes. After seven days, splenocytes were prepared and used in ELISpot assay. For this, Immobilon-P plates (Millipore) were activated and coated with 1—2μg/mL IFNγ capture monoclonal antibody (mAb; AN18; eBiosciences) overnight. Excess mAb was washed and blocked with 10% FCS in RPMI-1640. Meanwhile, $2.5—3\times10^5$ red blood cell-free immune splenocytes were stimulated with the indicated concentrations of peptides (see Table 2) in triplicate. After 48hrs, plates were washed first with Ca^{2+}- and Mg^{2+}-free PBS and then with PBS

containing 1% FBS and 0.05% Tween-20. Cytokine spots were detected with 1μg/mL IFNγ-specific biotinylated mAb (R4-6A2; eBiosciences). After ~3hrs at room temperature, excess mAb was washed away and Vectastain ABC peroxidase (Vector Laboratories) was added to each well. Spots were visualised by reacting 2.2-dimethyl-formamide and 3-amino-9-ethylcarbazole with 30% hydrogen peroxide (Sigma). Spots were counted using CTL ImmunoSpot analyzer and CTL ImmunoSpot software, version 3.2 (Cellular Technology).

The response of H3b^{a}-specific CD4 T cell clones, LPa/B10-B6 and LPa/B10-line, was determined by stimulating ~10^5 cells with increasing numbers of splenocytes isolated from the indicated mouse strains at 1:1; 1:2.5; 1:5; and 1:10 ratio of responder to stimulator. After 48hrs, IFNγ-secreting cells were detected by ELISpot assay as described above.

DC depletion

Vehicle (PBS) or diphteria toxin (DT) (Sigma) was administered i.p. to hemizygous hDTR^{tg} mice at 4ng/g body weight as previously described. After 18—24hrs, vehicle- or DT-treated mice were used either as recipients or to isolate donor splenocytes for immunisation. Flow cytometry analysis in pilot experiments and of donor hDTR^{tg} splenocytes indicated that DT-treated mice were depleted of ≥90% splenic CD11c^{+} cells within 18hrs and remained in this state for ~72hrs (45).

L. monocytogenes infection

To elicit primary CD4^{+} T cell responses, mice were inoculated retro-orbitally with ~5×10^4 cfu *L. monocytogenes*. After 12—14d, the response of 0.5—1×10^6 immune splenocytes to *L. monocytogenes*-derived peptide epitopes was determined by ELISpot assay as described above. To determine secondary CD4^{+} T cell responses, mice were inoculated i.p. with ~10^3 cfu *L. monocytogenes* in 0.2 ml PBS or with PBS alone. After 14 days, mice were boosted i.p. with ~10^6 cfu and analyzed 14d later by ELISpot assay. For this, 0.5—1×10^6 non-immune and immune splenocytes were stimulated with a panel of class II-restricted *L. monocytogenes*-derived peptide epitopes or negative control peptides (see Table S2).

RESULTS

Indirect presentation of HY alloantigen primes T_H cells in vivo

In order to study the mechanism(s) underlying indirect presentation of cytosolic MHC class II-restricted Ags, we first determined how the male H2A^{b}-restricted HY minor histocompatibility Ag (mHAg) is presented to T_H cells. The alloantigenic HY peptide (pHY) is derived from RNA helicase, a ubiquitously expressed nucleo-cytoplasmic protein encoded by the evolutionarily conserved *Dby* gene located on the Y-chromosome (6, 43). No other H2^{b}-restricted T cell epitopes are derived from this helicase (46). Thus, female C57BL/6 (B6) and B6.129-Ab^{0} mice were immunized with H2^{b}-compatible but mHAg-incompatible (Table S1) male 129 donor splenocytes. After 7d, the ability of mHAg-reactive T_H cells and CTL to produce interferon-γ (IFNγ) was determined by ELISpot assay.

Immunisation of B6 mice resulted in IFNγ-producing splenic T_H cells to pHY but not to the control *Dbx*-encoded self HX peptide (pHX; Fig. 1) expressed by both males and females. This response was specific because pHY did not elicit any IFNγ response from immune B6.129-Ab^{0} mice (Fig. 1). Moreover, female B6 mice immunized with male 129-Ab^{0} splenocytes also primed pHY-reactive T_H cells (Fig. 1). Therefore, we conclude that the H2A^{b}-restricted HY Ag is indirectly presented to T_H cells in vivo.

In the same experiment described above, the role of pHY-specific T_H response in eliciting CTL responses to class I-restricted mHAgs was determined. We found IFNγ-producing CTL responses to the immunodominant $H2K^b$-restricted, H60 and $H4^b$ alloantigens but not to control $H2K^b$-restricted, SV40 TAg (TAg) epitope-IV (epi-IV) in B6 mice immunized with either male 129 or 129-Ab^0 splenocytes (Fig. 1). Nonetheless, B6.129-Ab^0 recipients did not elicit CTL responses to class I-restricted pH60 and $pH4^b$ (Fig. 1). Furthermore, T_H and CTL responses similar to those described above were obtained using Ii-deficient recipients (data not shown). These data together suggest that the primary CTL response to mHAgs is entirely dependent on CD4 help.

Indirect presentation of pHY requires $CD8^+$ dendritic cells

Because *Dby* is broadly expressed (47), it was important to determine which donor cell type donates and which recipient APC type presents the alloantigen. For this, we took advantage of the $hDTR^{tg}$ mouse–in which the human DT receptor transgene expression is regulated by the *Cd11c* enhancer/promotor (48). Thus, DT administration renders $hDTR^{tg}$ mice conditionally deficient in $CD11c^+$ myeloid cells including DCs and splenic sub-capsular macrophages (48, 49). We previously reported that DT-treated B6.FVB-$hDTR^{tg}$ mice became DC-deficient within ~18hrs and remained so for 72hrs (45).

To determine which APC type presents donor mHAg, we treated B6.FVB-$hDTR^{tg}$ mice with PBS or DT and immunized them ~18 hrs later with male splenocytes from 129.FVB-$hDTR^{tg}$ mice that received PBS ~18 hrs earlier. On d7, pHY-specific T_H cell responses were monitored. Depletion of recipients' $CD11c^+$ cells dramatically tempered T_H cell responses to pHY compared to that observed in mice containing $CD11c^+$ cells (Fig. 2a). Similarly, depletion of donor $CD11c^+$ cells resulted in poor T_H cell responses to pHY (Fig. 2b) indicating a significant role for $CD11c^+$ cells in donating alloantigens for indirect presentation. As expected, depletion of both recipient and donor $CD11c^+$ cells resulted in no T_H cell response to pHY (Fig. 2a). Additional data revealed that both donor and recipient $CD11c^+$ cells were required to prime class I-restricted pH60 and $pH4^b$-CTL responses in vivo (unpublished data). Because DCs express high levels of CD11c, constitute the majority of $CD11c^+$ splenocytes and are critical for priming naïve T cells, the above data suggest that DCs are responsible for indirect presentation.

To firm the contribution of DCs in indirect presentation and to determine which DC subset is responsible, we used the recently reported 129-$Batf3^0$ mice, which are deficient in splenic $CD8^+$ DCs (50). Female 129 and 129-$Batf3^0$ mice were immunized with male B6, 129 or 129-$Batf3^0$ splenocytes and HY-specific T_H cell response was monitored 7d later. The data revealed that the lack of splenic $CD8^+$ DCs in the recipient dramatically reduced the T_H cell response to HY (Fig. 2c). Similarly, the lack of donor $CD8^+$ DCs resulted in much tempered T_H cell response to HY (Fig. 2d), which was completely lost upon immunising $CD8^+$ DC-deficient female recipients with male donor splenocytes lacking $CD8^+$ DCs (Fig. 2e).

We also monitored pH60-speicific CTL responses in the experiment described above. The data revealed a requirement for donor and recipient $CD8^+$ DCs for cross-priming CTL responses to pH60 (unpublished data). Together, these data suggest that $CD8^+$ DC play important roles in donating and indirectly presenting the HY alloantigen.

TAP and ERAAP regulate T_H cell responses to pHY

Because pHY is derived from nucleo-cytoplasmic RNA helicase, we predicted that components of the CAP machinery might have access to HY and potentially regulate its availability for indirect presentation. Therefore, we next determined whether TAP had any role in indirect presentation of pHY. Immunisation of female B6 mice with male splenocytes

derived from $H2^b$-compatible but mHAg-incompatible C.B10-$H2^b$ (BALB.B; Table S1) or B.129-TAP^0 (B stands for BALB.B) mice generated comparable pHY-specific T_H cell response (Fig. 3a, b). Similarly, B6.129-TAP^0 female mice immunised with C.B10-$H2^b$ male splenocytes elicited comparable pHY-specific T_H cell responses (Fig. 3a, b). Surprisingly, however, when B6.129-TAP^0 female recipients were immunised with B.129-TAP^0 male donor splenocytes, 2—3-fold increased T_H cell response against $H2A^b$-restricted pHY was observed (Fig. 3a). Thus, TAP function in both donor and recipient cells had a detrimental effect on the indirect presentation of class II-restricted cytoplasmic Ag that tempered the T_H cell response.

We considered the possibility that peptides translocated by TAP into the ER might become substrates for destruction by ERAAP, and hence unavailable for presentation. To test this possibility, B6, B6.129-TAP^0 and B6.129-$ERAAP^0$ female mice were immunised with C.B10-$H2^b$, B.129-TAP^0 or 129-$ERAAP^0$ male splenocytes. As with B6 and B6.129-TAP^0 mice, B6.129-$ERAAP^0$ female mice immunised with wt male splenocytes elicited similar pHY-specific T_H cell responses (Fig. 3b—d). In striking contrast, immunisation of B6.129-$ERAAP^0$ female mice with B.129-TAP^0 male splenocytes resulted in two-fold increases pHY-specific T_H cell responses (Fig. 3b). Similarly, immunisation of B6.129-TAP^0 or B6.129-$ERAAP^0$ mice with 129-$ERAAP^0$ male splenocytes resulted in a 2—3-fold increase in pHY-specific T_H cell response (Fig. 3c, d).

As a control for the above experiments, the monitoring of CTL response in wt mice immunised with male wt or TAP-deficient donor splenocytes revealed an identical CTL response to pH60 and $pH4^b$, suggesting that the two class I-restricted mHAgs are cross-presented (Fig. S1a). As expected, TAP-deficient recipient did not respond to class I-restricted mHAgs as they are devoid of $CD8^+$ T cells (Fig. S1a). We therefore, conclude that a pool of cytoplasmic class II-restricted Ags is pumped into the ER by TAP, thence destroyed by ERAAP.

TAP and ERAAP regulate indirect presentation of class II-restricted bacterial Ags in vivo

To determine the generality of TAP's and ERAAP's role in indirect Ag presentation, we tested whether the CAP pathway impacts indirect presentation of *L. monocytogenes*-derived class II-restricted Ags. *L. monocytogenes* listerolysin O (LLO) disrupts the phagolysosome to permit entry of the organism into the cytoplasm for its growth, and multiplication. Therefore, the priming of T_H cell responses against listerial Ags requires indirect presentation (51–53). Thus, B6, B6.129-TAP^0, B6.129-$ERAAP^0$ and B6.129-Ab^0 as well as 129S6/SvEvTac, 129-$ERAAP^0$, B6.129-Ab^0 and 129-Ab^0 mice were inoculated i.p. with bacteria, boosted 14d later and secondary T_H cell response to known $H2A^b$-restricted epitopes were monitored after an additional 14d. PBS-treated B6 and 129 mice served as negative controls. We observed a 2—5-fold increase in the secondary T_H cell response to $H2A^b$-restricted pLLO(190–201), p60(177–188), pLLO(318–329), and pLLO(253–264) in B6.129-TAP^0 mice compared to B6 mice (Fig. 4a, b). A similar pattern of increased T_H cell reactivity to pLLO(190–201), p60(177–188) and pLLO(318–329) was observed in ERAAP-deficient mice compared to B6 mice (Fig. 4a, b). In contrast, the response to pLLO(253–264) was indistinguishable between wt and ERAAP-deficient mice (Fig 4b). As expected, neither *Listeria*-inoculated $H2Ab^0$ nor PBS-treated wt mice responded to the three listerial peptides; none of the mice responded to irrelevant peptides (Fig. 4). In additional experiments, we also found that the primary T_H response to *L. monocytogenes* Ags—elicited by retro-orbital bacterial inoculation—yielded similar results as above (Fig. S2). Thus, the TAP and ERAAP effect on indirect presentation of cytoplasmic class II-restricted Ags appears to be a general principle as they impact T_H cell responses to mHAgs and bacterial Ags similarly.

Proteasomes regulate indirect presentation of HY mHAg

Several mechanisms can potentially explain the above finding: (a) competition between class I and class II molecules for Ag; (b) competition between $CD4^+$ and $CD8^+$ T cells; (c) enhanced autophagy and/or enhanced ER-associated degradation (ERAD); and (d) quantitative differences in the Ag(s) presented.

To test whether competition for Ag played a role, T_H response of female B6, B6.129-$\beta 2m^{-/-}$ and B6.129-$Tpn^{-/-}$ mice—which, akin to TAP deficiency, lack functional class I-assembly complex due to β2m and tapasin deficiency—was assessed after immunising with male C.B10-$H2^b$ or B.129-TAP^0 splenocytes. All three recipients elicited similar pHY-specific T_H cell responses (Fig. 5a), suggesting that simply lacking class I does not 'free up' more cytoplasmic Ags for presentation by class II molecules. In conjunction with the fact that no known CTL epitopes are derived from *Dby*-encoded helicase (46), competition for Ag is a less likely explanation for our finding.

To ascertain whether the increased T_H cell responses was a compensatory effect caused by the absence of recipient CTL, B6.129-$CD8\alpha^{-/-}$ female mice were immunised with either C.B10-$H2^b$ or B.129-TAP^0 male splenocytes. The resulting T_H cell response to pHY was comparable in both wt and $CD8^+$ T cell-deficient mice (Fig. 5b). As expected, female B6.129-$\beta 2m^0$, B6.129-Tpn^0 and B6.129-$CD8\alpha^0$ recipients did not elicit IFNγ response to class I-restricted mHAgs (Fig. S1b, c). Hence, competition between $CD4^+$ and $CD8^+$ T cells is unlikely to explain the increased T_H response in the absence of TAP or ERAAP.

TAP andβ2m deficiency enhances ERAD (54). ERAD can enhance autophagy (55), which is required for class II-restricted cytoplasmic Ag presentation (11–14, 23). Nevertheless, we found that immunisation of female B6 mice with male 129 splenocytes treated with tunicamycin—which induces ERAD due to stress from accumulating unfolded proteins—completely abrogated T_H response to pHY whilst DMSO-treated donor cells responded as expected (Fig. 5c). Similarly, induction of autophagy—by maintaining donor male splenocytes in nutrition-free conditions prior to immununisation of female B6 mice—did not enhance, but instead abrogated the T_H response to pHY (Fig. 5d). Additionally, constitutive autophagy was not enhanced in TAP^0 (TAPTAg) or $\beta 2m^0$ (β2mTAg) TAg-transformed fibroblast lines compared to similarly transformed wt fibroblasts (wtTAg; (56) as similar levels of LC3-I and LC3-II were detected in immunoblots of proteins extracted from wt and mutant lines (Fig. 5e). Together, these data discount a role for enhanced ERAD and autophagy in explaining the impact of TAP and ERAAP on indirect presentation of cytosolic Ags. If anything, the data argue that if autophagy is enhanced by TAP or ERAAP deficiency, it would destroy and not protect cytoplasmic Ags for indirect presentation by class II molecules.

As proteasomal degradation is enhanced by ERAD (55), we tested whether proteolysis within the cytosol of donor cells impacted indirect Ag presentation. If enhanced ERAD was the cause for the phenotype then proteasome inhibition should abrogate T_H cell response to HY. Conversely, if cytosolic degradation, rather than ERAD, was the mechanism, then proteasome inhibition should recapitulate the TAP and ERAAP effect. Thus, B6 mice were immunized with male 129 splenocytes that were treated for 2hr with either DMSO or the selective proteasome inhibitor epoxomicin (57, 58) and T_H cell responses were monitored. Surprisingly, in contrast to the negative outcome of immunisation with tunicamycin-treated cells, we found that irreversible proteasome inhibition of donor cells resulted in a two-fold increase in T_H cell responses to pHY when compared to that elicited by donor cells containing functional proteasomes (Fig. 6a). Thus, proteasomes negatively impact indirect presentation and the intact form of the HY alloantigen is perhaps donated to recipient $CD8^+$ DCs for indirect presentation.

If intact antigen is donated for indirect presentation, then it may require processing within recipient DCs. Because recipient TAP and ERAAP influenced indirect presentation of pHY, we reasoned that the recipient's proteasomes may be involved. Thus, immunisation of female B6.129-LMP2$^{-/-}$ mice with male 129 splenocytes resulted in tempered T_H response to pHY/A^b (Fig. 6b). Surprisingly however, the T_H response to pHY/A^b was completely lost if the donor splenocytes were treated with epoxomicin and then transferred into LMP2-deficient recipient (Fig. 6b). Consistent with this result is the finding that altered pH balance of the phagolysosome caused by a deficiency in donor and/or recipient gp91^{PHOX} did not affect T_H cell response to pHY/A^b (data not shown). These data suggest that the increased donation of intact HY Ag upon proteasomal inhibition of donor cells requires cytosolic processing within the recipient DCs.

Role for chaperones in indirect presentation of HY alloantigen

Cross-presentation of class I-restricted antigens require heat shock proteins (HSP) (59, 60). Because the HY alloantigen is a nucleo-cytoplasmic protein that is degraded by donor proteasomes (Fig. 6), we reasoned that donor HSP may play a role in indirect presentation of this antigen. This possibility was addressed in two ways: In the first approach, male 129 splenocytes were treated with pharmacologic HSP inhibitors, geldanamycin and KNK437, or DMSO for 4hrs and used to immunise B6 mice. Inhibition of HSP90 with either geldanamycin or KNK437 tempered T_H cell responses against pHY (Fig. 7a). This result suggested a role for HSP90 in indirect presentation of HY alloantigen.

To firm a role for HSP90, in the second approach, we employed mouse embryonic fibroblasts (MEF) deficient in heat shock factor protein 1 (Hsf1), a transcription factor that regulates the expression of members of the HSP90 family of heat shock proteins (42). We first generated HY$^+$Hsf1^0 and wt MEF by *Dby* cDNA transfer because these cells do not otherwise express HY mHAg (data not shown). Immunisation of B6 mice with HY$^+$Hsf1^0 MEF resulted in tempered T_H cell responses to HY compared to mice immunized with HY$^+$ wt MEF (Fig. 7b). These results imply a critical role for HSP90 in efficient indirect presentation of the HY alloantigen.

Calreticulin (CRT), an ER-resident chaperone, is implicated in cross-presentation of class I-restricted antigens (61). Therefore, we determined whether CRT expression by donor APC was essential for indirect presentation of HY alloantigen. For this, we first generated HY$^+$CRT^0 and HY$^+$CRT$^+$ MEF by *Dby* cDNA transfer. Immunisation of B6 mice with HY$^+$CRT^0 MEF resulted in tempered T_H cell responses to HY compared to mice immunized with HY$^+$CRT$^+$ MEF (Fig. 7c). These results imply a critical role for CRT in efficient indirect presentation of the HY alloantigen.

TAP regulates the quantity of class II-restricted Ags displayed

To test the idea that TAP and ERAAP regulate the quantitative aspects of class II-restricted Ag presentation, we determined the response of two distinct H3b^a mHAg-specific T_H cell lines, LPa/B10-B6 and LPa/B10-line. Akin to HY, the H3b^a mHAg is also derived from a cytoplasmic protein, ribosome binding protein-1 (RRBP1; AC Brown, GJC & DCR, in preparation). Moreover, the H3b^a-reactive T cell lines allowed us to address the direct role of TAP and ERAAP in class II-restricted cytosolic Ag presentation independently of any potential indirect effect TAP and ERAAP might have on responder T cells in the intact mouse. Thus, LPa/B10-B6 and LPa/B10-line were stimulated with B6, B6.129-TAP^0, C.B10-H2^b, B.129-TAP$^{-/-}$ or 129/SvJ splenocytes for 48 hrs and the number of IFNγ^+ spots determined. The data revealed that B6.129-TAP^0 splenocytes, compared to B6 splenocytes, induced 7—8-fold greater number of IFNγ^+ spots from the two H3b^a-specific T_H clones compared to B6 splenocytes (Fig. 8). This response was Ag-specific because the

T_H cell clones did not respond to negative control C.B10-H2^b, B.129-TAP^0 and 129/SvJ splenocytes (Fig. 8). Thus, TAP and ERAAP regulate the quantity of class II-restricted Ag presentation.

DISCUSSION

Despite the recognition that class II molecules present cytoplasmic Ags directly and indirectly, the principles underlying indirect presentation are poorly defined. Understanding this process is highly significant because T_H cells regulate antibody- and CTL-mediated adaptive immunity to pathogens, cancers, allografts and autoantigens. Such an understanding is especially important because many tumour and virus infected cells down regulate TAP gene expression to evade CTL-mediated immune surveillance. Furthermore, our findings will impact how we understand T cell responses in individuals that express TAP null and ERAAP variants (62–64), especially those that inhibit or alter peptide processing within the ER.

We have shown here that indirect presentation of class II-restricted Ags requires CD8$^+$ donor and recipient DCs. Within these cells, proteasomes, TAP and ERAAP—key components of the cytoplasmic Ag-processing pathway—regulate indirect presentation of class II-restricted Ags thereby impacting the magnitude of T_H cell responses to cytoplasmic alloantigens (HY and H3b) and bacterial (*L. monocytogenes*) Ags. Because these effects were observed with two distinct models, we suggest that the impact of the CAP machinery on indirect presentation of cytoplasmic Ags might be a general regulatory process, one that is of significant immunologic import.

TAP deficiency is known to alter NK cell development and function in both mice and humans (62, 63, 65). The altered NK cell function was previously shown to indirectly regulate CD4$^+$ T cell priming in a *Toxoplasma gondii* infection model (66). Hence, it was possible that the several fold increased T_H cell response to the HY alloantigen and listerial Ags in TAP-null recipients were indirectly regulated by NK cells. Therefore, we immunised both wt and NK cell-deficient IL-15^0 mice with male donor cells and found that the pHY/A^b-specific T_H response was similar in both recipients (data not shown). Thus, we conclude that NK cells contributed very little to the T_H cell response to HY.

Although TAP and β2-m deficiencies are known to cause ERAD (67), and ERAD enhances autophagy (55), we systematically ruled out a role for these degradative processes as mechanisms underlying our central observations. Note that we do not claim that autophagy per se is not involved. But we claim that because TAP-deficiency does not enhance autophagy, the increased class II-restricted Ag presentation in the absence of peptide transport to the ER is not due to overt autophagy. Furthermore, neither β2m nor tapasin deficiencies altered indirect Ag presentation. Their absence, akin to TAP deficiency, renders class I molecules unstable and also results in mice that lack CD8$^+$ T cells. Hence, competition between class I and class II molecules as well as competition between T_H cells and CTL for the same Ag is a most unlikely mechanism by which TAP and ERAAP deficiencies alter indirect presentation of cytosolic Ags.

Our data suggests that TAP and ERAAP are acting directly on class II-restricted cytoplasmic Ags. Such Ags are perhaps processed by the proteasome in the cytoplasm and transported to the ER lumen. Thus, TAP and ERAAP deficiency would prevent transport of processed cytoplasmic peptides into the ER lumen and their subsequent degradation. Such a process would then quantitatively increase the cytoplasmic Ag pool making it available for indirect presentation. Indeed, our data favours this role for TAP and ERAAP in indirect Ag

presentation as observed with the increased presentation of the H3b^a mHAg by TAP-deficient splenocytes.

Curiously, the effect of TAP and ERAAP on indirect presentation was only observed when both the donor and recipient APC were deficient in the CAP components. Therefore, one possibility is that the donated Ag escapes into the cytoplasm of the recipient APC upon donation by donor allogeneic cells. That such escape might occur is consistent with the need for recipient proteasome for indirect presentation of pHY and the lack of a role for gp91^{PHOX} for indirect presentation of the same Ag. The escape of Ags from the phagosome to the cytoplasm has been observed with several model and microbial Ags used for mechanistic studies of class I-restricted Ag cross-presentation (68–70). Thus, the CAP pathway can sculpt the repertoire of class II-restricted cytoplasmic Ags in both donor and recipient APC.

We view the data obtained with LMP2-deficient mice with caution as prior studies have shown that alterations in the immunoproteasomes can impact CTL repertoire as well as T cell activation (71, 72). We reported herein that LMP2 deficiency resulted in ~50% reduction in T_H cell response to pHY/A^b. This result could be explained entirely by deficiencies in T cell repertoire and/or activation in the LMP2-null recipients as suggested in previous reports (71, 72). Notwithstanding, the T_H response to pHY/A^b was completely lost when the LMP2-null recipients were immunised with donor cells in which the proteasomes were irreversibly inhibited. If processing of the donated Ag occurred independent of the recipient's proteasome, then one would have expected the same level of T_H cell response to pHY/A^b when LMP2^0 mice were immunised with untreated or epoxomicin-treated donor cells. But instead, the response to the latter was completely lost. Hence, we suggest that the HY alloantigen is donated as an intact protein, which is then processed by the recipient immunoproteasomes for indirect presentation. Furthermore, this finding suggests that the donor HY alloantigen accesses the recipient's cytoplasm as has been reported for HIV nef and HSV-1 glycoprotein B (68, 70). This is perhaps why TAP and ERAAP impact indirect presentation of the donated Ags by class II molecules.

It is noteworthy that the inhibition of constitutive and induced HSP90 function in the donor cells disrupted indirect presentation of class II-restricted Ags, and so did the absence of calreticulin. Both HSP90 and calreticulin are implicated as chaperonins for the donation of cross-presented Ags to presenting APC (59–61). Therefore, HSP90 and calreticulin may work together to chaperone Ags for indirect presentation of class II-restricted Ags as well. Although calreticulin deficiency could induce/enhance ERAD/autophagy, for afore discussed reasons, these processes do not explain the need for the two chaperonins in indirect presentation. Moreover, calreticulin is also known to act as an "eat me" signal for apoptotic cells, which express the otherwise ER-resident protein at the plasma membrane (73, 74). Therefore, calreticulin-deficiency may have resulted in poor phagocytosis of the allogeneic donor cells, thereby severely impeding indirect presentation.

Taken together, the model that emerges from the data presented herein is that proteasomes, TAP and ERAAP regulate the quantity of the class II-associated self (mHAgs) and non-self (listerial) peptide repertoire. The increased self-peptide presentation could alter the CD4$^+$ T cell repertoire in recipient cells. Nonetheless, current serological data indicates that the CD4$^+$ T cell repertoire is very similar between wt and TAP-deficient mice (54). Altered cytosolic Ag pool within donating cells coupled with altered Ag presentation by the APC could explain how the CAP machinery regulates T_H cell responses to indirectly presented cytosolic Ags.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S Roopenian for help with growth and maintenance of T cell clones; Dr. K Murphy for providing Batf3^{0} mice; and Dr I Benjamin for providing Hsf-1^{0} cell line.

References

1. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998; 392:245–252. [PubMed: 9521319]
2. Reis e Sousa C. Activation of dendritic cells: translating innate into adaptive immunity. Curr Opin Immunol. 2004; 16:21–25. [PubMed: 14734106]
3. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol. 2005; 23:975–1028. [PubMed: 15771591]
4. Cresswell P, Ackerman AL, Giodini A, Peaper DR, Wearsch PA. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. Immunol Rev. 2005; 207:145–157. [PubMed: 16181333]
5. Wilson NS, Villadangos JA. Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications. Adv Immunol. 2005; 86:241–305. [PubMed: 15705424]
6. Chen Y, Demir Y, Valujskikh A, Heeger PS. The male minor transplantation antigen preferentially activates recipient CD4+ T cells through the indirect presentation pathway in vivo. J Immunol. 2003; 171:6510–6518. [PubMed: 14662851]
7. Thery C, Duban L, Segura E, Veron P, Lantz O, Amigorena S. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. Nat Immunol. 2002; 3:1156–1162. [PubMed: 12426563]
8. Russmann H, Gerdemann U, Igwe EI, Panthel K, Heesemann J, Garbom S, Wolf-Watz H, Geginat G. Attenuated Yersinia pseudotuberculosis carrier vaccine for simultaneous antigen-specific CD4 and CD8 T-cell induction. Infect Immun. 2003; 71:3463–3472. [PubMed: 12761131]
9. Valentino MD, Hensley LL, Skrombolas D, McPherson PL, Woolard MD, Kawula TH, Frelinger JA, Frelinger JG. Identification of a dominant CD4 T cell epitope in the membrane lipoprotein Tul4 from Francisella tularensis LVS. Mol Immunol. 2009; 46:1830–1838. [PubMed: 19233475]
10. Matyszak MK, Young JL, Gaston JS. Uptake and processing of Chlamydia trachomatis by human dendritic cells. Eur J Immunol. 2002; 32:742–751. [PubMed: 11870618]
11. Dissanayake SK, Tuera N, Ostrand-Rosenberg S. Presentation of endogenously synthesized MHC class II-restricted epitopes by MHC class II cancer vaccines is independent of transporter associated with Ag processing and the proteasome. J Immunol. 2005; 174:1811–1819. [PubMed: 15699107]
12. Nimmerjahn F, Milosevic S, Behrends U, Jaffee EM, Pardoll DM, Bornkamm GW, Mautner J. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. Eur J Immunol. 2003; 33:1250–1259. [PubMed: 12731050]
13. Paludan C, Schmid D, Landthaler M, Vockerodt M, Kube D, Tuschl T, Munz C. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science. 2005; 307:593–596. [PubMed: 15591165]
14. Zhou D, Li P, Lin Y, Lott JM, Hislop AD, Canaday DH, Brutkiewicz RR, Blum JS. Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens. Immunity. 2005; 22:571–581. [PubMed: 15894275]
15. Jacobson S, Sekaly RP, Jacobson CL, McFarland HF, Long EO. HLA class II-restricted presentation of cytoplasmic measles virus antigens to cytotoxic T cells. J Virol. 1989; 63:1756–1762. [PubMed: 2784508]

16. Jaraquemada D, Marti M, Long EO. An endogenous processing pathway in vaccinia virus-infected cells for presentation of cytoplasmic antigens to class II-restricted T cells. J Exp Med. 1990; 172:947–954. [PubMed: 2388037]
17. Wang RF, Wang X, Atwood AC, Topalian SL, Rosenberg SA. Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science. 1999; 284:1351–1354. [PubMed: 10334988]
18. Brooks A, Hartley S, Kjer-Nielsen L, Perera J, Goodnow CC, Basten A, McCluskey J. Class II-restricted presentation of an endogenously derived immunodominant T-cell determinant of hen egg lysozyme. Proc Natl Acad Sci U S A. 1991; 88:3290–3294. [PubMed: 1707537]
19. Chicz RM, Urban RG, Gorga JC, Vignali DA, Lane WS, Strominger JL. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. J Exp Med. 1993; 178:27–47. [PubMed: 8315383]
20. Dongre AR, Kovats S, deRoos P, McCormack AL, Nakagawa T, Paharkova-Vatchkova V, Eng J, Caldwell H, Yates JR 3rd, Rudensky AY. In vivo MHC class II presentation of cytosolic proteins revealed by rapid automated tandem mass spectrometry and functional analyses. Eur J Immunol. 2001; 31:1485–1494. [PubMed: 11465105]
21. Rudensky A, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA Jr. Sequence analysis of peptides bound to MHC class II molecules. Nature. 1991; 353:622–627. [PubMed: 1656276]
22. Bird PI, Trapani JA, Villadangos JA. Endolysosomal proteases and their inhibitors in immunity. Nat Rev Immunol. 2009; 9:871–882. [PubMed: 19935806]
23. Lee HK, Mattei LM, Steinberg BE, Alberts P, Lee YH, Chervonsky A, Mizushima N, Grinstein S, Iwasaki A. In vivo requirement for Atg5 in antigen presentation by dendritic cells. Immunity. 2011; 32:227–239. [PubMed: 20171125]
24. Michalek MT, Benacerraf B, Rock KL. The class II MHC-restricted presentation of endogenously synthesized ovalbumin displays clonal variation, requires endosomal/lysosomal processing, and is up-regulated by heat shock. J Immunol. 1992; 148:1016–1024. [PubMed: 1737924]
25. Reed AJ, Noorchashm H, Rostami SY, Zarrabi Y, Perate AR, Jeganathan AN, Caton AJ, Naji A. Alloreactive CD4 T cell activation in vivo: an autonomous function of the indirect pathway of alloantigen presentation. J Immunol. 2003; 171:6502–6509. [PubMed: 14662850]
26. Richards DM, Dalheimer SL, Ehst BD, Vanasek TL, Jenkins MK, Hertz MI, Mueller DL. Indirect minor histocompatibility antigen presentation by allograft recipient cells in the draining lymph node leads to the activation and clonal expansion of CD4+ T cells that cause obliterative airways disease. J Immunol. 2004; 172:3469–3479. [PubMed: 15004147]
27. Hammer GE, Gonzalez F, Champsaur M, Cado D, Shastri N. The aminopeptidase ERAAP shapes the peptide repertoire displayed by major histocompatibility complex class I molecules. Nat Immunol. 2006; 7:103–112. [PubMed: 16299505]
28. Hammer GE, Gonzalez F, James E, Nolla H, Shastri N. In the absence of aminopeptidase ERAAP, MHC class I molecules present many unstable and highly immunogenic peptides. Nat Immunol. 2007; 8:101–108. [PubMed: 17128277]
29. Yan J V, Parekh V, Mendez-Fernandez Y, Olivares-Villagomez D, Dragovic S, Hill T, Roopenian DC, Joyce S, Van Kaer L. In vivo role of ER-associated peptidase activity in tailoring peptides for presentation by MHC class Ia and class Ib molecules. J Exp Med. 2006; 203:647–659. [PubMed: 16505142]
30. York IA, Brehm MA, Zendzian S, Towne CF, Rock KL. Endoplasmic reticulum aminopeptidase 1 (ERAP1) trims MHC class I-presented peptides in vivo and plays an important role in immunodominance. Proc Natl Acad Sci U S A. 2006; 103:9202–9207. [PubMed: 16754858]
31. Lich JD, Elliott JF, Blum JS. Cytoplasmic processing is a prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins. J Exp Med. 2000; 191:1513–1524. [PubMed: 10790426]
32. Tewari MK, Sinnathamby G, Rajagopal D, Eisenlohr LC. A cytosolic pathway for MHC class II-restricted antigen processing that is proteasome and TAP dependent. Nat Immunol. 2005; 6:287–294. [PubMed: 15711549]
33. Mukherjee P, Dani A, Bhatia S, Singh N, Rudensky AY, George A, Bal V, Mayor S, Rath S. Efficient presentation of both cytosolic and endogenous transmembrane protein antigens on MHC

class II is dependent on cytoplasmic proteolysis. J Immunol. 2001; 167:2632–2641. [PubMed: 11509605]

34. Testa JS, Apcher GS, Comber JD, Eisenlohr LC. Exosome-driven antigen transfer for MHC class II presentation facilitated by the receptor binding activity of influenza hemagglutinin. J Immunol. 2011; 185:6608–6616. [PubMed: 21048109]
35. Malnati MS, Marti M, LaVaute T, Jaraquemada D, Biddison W, DeMars R, Long EO. Processing pathways for presentation of cytosolic antigen to MHC class II-restricted T cells. Nature. 1992; 357:702–704. [PubMed: 1614517]
36. Dani A, Chaudhry A, Mukherjee P, Rajagopal D, Bhatia S, George A, Bal V, Rath S, Mayor S. The pathway for MHCII-mediated presentation of endogenous proteins involves peptide transport to the endo-lysosomal compartment. J Cell Sci. 2004; 117:4219–4230. [PubMed: 15316082]
37. Gueguen M, Long EO. Presentation of a cytosolic antigen by major histocompatibility complex class II molecules requires a long-lived form of the antigen. Proc Natl Acad Sci U S A. 1996; 93:14692–14697. [PubMed: 8962116]
38. Oxenius A, Bachmann MF, Ashton-Rickardt PG, Tonegawa S, Zinkernagel RM, Hengartner H. Presentation of endogenous viral proteins in association with major histocompatibility complex class II: on the role of intracellular compartmentalization, invariant chain and the TAP transporter system. Eur J Immunol. 1995; 25:3402–3411. [PubMed: 8566030]
39. Loss GE Jr, Elias CG, Fields PE, Ribaudo RK, McKisic M, Sant AJ. Major histocompatibility complex class II-restricted presentation of an internally synthesized antigen displays cell-type variability and segregates from the exogenous class II and endogenous class I presentation pathways. J Exp Med. 1993; 178:73–85. [PubMed: 8315396]
40. Heath WR, Carbone FR. Cross-presentation, dendritic cells, tolerance and immunity. Annu Rev Immunol. 2001; 19:47–64. [PubMed: 11244030]
41. Gao B, Adhikari R, Howarth M, Nakamura K, Gold MC, Hill AB, Knee R, Michalak M, Elliott T. Assembly and antigen-presenting function of MHC class I molecules in cells lacking the ER chaperone calreticulin. Immunity. 2002; 16:99–109. [PubMed: 11825569]
42. Xiao X, Zuo X, Davis AA, McMillan DR, Curry BB, Richardson JA, Benjamin IJ. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. Embo J. 1999; 18:5943–5952. [PubMed: 10545106]
43. Scott D, Addey C, Ellis P, James E, Mitchell MJ, Saut N, Jurcevic S, Simpson E. Dendritic cells permit identification of genes encoding MHC class II-restricted epitopes of transplantation antigens. Immunity. 2000; 12:711–720. [PubMed: 10894170]
44. Yoshimura Y, Yadav R, Christianson GJ, Ajayi WU, Roopenian DC, Joyce S. Duration of alloantigen presentation and avidity of T cell antigen recognition correlate with immunodominance of CTL response to minor histocompatibility antigens. J Immunol. 2004; 172:6666–6674. [PubMed: 15153482]
45. Bezbradica JS, Stanic AK, Matsuki N, Bour-Jordan H, Bluestone JA, Thomas JW, Unutmaz D, Van Kaer L, Joyce S. Distinct roles of dendritic cells and B cells in Va14Ja18 natural T cell activation in vivo. J Immunol. 2005; 174:4696–4705. [PubMed: 15814694]
46. Spencer CT, Gilchuk P, Dragovic SM, Joyce S. Minor histocompatibility antigens: presentation principles, recognition logic and the potential for a healing hand. Curr Opin Organ Transplant. 2010; 15:512–525. [PubMed: 20616723]
47. Mazeyrat S, Saut N, Sargent CA, Grimmond S, Longepied G, Ehrmann IE, Ellis PS, Greenfield A, Affara NA, Mitchell MJ. The mouse Y chromosome interval necessary for spermatogonial proliferation is gene dense with syntenic homology to the human AZFa region. Hum Mol Genet. 1998; 7:1713–1724. [PubMed: 9736773]
48. Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity. 2002; 17:211–220. [PubMed: 12196292]
49. Aoshi T, Zinselmeyer BH, Konjufca V, Lynch JN, Zhang X, Koide Y, Miller MJ. Bacterial entry to the splenic white pulp initiates antigen presentation to CD8+ T cells. Immunity. 2008; 29:476–486. [PubMed: 18760639]

50. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, Calderon B, Schraml BU, Unanue ER, Diamond MS, Schreiber RD, Murphy TL, Murphy KM. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science. 2008; 322:1097–1100. [PubMed: 19008445]

51. Skoberne M, Schenk S, Hof H, Geginat G. Cross-presentation of Listeria monocytogenes-derived CD4 T cell epitopes. J Immunol. 2002; 169:1410–1418. [PubMed: 12133966]

52. Lara-Tejero M, Pamer EG. T cell responses to Listeria monocytogenes. Curr Opin Microbiol. 2004; 7:45–50. [PubMed: 15036139]

53. Geginat G, Schenk S, Skoberne M, Goebel W, Hof H. A novel approach of direct ex vivo epitope mapping identifies dominant and subdominant CD4 and CD8 T cell epitopes from Listeria monocytogenes. J Immunol. 2001; 166:1877–1884. [PubMed: 11160235]

54. Tourne S, van Santen HM, van Roon M, Berns A, Benoist C, Mathis D, Ploegh H. Biosynthesis of major histocompatibility complex molecules and generation of T cells in Ii TAP1 double-mutant mice. Proc Natl Acad Sci U S A. 1996; 93:1464–1469. [PubMed: 8643655]

55. Kroeger H, Miranda E, MacLeod I, Perez J, Crowther DC, Marciniak SJ, Lomas DA. Endoplasmic reticulum-associated degradation (ERAD) and autophagy cooperate to degrade polymerogenic mutant serpins. J Biol Chem. 2009; 284:22793–22802. [PubMed: 19549782]

56. Mylin LM, Schell TD, Roberts D, Epler M, Boesteanu A, Collins EJ, Frelinger JA, Joyce S, Tevethia SS. Quantitation of CD8(+) T-lymphocyte responses to multiple epitopes from simian virus 40 (SV40) large T antigen in C57BL/6 mice immunized with SV40, SV40 T-antigen-transformed cells, or vaccinia virus recombinants expressing full-length T antigen or epitope minigenes. J Virol. 2000; 74:6922–6934. [PubMed: 10888631]

57. Meng L, Mohan R, Kwok BH, Elofsson M, Sin N, Crews CM. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. Proc Natl Acad Sci U S A. 1999; 96:10403–10408. [PubMed: 10468620]

58. Schwarz K, de Giuli R, Schmidtke G, Kostka S, van den Broek M, Kim KB, Crews CM, Kraft R, Groettrup M. The selective proteasome inhibitors lactacystin and epoxomicin can be used to either up-or down-regulate antigen presentation at nontoxic doses. J Immunol. 2000; 164:6147–6157. [PubMed: 10843664]

59. Lev A, Takeda K, Zanker D, Maynard JC, Dimberu P, Waffarn E, Gibbs J, Netzer N, Princiotta MF, Neckers L, Picard D, Nicchitta CV, Chen W, Reiter Y, Bennink JR, Yewdell JW. The exception that reinforces the rule: crosspriming by cytosolic peptides that escape degradation. Immunity. 2008; 28:787–798. [PubMed: 18549799]

60. Srivastava P. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. Annu Rev Immunol. 2002; 20:395–425. [PubMed: 11861608]

61. Berwin B, Hart JP, Rice S, Gass C, Pizzo SV, Post SR, Nicchitta CV. Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. Embo J. 2003; 22:6127–6136. [PubMed: 14609958]

62. de la Salle H, Hanau D, Fricker D, Urlacher A, Kelly A, Salamero J, Powis SH, Donato L, Bausinger H, Laforet M, et al. Homozygous human TAP peptide transporter mutation in HLA class I deficiency. Science. 1994; 265:237–241. [PubMed: 7517574]

63. Matamoros N, Mila J, Llano M, Balas A, Vicario JL, Pons J, Crespi C, Martinez N, Iglesias-Alzueta J, Lopez-Botet M. Molecular studies and NK cell function of a new case of TAP2 homozygous human deficiency. Clin Exp Immunol. 2001; 125:274–282. [PubMed: 11529920]

64. Andres AM, Dennis MY, Kretzschmar WW, Cannons JL, Lee-Lin SQ, Hurle B, Schwartzberg PL, Williamson SH, Bustamante CD, Nielsen R, Clark AG, Green ED. Balancing selection maintains a form of ERAP2 that undergoes nonsense-mediated decay and affects antigen presentation. PLoS Genet. 2010; 6:e1001157. [PubMed: 20976248]

65. Ljunggren HG, Van Kaer L, Ploegh HL, Tonegawa S. Altered natural killer cell repertoire in Tap-1 mutant mice. Proc Natl Acad Sci U S A. 1994; 91:6520–6524. [PubMed: 8022815]

66. Goldszmid RS, Bafica A, Jankovic D, Feng CG, Caspar P, Winkler-Pickett R, Trinchieri G, Sher A. TAP-1 indirectly regulates CD4+ T cell priming in Toxoplasma gondii infection by controlling NK cell IFN-gamma production. J Exp Med. 2007; 204:2591–2602. [PubMed: 17923502]

67. Raposo G, van Santen HM, Leijendekker R, Geuze HJ, Ploegh HL. Misfolded major histocompatibility complex class I molecules accumulate in an expanded ER-Golgi intermediate compartment. J Cell Biol. 1995; 131:1403–1419. [PubMed: 8522600]

68. Ackerman AL, Giodini A, Cresswell P. A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. Immunity. 2006; 25:607–617. [PubMed: 17027300]

69. Singh R, Jamieson A, Cresswell P. GILT is a critical host factor for Listeria monocytogenes infection. Nature. 2008; 455:1244–1247. [PubMed: 18815593]

70. Singh R, Cresswell P. Defective cross-presentation of viral antigens in GILT-free mice. Science. 2010; 328:1394–1398. [PubMed: 20538950]

71. Chen W, Norbury CC, Cho Y, Yewdell JW, Bennink JR. Immunoproteasomes shape immunodominance hierarchies of antiviral CD8(+) T cells at the levels of T cell repertoire and presentation of viral antigens. J Exp Med. 2001; 193:1319–1326. [PubMed: 11390439]

72. Hensley SE, Zanker D, Dolan BP, David A, Hickman HD, Embry AC, Skon CN, Grebe KM, Griffin TA, Chen W, Bennink JR, Yewdell JW. Unexpected role for the immunoproteasome subunit LMP2 in antiviral humoral and innate immune responses. J Immunol. 2011; 184:4115–4122. [PubMed: 20228196]

73. Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, Bratton DL, Oldenborg PA, Michalak M, Henson PM. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell. 2005; 123:321–334. [PubMed: 16239148]

74. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Metivier D, Larochette N, van Endert P, Ciccosanti F, Piacentini M, Zitvogel L, Kroemer G. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med. 2007; 13:54–61. [PubMed: 17187072]

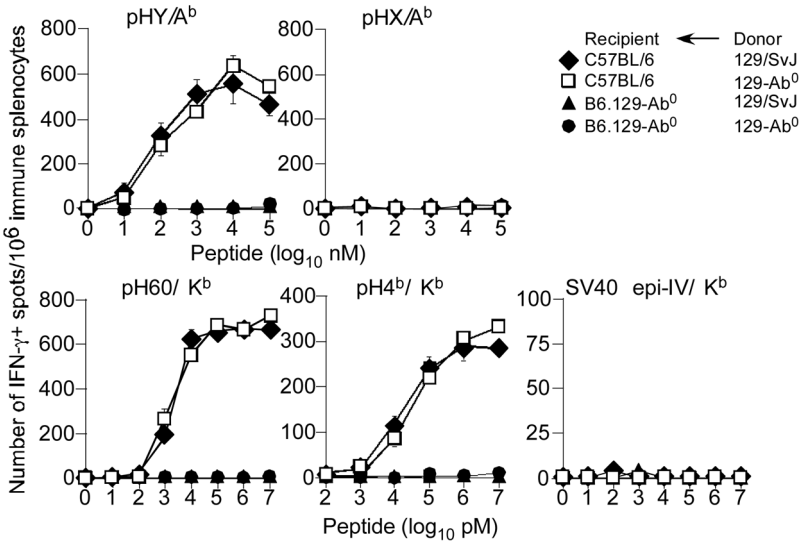

Figure 1. Indirect presentation of the male HY alloantigen

B6 and B6.129-IA$^{b-/-}$ female mice were immunised with either male donor 129/SvJ or 129-Ab^0 splenocytes. After 7d, IFNγ response by T_H cells to pHY/A^b or negative control pHX/A^b was assessed by ex vivo ELISpot assay. At the same time, IFNγ response by CTL to pH60/K^b, pH4^b/K^b and negative control SV40 epi IV/K^b was similarly determined. Data represent 6 similar experiments using ~4 recipient mice per group per experiment; ± sem (standard error of mean).

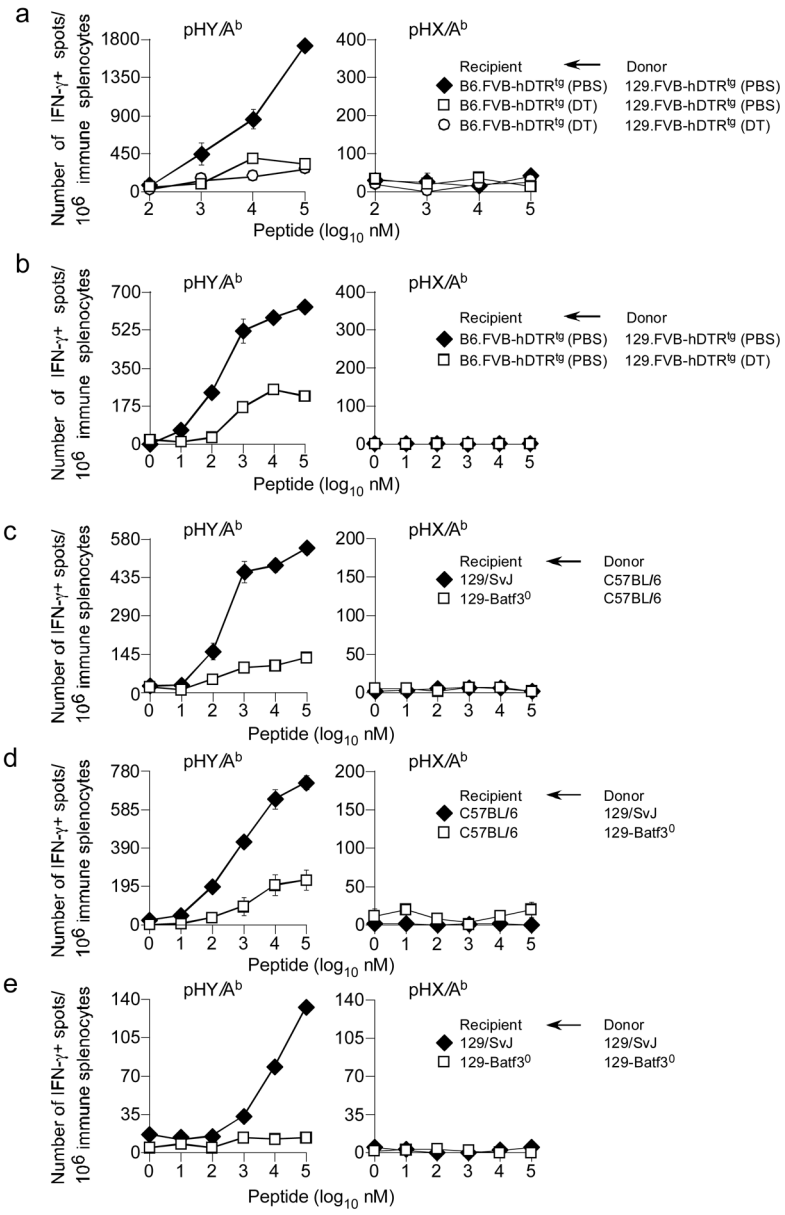

Figure 2. Indirect presentation of the class II-restricted HY alloantigen requires CD8$^+$ DCs

(a) Female recipient B6.FVB-hDTR^{tg} mice treated with vehicle (PBS) or DT and immunised 24 hrs later with male donor 129.FVB-hDTR^{tg} splenocytes from mice that were either PBS- or DT-treated 24 hrs earlier. After 7d, IFNγ response by T_H cells to pHY/A^b or negative control pHX/A^b was assessed by ex vivo ELISpot assay. Data represent 6 similar experiments using ~3 recipient mice per group per experiment; ± sem. **(b)** Male donor 129.FVB-hDTR^{tg} mice treated with vehicle or DT and used 24 hrs later to immunise female B6.FVB-hDTR^{tg} mice. After 7d, IFNγ response by T_H cells to pHY/A^b and pHX/A^b was determined by ex vivo ELISpot assay. Data represent 6 similar experiments using ~3 recipient mice per group per experiment; ± sem. **(c)** 129/SvJ and 129-Batf3^0 female recipients were immunized with male C57BL/6 donor splenocytes. After 7d, IFNγ response by T_H cells to pHY/A^b and pHX/A^b was determined by ex vivo ELISpot assay. Data represents 2 similar experiments using ~2—4 recipient per group per experiment; ± sem. **(d)**

B6 female recipients were immunized with male 129/SvJ or 129-Batf3^0 donor splenocytes. After 7d, IFNγ response by T_H cells to pHY/A^b and pHX/A^b was determined by ex vivo ELIspot assay. Data represents 2 similar experiments using ~2—4 recipient per group per experiment; ± sem. **(e)** 129/SvJ and 129-Batf3^0 female recipients were immunized with either male 129/SvJ or 129-Batf3^0 donor splenocytes. After 7d, IFN-γ response by T_H cells to pHY/A^b and pHX/A^b was determined by ex vivo ELIspot assay. Data represents 2 similar experiments using ~2—4 recipient per group per experiment; ± sem.

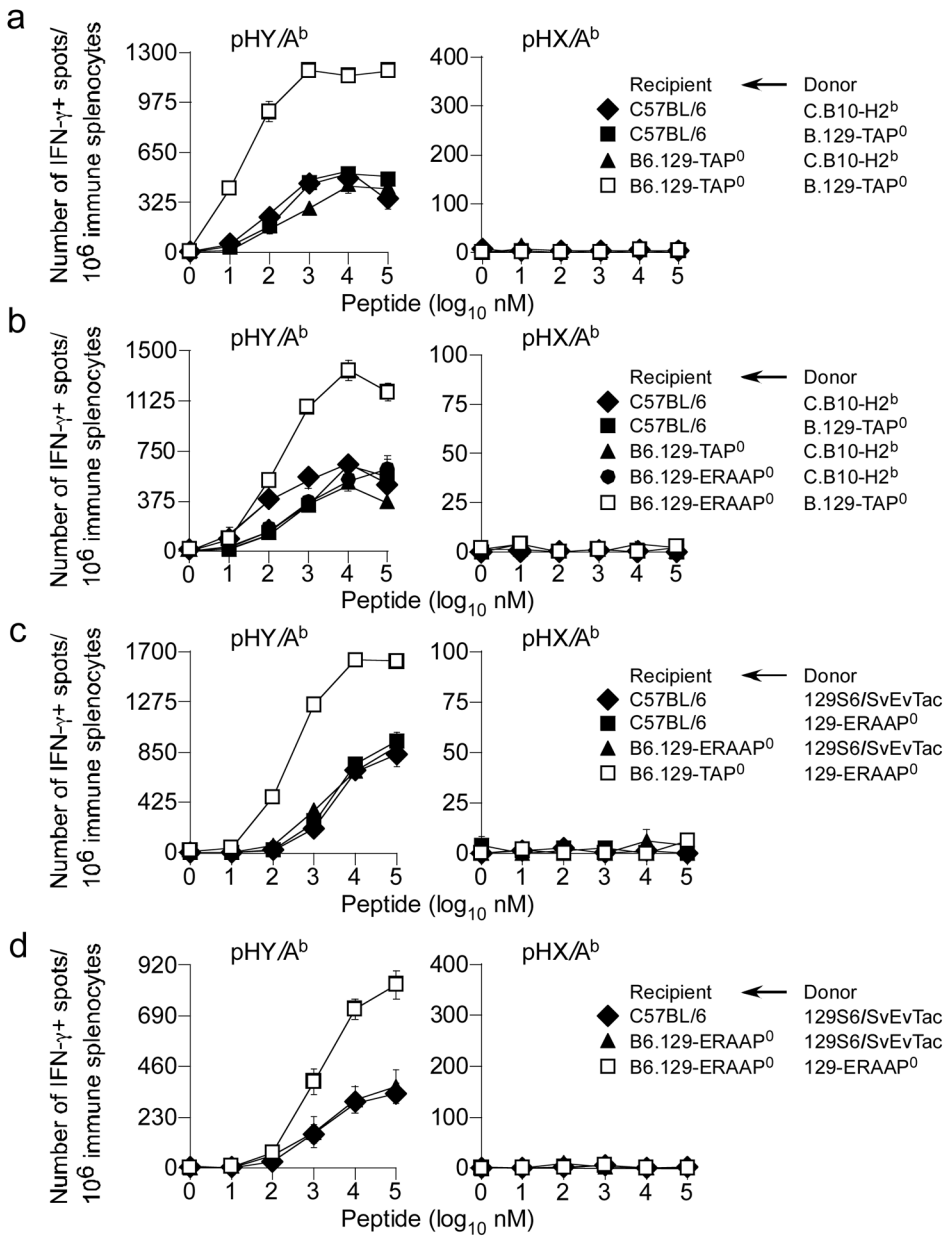

Figure 3. ERAAP and TAP impact indirect presentation of class II-restricted HY alloantigen

(a) B6 and B6.129-TAP^0 female mice were immunised with either male donor C.B10-H2^b or B.129-TAP^0 splenocytes. IFNγ response by T_H cells to pHY/A^b and pHX/A^b was determined by ex vivo ELISpot assay after 7d. Data represent 8 similar experiments using ~3—4 recipient mice per group per experiment; ± sem. **(b)** B6, B6.129-TAP^0 and B6.129-ERAAP^0 female mice were immunised with either male donor C.B10-H2^b or B.129-TAP^0 splenocytes and IFNγ response by T_H cells to pHY/A^b and pHX/A^b was assessed 7d later by ex vivo ELISpot assay. Data represent 7 similar experiments using ~4 recipient mice per group per experiment; ± sem. **(c)** B6, B6.129-TAP^0 and B6.129-ERAAP^0 female mice were immunised with either male donor 129S6/SvEvTac or 129-ERAAP^0 splenocytes. IFNγ response by T_H cells to pHY/A^b and pHX/A^b was determined 7d later by ex vivo ELISpot assay. Data represent 7 similar experiments using ~2—3 recipient mice per group per mice;

± sem. **(d)** B6 and B6.129-ERAAP^0 female mice were immunized with male donor 129S6/SvEvTac or 129-ERAAP^0 splenocytes. After 7d, IFNγ response by T_H cells to pHY/A^b and pHX/A^b was determined by ex vivo ELISpot assay. Data represent 3 similar experiments using ~2—3 recipient mice per group per experiment; ± sem.

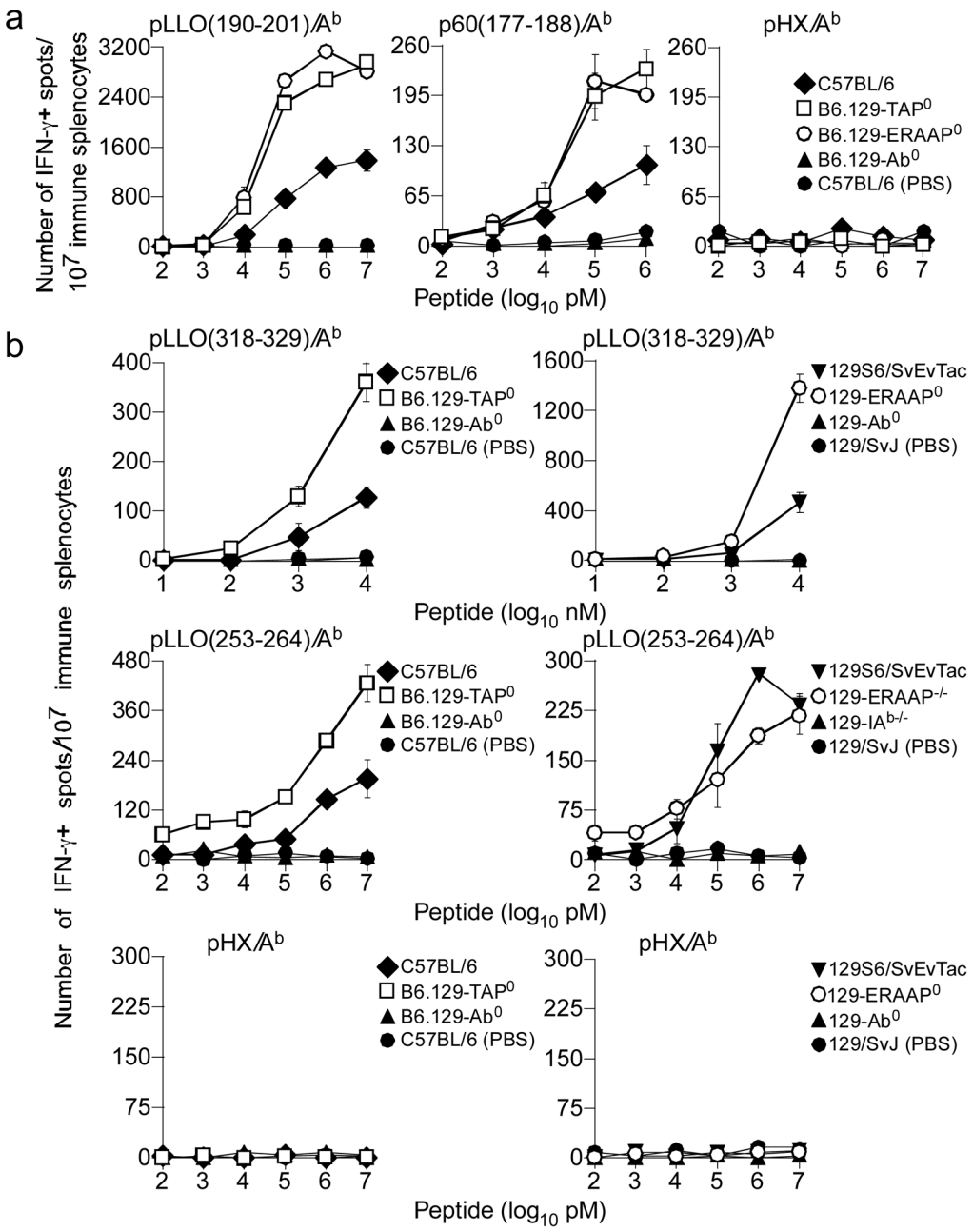

Figure 4. TAP and ERAAP regulate class II-restricted listerial Ag presentation

(a) B6, B6.129-TAP^0, B6.129-ERAAP^0 or B6-129-Ab^0 mice were inoculated with *L. monocytogenes* or delivered PBS i.p. and boosted 2 weeks later. Two weeks after boost, immune splenocytes were stimulated with the indicated peptides for 48hrs and IFN-γ response by T_H cells was monitored by ELIspot assay. **(b)** 129S6/SvEvTac, B.129-TAP^0, 129-ERAAP^0 or 129-Ab^0 mice were primed and boosted as in **(a)**. Two weeks after boost, immune splenocytes were stimulated with the indicated peptides for 48hrs and IFN-γ was monitored by ELIspot assay. Data represents 2 similar experiments using 3—4 mice/group; ± sem.

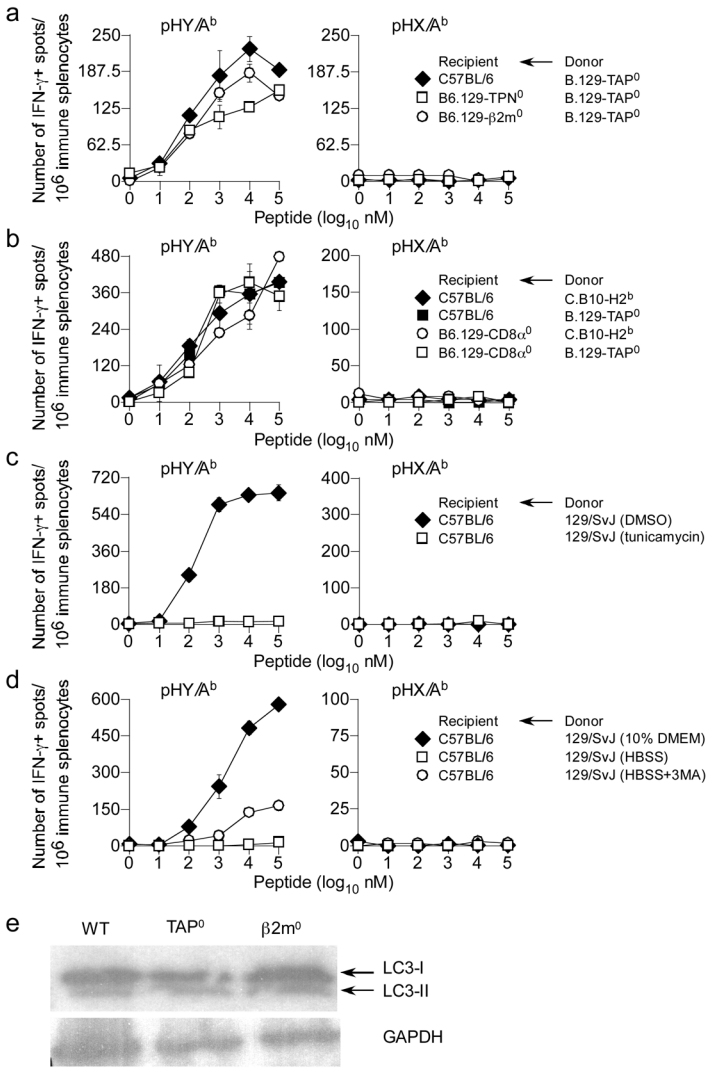

Figure 5. Neither Ag competition, T cell competition, ERAD nor enhanced autophagy explain increased T_H cell response to pHY in TAP^0 mice

(a) B6, B6.129-TPN^0 and B6.129-$\beta2m^0$ female mice were immunised with male donor B.129-TAP^0 splenocytes and IFN-γ response by CD4 T cells to pHY/A^b and pHX/A^b was assessed 7d later by ex vivo ELIspot assay. Data represents 3 similar experiments using 27 recipient mice; ± sem. (b) B6 and B6.129-$CD8\alpha^0$ female mice were immunised with either male donor C.B10-$H2^b$, or B.129-TAP^0 splenocytes. IFN-γ response by CD4 T cells to pHY/A^b and pHX/A^b was determined by ex vivo ELIspot assay 7d later. Data represents 4 similar experiments using 24 recipient mice; ± sem. (c) Female B6 mice were immunised with male donor 129 splenocytes treated with either DMSO or tunicamycin for ~2hrs. IFN-γ response by T_H cells to pHY/A^b and pHX/A^b was determined 7d later as described above. Data represents 2 similar experiments using 12 recipient mice; ± sem. (d) Female B6 mice were immunised with male donor 129 splenocytes incubated in either nutrition-rich medium or HBSS for ~3hrs and IFN-γ response by T_H cells to pHY/A^b and pHX/A^b was assessed 7d later as above. Data represent 2 similar experiments using 12 recipient mice; ± sem. (e) Protein extracts from SV40 TAg-transformed wt, TAP^0 and $\beta2m^0$ kidney fibroblast lines were separated by SDS-PAGE, transferred onto PVDF membrane and probed with LC3- and GADPH-specific mAbs.

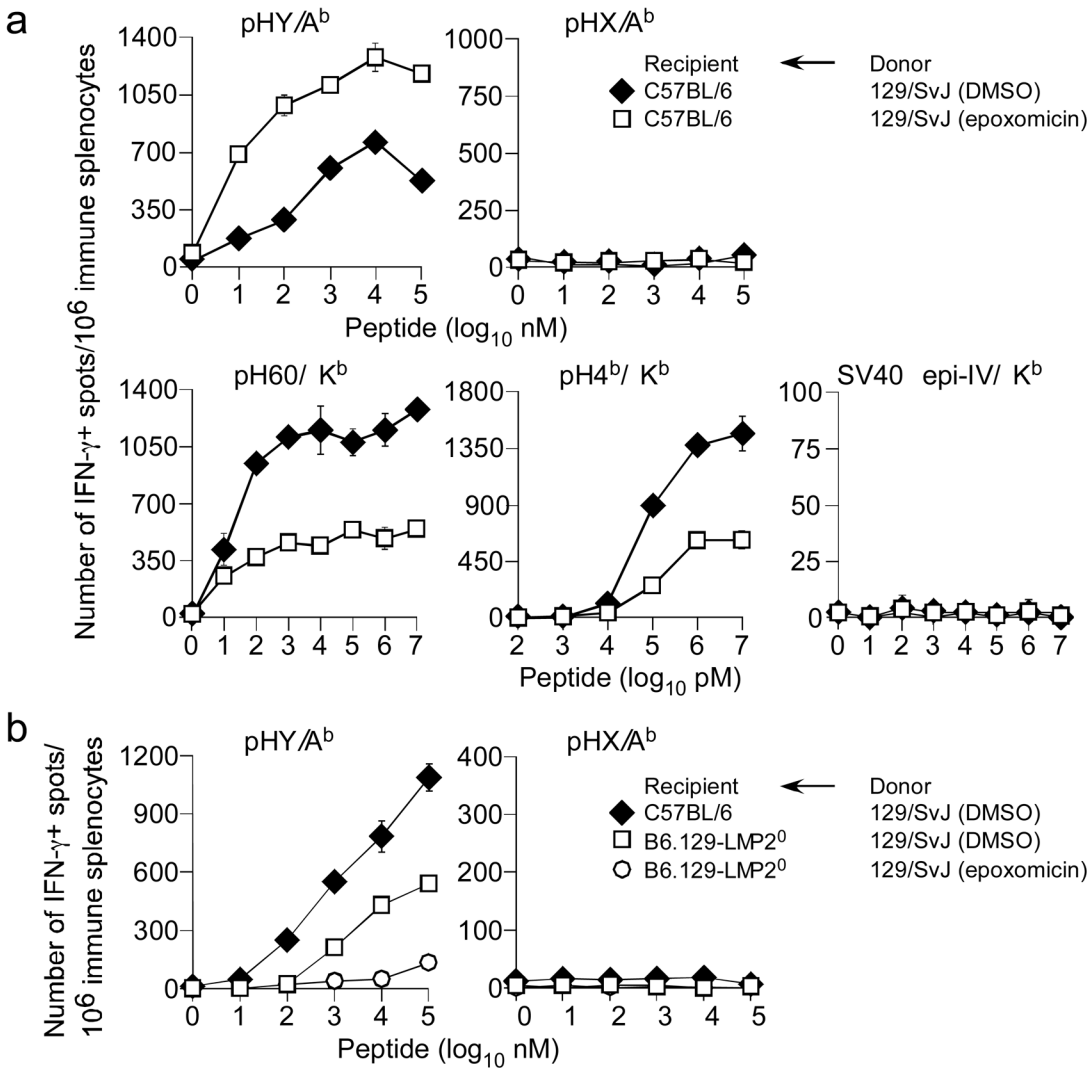

Figure 6. Proteasomes regulate indirect presentation of the class II-restricted pHY alloantigen

(a) B6 female mice were immunised with either DMSO (vehicle)- or epoxomicin-treated male donor 129/SvJ splenocytes. After 7d, IFNγ response by T_H cells to pHY/A^b and pHX/A^b and by CTL to pH60/K^b, pH4^b/K^b and SV40 epi IV/K^b was determined by ex vivo ELISpot assay. Data represent 5 similar experiments using ~2—3 recipient mice per group per experiment; ± sem. **(b)** 129/SvJ male donor splenocytes were treated for 2 hours with either DMSO (vehicle) or epoxomicin. B6 and B6.129-LMP2^0 female mice were immunised with extensively washed male donor 129/SvJ splenocytes. After 7d, IFNγ response T_H cells to pHY/A^b and pHX/A^b was assessed by ex vivo ELISpot assay. Data represent 2 similar experiments using ~3 recipient mice per group per experiment; ± sem.

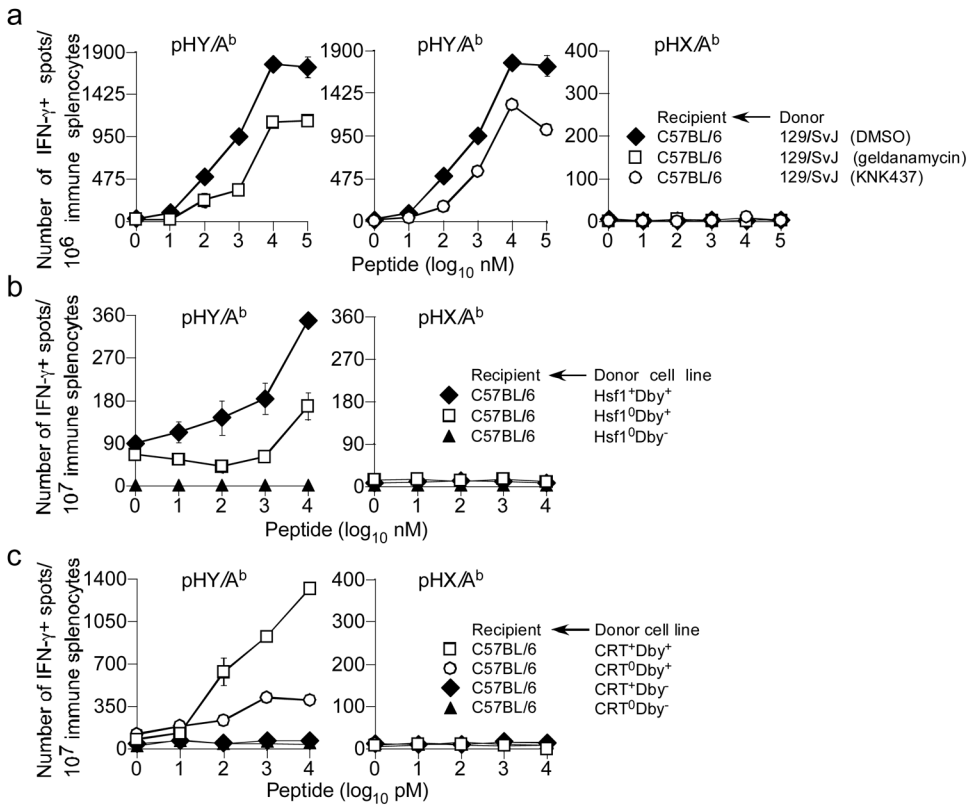

Figure 7. Donor HSP90, HSP70 and calreticulin facilitate indirect presentation of class II-restricted HY alloantigen

(a) B6 female mice were immunised with Hsf1^{0}Dby$^+$, Hsf1$^+$Dby$^+$ or Hsf1^{0}Dby$^-$ MEF. After 7d, IFNγ response by Th cells to pHY/A^b and pHX/A^b was determined by ex vivo ELISpot assay. Data represent 2 similar experiments using ~2—3 recipient mice per group per experiment; ± sem. **(b)** Donor 129/SvJ splenocytes were treated with pharmacological inhibitors for 3 hours and next extensively washed with PBS. B6 female mice were immunised with either DMSO (vehicle), geldanamycin (HSP90 inhibitor) or KNK437 (HSP70 inhibitor) treated male donor 129/SvJ splenocytes. After 7d, IFNγ response Th cells to pHY/A^b and pHX/A^b was assessed by ex vivo ELISpot assay. Data represent 3 similar experiments using ~2—3 recipient mice per group per experiment; ± sem. **(c)** Female B6 mice were immunised with CRT$^+$Dby$^+$, CRT^{0}Dby$^+$, CRT$^+$Dby$^-$ or CRT^{0}Dby$^-$ MEF. After 7d, IFNγ response by T$_H$ cells to pHY/A^b and pHX/A^b was determined as above. Data represent 3 similar experiments using ~3—4 recipient mice per group per experiment; ± sem.

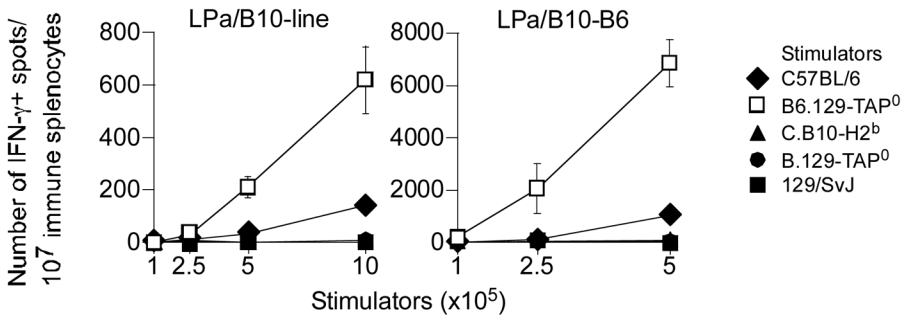

Figure 8. TAP affects the quantitative aspects of the direct presentation of class II-restricted H3b^a mHAg

H3b^a-specific T helper clones LPa/B10-line and LPa/B10-B6 were directly stimulated with the indicated numbers of B6, B6.129-TAP^0, C.B10-H2^b, B.129-TAP^0 or 129/SvJ splenocytes. After 48hrs, IFN-γ response by the T_H clones was determined by ELISpot assay. Data represent 2 similar experiments; ± sem.