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Proteasomes, TAP, and Endoplasmic Reticulum-Associated Aminopeptidase Associated with Antigen Processing Control CD4+ Th Cell Responses by Regulating Indirect Presentation of MHC Class II-Restricted Cytoplasmic Antigens

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Cytoplasmic Ags derived from viruses, cytosolic bacteria, tumors, and allografts are presented to T cells by MHC class I or class II molecules. In the case of class II-restricted Ags, professional APCs 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 endoplasmic reticulum-associated aminopeptidase associated with Ag processing, but not tapasin, partially destroyed or removed cytoplasmic class II-restricted Ags, such that their inhibition or deficiency led to dramatically increased Th cell responses to allograft (HY) and microbial (Listeria monocytogenes) Ags, both of which are indirectly presented. This effect was neither due to enhanced endoplasmic reticulum-associated degradation nor competition for Ag between class I and class II molecules. From these findings, a novel model emerged in which the cytosolic Ag-processing machinery regulates the quantity of cytoplasmic peptides available for presentation by class II molecules and, hence, modulates Th cell responses. *The Journal of Immunology, 2011, 186: 6683–6692.

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Abbreviations used in this article: CAP, cytosolic Ag processing; CRT, calreticulin; DC, dendritic cell; DT, diphtheria toxin; ER, endoplasmic reticulum; ERAAP, endoplasmic reticulum-associated aminopeptidase associated with Ag processing; ERAD, endoplasmic reticulum-associated degradation; Hsfl, heat shock factor protein 1; HSP, heat shock protein; LC3, microtubule-associated protein L chain 3; LLO, Listeria monocytogenes listeriolysin O; MEF, mouse embryonic fibroblast; mHAg, minor histocompatibility Ag; WT, wild type.

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peptides to a length conducive for binding to class I molecules or degrade those that are incompatible with class I binding (27–30). Therefore, one would predict that all cyttoplasmic proteins, including those from which class II-restricted Ags are generated, could become substrates for degradation by the CAP machinery. If such cyttoplasmic degradation occurs, it would regulate the quantity of cyttoplasmic proteins reaching the endolysosomes for presentation by class II molecules.

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

To acquire Ags for indirect presentation, APCs are thought to phagocytose dying cells from which derived Ags are presented by class II molecules (3, 5, 40). Thus, it is 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 shaping the repertoire of cyttoplasmic Ags indirectly presented by class II molecules and its impact on Th cell responses. Toward this goal, we defined the cellular and molecular bases for class II-restricted presentation of cytotoxic 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 cyttoplasmic Ags indirectly presented by class II molecules. Such alteration in Ag presentation modulated the magnitude of Th cell responses to cyttoplasmic Ags in vivo.

**Materials and Methods**

**Mice**

All mouse strains, their histocompatibility genotype, and sources are described in Supplemental Table I. 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 (WT) K41 mouse embryonic fibroblasts (MEFs), calreticulin (CRT)-null K42 MEFs (41), and heat shock factor protein 1 (Hsf1)-null MEFs (42) were maintained in RPMI 1640 (Cellgro) supplemented with 5% FCS (HyClone), α-glutamine, HEPES, and antibiotics (Cellgro). These MEFs were transfected with Dby cDNA (43) and selected with 0.5 mg/ml G418 for 4–8 wk to express the HY alloantigen. Dby expression was verified by RT-PCR using forward (5'-GGCTGGAAAAGAACGTCCG-3') and reverse (5'-TTGGTGCAATTTGTCCTG-3') 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, tunicamycin (Sigma) for 2 or 3 h, respectively, at 37°C. In other experiments, donor splenocytes were starved for 2 h in HBSS (Cellgro) or maintained in DMEM containing 10% FCS, penicillin, streptomycin, l-glutamine, sodium bicarbonate, and HEPES buffer. Cells were washed thoroughly, resuspended at ~2 × 10^7 cells/ml, and used for immunization.

**Peptides**

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

**Immunization and ELISPOT assay**

Recipient mice were immunized i.p. with 2 × 10^7 donor splenocytes. After 7 d, splenocytes were prepared and used in ELISPOT assay. For this, Immobilon-P plates (Millipore) were activated and coated with 1–2 μg/ml IFN-γ capture mAb (AN18; E Biosciences) overnight. Excess mAb was washed and blocked with 10% FCS in RPMI 1640. Meanwhile, 2.5–3 × 10^5 IFN-free immune splenocytes were stimulated with the indicated concentrations of peptides (Supplemental Table II) in triplicate. After 48 h, 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 1 μg/ml IFN-γ–specific biotinylated mAb (RA4-6A2; E Biosciences). After ~3 h at room temperature, excess mAb was washed away, and Vectastain ABC peroxidase (Vector Laboratories) was added to each well. Spots were visualized by reacting 2.2-dimethyl-formamide and 3-amino-9-ethylcarbazole with 30% H_2O_2 (Sigma). Spots were counted using CTL ImmunoSpot analyzer and CTL ImmunoSpot software, version 3.2 (Cellular Technology).

The response of H3b-specific 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 ratios of responder/stimulator. After 48 h, IFN-γ-secreting cells were detected by ELISPOT assay, as described above.

**Dendritic cell depletion**

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

**L. monocytogenes infection**

To elicit primary CD4^+ T cell responses, mice were inoculated retroorbitally with ~5 × 10^5 CFU L. monocytogenes. After 12–14 d, 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 ~10^5 CFU L. monocytogenes in 0.2 ml PBS or with PBS alone. After 14 d, mice were boosted i.p. with ~10^6 CFU and analyzed 14 d later by ELISPOT assay. For this, 0.5–1 × 10^6 nonimmune and immune splenocytes were stimulated with a panel of class II-restricted L. monocytogenes-derived peptide epitopes or negative control peptides (Supplemental Table II).

**Results**

**Indirect presentation of HY alloantigen primes Th cells in vivo**

To study the mechanism(s) underlying indirect presentation of cytotoxic MHC class II-restricted Ags, we first determined how the male H2A^b–restricted HY minor histocompatibility Ag (mHAg) is presented to Th cells. The alloantigenic HY peptide (pHY) is derived from RNA helicase, a ubiquitously expressed nucleocytoplasmic 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^b mice were immunized with H2^b–compatible, but mHAg-incompatible (Supplemental Table I), male 129 donor splenocytes. After 7 d, the ability of mHAg-reactive Th cells and CTLs to produce IFN-γ was determined by ELISPOT assay.
Immunization of B6 mice resulted in IFN-γ-producing splenic Th 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 IFN-γ response from immune B6.129-Ab5 mice (Fig. 1). Moreover, female B6 mice immunized with male 129-Ab5 splenocytes also primed pHY-reactive Th cells (Fig. 1). Therefore, we concluded that the H2Aβ-restricted HY Ag is indirectly presented to Th cells in vivo.

In the same experiment described above, the role of pHY-specific Th cell responses in eliciting CTL responses to class I-restricted mHAgs was determined. We found IFN-γ–producing CTL responses to the immunodominant H2Kb-restricted H60 and H4b alloantigens but not to control H2Kb-restricted SV40 T-Ag epitope-IV in B6 mice immunized with either male 129 or 129-Ab5 splenocytes (Fig. 1). Nonetheless, B6.129-Ab5 recipients did not elicit CTL responses to class I-restricted pH60 and pH4b (Fig. 1). Furthermore, Th and CTL responses, similar to those described above, were obtained using II-deficient recipients (data not shown). Together, these data suggested 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 hDTRtg mouse, in which the human DT receptor transgene expression is regulated by the Cd11c enhancer/promoter (48). Thus, DT administration renders hDTRtg mice conditionally deficient in CD11c+ myeloid cells, including dendritic cells (DCs) and splenic subcapsular macrophages (48, 49). We previously reported that DT-treated B6.FVB-hDTRtg mice became DC deficient within ~18 h and remained so for 72 h (45).

To determine which APC type presents donor mHAg, we treated B6.FVB-hDTRtg mice with PBS or DT and immunized them ~18 h later with male splenocytes from 129.FVB-hDTRtg mice that received PBS ~18 h earlier. On day 7, pHY-specific Th cell responses were monitored. Depletion of recipients’ CD11c+ cells dramatically tempered Th cell responses to pHY compared with those observed in mice containing CD11c+ cells (Fig. 2A). Similarly, depletion of donor CD11c+ cells resulted in poor Th cell responses to pHY (Fig. 2B), indicating a significant role for CD11c+ cells in donating alloantigens for indirect presentation.

**FIGURE 1.** Indirect presentation of the male HY alloantigen. B6 and B6.129-Aβ6 female mice were immunized with either male donor 129/SvJ or 129-Ab5 splenocytes. After 7 d, IFN-γ response by Th cells to pHY/A6 or negative control pHX/A6 was assessed by ex vivo ELISPOT assay. At the same time, IFN-γ response by CTLs to pH60/K6, pH4b/K6, and negative control SV40 epitope-IV/K6 was similarly determined. Data represent six similar experiments using four recipient mice/group/experiment (± SEM).

**FIGURE 2.** Indirect presentation of the class II-restricted HY alloantigen requires CD8+ DCs. A. Female recipient B6.FVB-hDTRtg mice were treated with vehicle (PBS) or DT and immunized 24 h later with male donor 129.FVB-hDTRtg splenocytes from mice that were treated with PBS or DT 24 h earlier. After 7 d, IFN-γ response by Th cells to pHY/A6 or negative control pHX/A6 was assessed by ex vivo ELISPOT assay. Data represent six similar experiments using three recipient mice/group/experiment (± SEM). B. Male donor 129.FVB-hDTRtg mice were treated with vehicle or DT and used 24 h later to immunize female B6.FVB-hDTRtg mice. After 7 d, IFN-γ response by Th cells to pHY/A6 and pHX/A6 was determined by ex vivo ELISPOT assay. Data represent six similar experiments using three recipient mice/group/experiment (± SEM). C. 129/SvJ and 129-Batf3 female recipients were immunized with male C57Bl/6 donor splenocytes. After 7 d, IFN-γ response by Th cells to pHY/A6 and pHX/A6 was determined by ex vivo ELISPOT assay. Data represent two similar experiments using two to four recipient mice/group/experiment (± SEM). D. B6 female recipients were immunized with male 129/SvJ or 129-Batf3 donor splenocytes. After 7 d, IFN-γ response by Th cells to pHY/A6 and pHX/A6 was determined by ex vivo ELISPOT assay. Data represent two similar experiments using two to four recipient mice/group/experiment (± SEM). E. 129/SvJ and 129-Batf3 female recipients were immunized with either male 129/SvJ or 129-Batf3 donor splenocytes. After 7 d, IFN-γ response by Th cells to pHY/A6 and pHX/A6 was determined by ex vivo ELISPOT assay. Data represent two similar experiments using two to four recipient mice/group/experiment (± SEM).
to prime class I-restricted pH60 and pH4b-CTL responses in vivo (S. Dragovic, T. Hill, and S. Joyce, unpublished observations). Because DCs express high levels of CD11c, constitute the majority of CD11c+ splenocytes, and are critical for priming naive T cells, the above data suggested that DCs are responsible for indirect presentation.

To firmly establish the contribution of DCs in indirect presentation and to determine which DC subset is responsible, we used the recently reported 129-Batf3<sup>−/−</sup> mouse, which is deficient in splenic CD8<sup>+</sup> DCs (50). Female 129 and 129-Batf3<sup>−/−</sup> mice were immunized with male B6, 129, or 129-Batf3<sup>−/−</sup> splenocytes, and HY-specific Th cell responses were monitored 7 d after the immunization. The data revealed that the lack of splenic CD8<sup>+</sup> DCs in the recipient dramatically reduced the Th cell response to HY (Fig. 2C). Similarly, the lack of donor CD8<sup>+</sup> DCs resulted in much tempered Th cell responses to HY (Fig. 2D), which was completely lost upon immunizing CD8<sup>+</sup>-deficient female recipients with male donor splenocytes lacking CD8<sup>+</sup> DCs (Fig. 2E).

We also monitored pH60-specific CTL responses in the experiment described above. The data revealed a requirement for donor and recipient CD8<sup>+</sup> DCs for cross-priming CTL responses to pH60 (S. Dragovic and S. Joyce, unpublished observations). Together, these data suggested that CD8<sup>+</sup> DCs play important roles in donating and indirectly presenting the HY alloantigen.

**TAP and ERAAP regulate Th 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. Immunization of female B6 mice with male splenocytes derived from H2<sup>b</sup>-compatible, but mHAg-incompatible, C.B10-H2<sup>b</sup> (BALB.B; Supplemental Table I) or B.129-TAP<sup>−/−</sup> (B stands for BALB.B) mice generated comparable pHY-specific Th cell responses (Fig. 3A, 3B). Similarly, B6.129-TAP<sup>−/−</sup> female mice immunized with C.B10-H2<sup>b</sup> male splenocytes elicited comparable pHY-specific Th cell responses (Fig. 3A, 3B). Surprisingly, however, when B6.129-TAP<sup>−/−</sup> female recipients were immunized with B.129-TAP<sup>−/−</sup> male donor splenocytes, a 2–3-fold increased Th cell response against H2A<sup>b</sup>-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 Th cell response.

We considered the possibility that peptides translocated by TAP into the ER might become substrates for destruction by ERAAP and, hence, be unavailable for presentation. To test this possibility, B6, B6.129-TAP<sup>−/−</sup>, and B6.129-ERAAP<sup>−/−</sup> female mice were immunized with C.B10-H2<sup>b</sup>, B.129-TAP<sup>−/−</sup>, or 129-ERAAP<sup>−/−</sup> male splenocytes. As with B6 and B6.129-TAP<sup>−/−</sup> mice, B6.129-ERAAP<sup>−/−</sup> female mice immunized with WT male splenocytes elicited similar pHY-specific Th cell responses (Fig. 3B–D). In striking contrast, immunization of B6.129-ERAAP<sup>−/−</sup> female mice with B.129-TAP<sup>−/−</sup> male splenocytes resulted in a 2-fold increase in pHY-specific Th cell responses (Fig. 3B). Similarly, immunization of B6.129-TAP<sup>−/−</sup> or B6.129-ERAAP<sup>−/−</sup> mice with 129-ERAAP<sup>−/−</sup> male splenocytes resulted in a 2–3-fold increase in pHY-specific Th cell responses (Fig. 3C, 3D).

As a control for the above experiments, the monitoring of CTL response in WT mice immunized with male WT or TAP-deficient donor splenocytes revealed an identical CTL response to pH60 and pH4<sup>b</sup>, suggesting that the two class I-restricted mHAgs are cross-presented (Supplemental Fig. 1A). As expected, TAP-deficient recipients did not respond to class I-restricted mHAgs because they are devoid of CD8<sup>+</sup> T cells (Supplemental Fig. 1A). Therefore, we concluded 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 listeriolysin O (LLO) disrupts the phagolysosome to permit entry of the organism into the cytoplasm for its growth and multiplication. Therefore, the priming of Th cell responses against listeriolysin O (LLO) was determined 7 d after the immunizing CD8<sup>+</sup> DC-deficient female recipients with male donor splenocytes. As with B6 and B6.129-TAP<sup>−/−</sup> mice, B6.129-ERAAP<sup>−/−</sup> female mice were immunized with either male donor C.B10-H2<sup>b</sup> or B.129-TAP<sup>−/−</sup> splenocytes. IFN-γ response by Th cells to pHYA<sup>a</sup> and pHXA<sup>a</sup> was determined by ex vivo ELISPOT assay after 7 d. Data represent eight similar experiments using three or four recipient mice/group/experiment (± SEM). B, B6, B6.129-TAP<sup>−/−</sup>, and B6.129-ERAAP<sup>−/−</sup> female mice were immunized with either male donor C.B10-H2<sup>b</sup> or B.129-TAP<sup>−/−</sup> splenocytes, and IFN-γ response by Th cells to pHYA<sup>a</sup> and pHXA<sup>a</sup> was assessed 7 d later by ex vivo ELISPOT assay. Data represent seven similar experiments using four recipient mice/group/experiment (± SEM). C, B6, B6.129-TAP<sup>−/−</sup>, and B6.129-ERAAP<sup>−/−</sup> female mice were immunized with either male donor 129S6/SvEvTac or 129-ERAAP<sup>−/−</sup> splenocytes. IFN-γ response by Th cells to pHYA<sup>a</sup> and pHXA<sup>a</sup> was determined 7 d later by ex vivo ELISPOT assay. Data represent seven similar experiments using two or three recipient mice/group/experiment (± SEM). D, B6 and B6.129-ERAAP<sup>−/−</sup> female mice were immunized with male donor 129S6/SvEvTac or 129-ERAAP<sup>−/−</sup> splenocytes. After 7 d, IFN-γ response by Th cells to pHYA<sup>a</sup> and pHXA<sup>a</sup> was determined by ex vivo ELISPOT assay. Data represent three similar experiments using two or three recipient mice/group/experiment (± SEM).

**FIGURE 3. ERAAP and TAP impact indirect presentation of class II-restricted HY alloantigen.** A, B6 and B6.129-TAP<sup>−/−</sup> female mice were immunized with either male donor C.B10-H2<sup>b</sup> or B.129-TAP<sup>−/−</sup> splenocytes. IFN-γ response by Th cells to pHYA<sup>a</sup> and pHXA<sup>a</sup> was determined by ex vivo ELISPOT assay after 7 d. Data represent eight similar experiments using three or four recipient mice/group/experiment (± SEM). B, B6, B6.129-TAP<sup>−/−</sup>, and B6.129-ERAAP<sup>−/−</sup> female mice were immunized with either male donor C.B10-H2<sup>b</sup> or B.129-TAP<sup>−/−</sup> splenocytes, and IFN-γ response by Th cells to pHYA<sup>a</sup> and pHXA<sup>a</sup> was assessed 7 d later by ex vivo ELISPOT assay. Data represent seven similar experiments using four recipient mice/group/experiment (± SEM). C, B6, B6.129-TAP<sup>−/−</sup>, and B6.129-ERAAP<sup>−/−</sup> female mice were immunized with either male donor 129S6/SvEvTac or 129-ERAAP<sup>−/−</sup> splenocytes. IFN-γ response by Th cells to pHYA<sup>a</sup> and pHXA<sup>a</sup> was determined 7 d later by ex vivo ELISPOT assay. Data represent seven similar experiments using two or three recipient mice/group/experiment (± SEM). D, B6 and B6.129-ERAAP<sup>−/−</sup> female mice were immunized with male donor 129S6/SvEvTac or 129-ERAAP<sup>−/−</sup> splenocytes. After 7 d, IFN-γ response by Th cells to pHYA<sup>a</sup> and pHXA<sup>a</sup> was determined by ex vivo ELISPOT assay. Data represent three similar experiments using two or three recipient mice/group/experiment (± SEM).
were inoculated i.p. with bacteria and boosted 14 d later, and secondary Th cell responses to known H2Aβ-restricted epitopes were monitored after an additional 14 d. PBS-treated B6 and 129 mice served as negative controls. We observed a 2–5-fold increase in the secondary Th cell response to H2Aβ-restricted pLLO(190–201), p60(177–188), pLLO(318–329), and pLLO(253–264) in B6.129-TAPβ mice compared with WT mice (Fig. 4A, 4B). A similar pattern of increased Th cell reactivity to pLLO(190–201), p60 (177–188), and pLLO(318–329) was observed in ERAAP-deficient mice compared with B6 mice (Fig. 4A, 4B). In contrast, the response to pLLO(253–264) was indistinguishable between WT and ERAAP-deficient mice (Fig. 4B). As expected, neither Listeria-inoculated H2Abβ 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 Th response to L. monocytogenes Ags, elicited by retro-orbital bacterial inoculation, yielded similar results as above (Supplemental Fig. 2). Thus, the effect of TAP and ERAAP on indirect presentation of cytoplasmic class II-restricted Ags seems to be a general principle, because they impact Th cell responses to mHAgs and bacterial Ags similarly.

**Proteasomes regulate indirect presentation of HY mHAgs**

Several mechanisms can potentially explain how the CAP regulates CD4+ T cell response: competition between class I and class II molecules for Ag, competition between CD4+ and CD8+ T cells, enhanced autophagy and/or enhanced ER-associated degradation (ERAD), and quantitative differences in the Ag(s) presented.

To test whether competition for Ag played a role, the Th response of female B6, B6.129-β2mβ, and B6.129-Tpβ mice, which, similar to TAP deficiency, lack a functional class I-assembly complex because of β2m and tapasin deficiency, was assessed after immunizing with male C.B10-H2β or B.129-TAPβ splenocytes. All three recipients exhibited similar pHY-specific Th cell responses (Fig. 5A), suggesting that a lack of 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 Th cell response was a compensatory response caused by the absence of recipient CTL, B6.129-CD8αβ female mice were immunized with either C.B10-H2β or B.129-TAPβ male splenocytes. The resulting Th cell response to pHY was comparable in both WT and CD8+ T cell-deficient mice (Fig. 5B). As expected, female B6.129-β2mβ, B6.129-Tpβ, and B6.129-CD8αβ recipients did not exhibit an IFN-γ response to class I-restricted mHAgs (Supplemental Fig. 1B, 1C). Hence, competition between CD4+ and CD8+ T cells is unlikely to explain the increased Th response in the absence of TAP or ERAAP.

TAP and β2m 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 immunization of female B6 mice with male 129 splenocytes treated with tunicamycin, which induces ERAD as a result of stress from accumulating unfolded proteins, completely abrogated Th response to pHY, whereas DMSO-treated donor cells responded as expected (Fig. 5C). Similarly, induction of autophagy, by maintaining donor male splenocytes in nutrition-free conditions prior to immunization of female B6 mice, did not enhance, but instead abrogated the Th response to pHY (Fig. 5D). Additionally, constitutive autophagy was not enhanced in TAPβ (TAPTAg) or β2mβ (β2mTag) Tag-transformed fibroblast lines compared with similarly transformed WT fibroblasts (WTTag) (56), because similar levels of microtubule-associated protein L chain 3 (LC3-I and LC3-II) were detected in immunoblots of proteins extracted from WT and mutant lines (Fig. 5E). Together, these data discounted 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 argued that if autophagy is enhanced by TAP or ERAAP deficiency, it would destroy and not protect cytosplasmic Ags for indirect presentation by class II molecules.

Because 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 Th 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 2 h with either DMSO or the selective proteasome inhibitor epoxomicin (57, 58), and Th cell responses were monitored. Surprisingly, in contrast to the negative outcome of immunization with tunicamycin-treated cells, we found that irreversible proteasome inhibition of donor cells resulted in a 2-fold increase in Th cell responses to pHY compared with that elicited by donor cells containing functional proteasomes (Fig. 6A). Thus, proteasomes negatively impact indirect presentation.
and the intact form of the HY alloantigen might be donated to recipient CD8+ DCs for indirect presentation. If intact Ag 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, immunization of female B6.129-LMP20 mice with male 129 splenocytes resulted in tempered Th responses to pHY/A^b (Fig. 6B).

Surprisingly, however, the Th response to pHY/A^b was completely lost when the donor splenocytes were treated with epoxomicin and then transferred into LMP2-deficient recipients (Fig. 6B). Consistent with this result was the finding that altered pH balance of the phagolysosome caused by a deficiency in donor and/or recipient gp91PHOX did not affect Th cell responses to pHY/Ab (data not shown). These data suggested 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 Ags requires heat shock proteins (HSPs) (59, 60). Because the HY alloantigen is a nucleocytoplasmic protein that is degraded by donor proteasomes (Fig. 6), we reasoned that donor HSP may play a role in indirect presentation of this Ag. 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 2 h and used to immunize B6 mice. Inhibition of HSP90 with either geldanamycin or KNK437 tempered Th cell responses against pHY (Fig. 7A). This result suggested a role for HSP90 in indirect presentation of HY alloantigen.

To confirm a role for HSP90, in the second approach, we used MEFs deficient in Hsf1, a transcription factor that regulates the expression of members of the HSP90 family of HSPs (42). We first generated HY+Hsf1^0 and WT MEFs by cDNA transfer because these cells do not otherwise express HY mHAg (data not shown). Immunization of B6 mice with HY+Hsf1^0 MEFs resulted in tempered Th cell responses to HY compared with mice im-

FIGURE 5. Ag competition, T cell competition, ERAD, or enhanced autophagy does not explain increased Th cell response to pHY in TAP^0 mice. A, B6, B6.129-TPN^0, and B6.129-β2m^0 female mice were immunized with male donor B.129-TAP^0 splenocytes, and IFN-γ response by CD4 T cells to pHY/A^b and pHX/A^a was assessed 7 d later by ex vivo ELISPOT assay. Data represent three similar experiments using 27 recipient mice (± SEM). B, B6 and B6.129-CD8a^0 female mice were immunized with either male donor B.129-H2^b or B.129-TAP^0 splenocytes. IFN-γ response by CD4 T cells to pHY/A^b and pHX/A^a was determined by ex vivo ELISPOT assay 7d later. Data represent four similar experiments using 24 recipient mice (± SEM). C, Female B6 mice were immunized with male donor 129 splenocytes treated with either DMSO or tunicamycin for ~2 h. IFN-γ response by Th cells to pHY/A^b and pHX/A^a was determined 7 d later, as described above. Data represent two similar experiments using 12 recipient mice (± SEM). D, Female B6 mice were immunized with male donor 129 splenocytes incubated in either nutrition-rich medium or HBSS for ~3 h, and IFN-γ response by Th cells to pHY/A^b and pHX/A^a was assessed 7 d later, as above. Data represent two similar experiments using 12 recipient mice (± SEM). E, Protein extracts from SV40 TAg-transformed WT, TAP^0, and β2m^0 kidney fibroblast lines were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and probed with LC3- and GAPDH-specific mAbs.

FIGURE 6. Proteasomes regulate indirect presentation of the class II-restricted pHY alloantigen. A, B6 female mice were immunized with either DMSO (vehicle)- or epoxomicin-treated male donor 129/SvJ splenocytes. After 7 d, IFN-γ response by Th cells to pHY/A^b and pHX/A^a, as well as by CTLs to pH60/K^b, pH4b/K^b, and SV40 epitope-I/IV/K^b, was determined by ex vivo ELISPOT assay. Data represent five similar experiments using two or three recipient mice/group/experiment (± SEM). B, 129/SvJ male donor splenocytes were treated for 2 h with either DMSO (vehicle) or epoxomicin. B6 and B6.129-LMP20^0 female mice were immunized with extensively washed male donor 129/SvJ splenocytes. After 7 d, IFN-γ response by Th cells to pHY/A^b and pHX/A^a was assessed by ex vivo ELISPOT assay. Data represent two similar experiments using three recipient mice/group/experiment (± SEM).
munized with HY\(^+\) WT MEFs (Fig. 7B). These results implied a critical role for HSP90 in efficient indirect presentation of the HY alloantigen.

CRT, an ER-resident chaperone, is implicated in cross-presentation of class I-restricted Ags (61). Therefore, we determined whether CRT expression by donor APCs was essential for indirect presentation of HY alloantigen. For this, we first generated HY\(^+\)CRT\(^-\) and HY\(^+\)CRT\(^+\) MEFs by Dby cDNA transfer. Immunization of B6 mice with HY\(^+\)CRT\(^-\) MEFs resulted in tempered Th cell responses to HY compared with mice immunized with HY\(^+\)WT MEFs (Fig. 7B). These results implied 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 H3ba\(^+\) mHAgs-specific Th cell lines: LPa/B10.B6 and LPa/B10.10-line. Similar to HY, the H3ba\(^+\) mHAgs is derived from a cytoplasmic protein, ribosome binding protein-I (A.C. Brown, G.J. Christianson, and D.C. Roopenian, manuscript in preparation). Moreover, the H3ba\(^+\)-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\(^0\), or 129/SvJ splenocytes for 48 h, and the number of IFN-\(\gamma\) spots was determined. The data revealed that compared with B6 splenocytes, B6.129-TAP\(^0\) splenocytes induced a 7–8-fold greater number of IFN-\(\gamma\) spots from the two H3ba\(^+\)-specific Th clones (Fig. 8). This response was Ag-specific because the Th cell clones did not respond to negative control C.B10-H2\(^b\), B.129-TAP\(^0\), or 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 Th cells regulate Ab- and CTL-mediated adaptive immunity to pathogens, cancers, allografts, and autoantigens. Such an understanding is especially important because many tumor- and virus-infected cells downregulate TAP gene expression to evade CTL-mediated immune surveillance. Furthermore, our findings will impact how we understand T cell responses in individuals who express TAP-null and ERAAP variants (62–64), especially those that inhibit or alter peptide processing within the ER.

We showed in this study 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 Th cell responses to cytoplasmic alloantigens (HY and H3ba\(^+\)) 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 shown to indirectly regulate CD4\(^+\) T cell priming in a Toxoplasma gondii infection model (66). Hence, it was possible that the several-fold-increased Th cell response to the HY alloantigen and listerial Ags in TAP-null recipients were indirectly regulated by NK cells. Therefore, we immunized both WT and NK cell-deficient IL-15\(^-\) mice with male donor cells and found that the HY\(^A\)-specific Th response was similar in both recipients (data not shown). Thus, we concluded that NK cells contributed very little to the Th cell response to HY.

Although TAP and \(\beta\)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. 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, similar to TAP deficiency, renders class I molecules unstable and results in mice that lack CD8+ T cells. Hence, competition between class I and II molecules, as well as competition between Th cells and CTLs, for the same Ag is a most unlikely mechanism by which TAP and ERAAP deficiencies alter indirect presentation of cytotoxic Ags.

Our data suggested that TAP and ERAAP act directly on class II-restricted cytoplasmic Ags. Such Ags might be 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 quantitatively increase the cytoplasmic Ag pool, making it available for indirect presentation. Indeed, our data favored this role for TAP and ERAAP 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, CRT 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, CRT 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 from this study is that proteasomes, TAP, and ERAAP regulate the quantity of the class II-associated self (mHAg) 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 indicate that the CD4+ T cell repertoire is very similar between WT and TAP-deficient mice (54). Altered cytotoxic Ag pool within donating cells, coupled with altered Ag presentation by the APCs, could explain how the CAP machinery regulates Th cell responses to indirectly presented cytotoxic Ags.

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