PROTEASOMES, TAP AND ERAAP CONTROL CD4$^+$ TH CELL RESPONSES BY REGULATING INDIRECT PRESENTATION OF MHC CLASS II-RESTRICTED CYTOPLASMIC ANTIGENS

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Srdan M. Dragovic

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Approved:

Professor Sebastian Joyce

Professor Luc Van Kaer

Professor Mark R. Boothby

Professor Eric P. Skaar

Professor Claudio A. Mosse

To my loving family, mother Miladinka, father Miodrag and my sister Anica

for their enormous love and support

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LIST OF ABBREVIATIONS

3-MA- 3-methyladenine,

β2m- β2-microglobulin,

ABC- ATP-binding cassette,

Ag- antigen,

APC- antigen presenting cell,

Atg- autophagy related,

BiP- immunoglobulin binding protein,

CIIV- MHC class II vesicles,

CLIP- class II-associated Ii peptide,

CMA- chaperone mediated autophagy,

CNX- calnexin,

cTECs- cortical thymic epithelial cells,

CTL- cytotoxic T lymphocytes,

CRT- calreticulin,

Cx43- Connexin 43,

DC- dendritic cell,

DCIR-2- dendritic cell inhibitory receptor-2,

DN- double negative,

DP- double positive,

DRiPs- defective ribosomal proteins,

EEA1- early endosome antigen 1,

ER- endoplasmic reticulum,

ERAAP- ER-associated aminopeptidase associated with antigen processing,

GILT- γ-interferon-inducible lysosomal thiolreductase,

GVHD- graft-versus-host disease,

H-2DM (HLA-DM)- MHC class II-like molecule,

HCMV- human cytomegalovirus,

HLA- human leukocyte antigen,

Hsc70- heat shock protein 70 complex,

HSP- heat shock protein,

HSV-1- Herpes simplex virus-1,

IFN-γ- interferon-γ,

Ii- invariant chain,

IL- interleukin,

IRAP- insulin-regulated aminopeptidase,

kD- kiloDalton,

LAMP-2a- lysosomal-associated membrane protein-2a,

LC3-II- light chain 3-II,

LCMV- Lymphocytic Choriomeningitis Virus,

LMP- low molecular weight protein,

LPS- lipopolysaccharide,

MIIC- MHC class II compartment,

Mϕ- macrophage,

MECL-1-multicatalytic endopeptidase complex-like 1,

MHC- major histocompatibility complex,

Minor HAgs- minor histocompatibility alloantigens,

MR- mannose-receptor,

mTECs- medullary thymic epithelial cells,

NBD- nucleotide binding domain,

NLRs- NOD-like receptors,

NOX2- NADPH oxidase complex 2,

OVA- ovalbumin,

p- peptide,

PLC- peptide loading complex,

PRRs- pattern recognition receptors,

RNS- reactive nitrogen species,

ROS- reactive oxygen species,

SP- single positive,

SR- scavenger receptor,

TAP- transporter associated with antigen processing,

TCR- T cell receptor,

TGF-β- transforming growth factor-β,

TGN- trans-Golgi network,

T_H cells- T helper cells,

TLR- Toll-like receptor,

TNF-α- tumour necrosis factor-α,

T_{regs} - regulatory T cells.

CHAPTER I

INTRODUCTION

Evolutionary development of innate and adaptive immunity

The immune system evolved with the decent of colonial metazoans as a means to recognize members of the species (sometimes allotypes, e.g., ascidians) and in defence against toxic substances, parasites and pathogens (1). Consequently, the immune system of each species coevolved with the environmental factors of the niche they occupy. This has led to specialization such that immune response outcomes often vary between different organisms even when basic mechanisms of immune recognition have remained the same (2). For example, the human immune system is further specialized by virtue of the signature microbiome with which the individual lives in commensal or symbiotic harmony. Nonetheless, studies on mice and nonhuman primates have significantly advanced our understanding of the basic principles of immune recognition and response that have laid the foundation for understanding human immunology (3-5).

Vertebrates utilize innate and adaptive defence mechanisms against foreign microbes and pathogens as well as toxic substances. Although the innate mechanisms are ancient and predate vertebrate descent, the adaptive immune system is more recent and appeared with the descent of aquatic vertebrates around 500 million years ago (6). The two systems, innate and adaptive,

nonetheless depend on each other to orchestrate an effective antigen-specific effector response (3).

But for the discovery of the genetic restriction of histocompatibility (blood groups and major histocompatibility complex (MHC), alloantigens) and immunosuppression, every human transplant would have remained an experiment whose outcome could seldom be predicted with certainty (7). Nonetheless, despite histocompatibility testing and immunosuppression, allograft outcome in many recipients remains uncertain. The innate immune responses early in allograft life and immunity to minor histocompatibility alloantigens (HAgs) affect greatly the function of transplanted organs (8).

T cell responses are primed when the innate immune response culminates in the display of processed antigens by antigen presenting cells (APCs) in the context of MHC-encoded class I and class II molecules (9). Class I molecules, which regulate cytotoxic T lymphocyte (CTL) functions, present endogenous/cytoplasmic antigens, whereas class II molecules, which control T helper (T_H) cell functions, present exogenous/extracellular antigens (10, 11). Notwithstanding this dichotomy, CTL responses can also be primed by class I-restricted extracellular antigens in a previously described, but still not entirely defined process termed cross-presentation (11, 12). Likewise, studies of T_H cell responses to alloantigens, cancers, viruses and cytosolic bacteria have suggested that class II molecules present certain cytoplasmic antigens (13-24) in a poorly defined process termed indirect presentation.

MHC class II processing pathway displays antigens originating from endogenous (direct) and indirect (dead or dying cells) sources. Direct antigen presentation is dependent on several mechanisms which facilitate cytoplasmic antigen supply to the lysosomal compartment. Such mechanisms include autophagy and, for few reported antigens, proteasome-dependent processing pathway (20, 25, 26). On the other hand, indirect presentation is the major pathway for antigen supply for loading class II molecules with antigenic peptides. According to the consensus based on available evidence, exogenous antigens that are captured through endocytosis or phagocytosis travel to the lysosomes where resident proteases facilitate antigen processing (27). In this regard, the characterization of minor HAgs, bacterial and tumour antigens have unravelled novel processing mechanisms as well as a better understanding of indirect presentation. Indirect presentation of class II antigens is a frequently occurring and fundamental process of immune recognition.

The class II processing pathway is primarily specialized for presentation of exogenous antigens, which, upon internalization, are targeted to lysosomes (28, 29). However, recent studies have suggested that a large number of class II-restricted antigens originate from cytoplasmic proteins derived from infected, or tumour cells which do not express class II molecules (30). Such cytoplasmic antigens require indirect presentation by professional APC in order to elicit T_H cell responses (31). However, internalized cytoplasmic antigens are frequently targeted to the proteasomes and transported to the lumen of the endoplasmic reticulum (ER) via transporter associated with antigen processing (TAP).

Therefore, internalized antigens need to seep into the cytoplasm and escape lysosomal processing through the process of cross-presentation (32, 33).

Even though a lot is known regarding cross-presentation, mechanisms are incompletely understood. Interestingly, very little is known about rules that govern indirect presentation of self and foreign cytoplasmic antigens that are from dead, dying and tumour cells by class II molecules in professional APCs. The ensuing overview discusses the currently understood principles by which antigens are processed and assembled with class II molecules.

MHC class II-restricted antigen processing pathway

MHC class II $\alpha\beta$ heterodimers assemble in the lumen of thr ER with the help of calnexin (CNX) and invariant chain (Ii) (34, 35). Class II molecules critically depend on the associated Ii to facilitate their transport to the Golgi apparatus where their N-glycans are trimmed and modified prior to egress and transport to the lysosomal compartment (36).

Class II $\alpha\beta$ heterodimers couple with a trimeric Ii scaffold, which contains a di-leucinal or leucinal-isoleucine targeting motif within its cytoplasmic tail that directs the class II-Ii complexes to the late endosomal/lysosomal compartment. Ii is a non-polymorphic, type II transmembrane protein consisting of a short N-terminal cytoplasmic domain, ER transmembrane domain, a disordered segment, which binds and shields the class II peptide binding groove, and a C-terminal trimerization motif. Upon Ii trimerization, three class II $\alpha\beta$ heterodimers bind to the trimeric Ii scaffold, thus forming a trimer of trimeric (class II $\alpha\beta$+Ii) complexes.

Ii not only prevents peptide binding to class II molecules in the ER, but also prevents other ER chaperones from binding to the heterodimers and retaining them in the ER (36-38). Finally, upon entry into the lysosomes, Ii is cleaved with the help of various peptidases through a series of cleavage events (discussed below).

Upon exit from the ER, class II-Ii complexes negotiate the Golgi apparatus and traffic into the TGN (39). There are several paths that the class II $\alpha\beta$+Ii complexes can take. First, a small fraction of the complexes exit directly to the cell surface, where they are rapidly internalized to early endosomes and loaded with class II-restricted peptides in a H-2DM-independent manner (40, 41).

Second, class II-Ii complexes may traverse the early endosomal compartment to acquire class II-restricted peptide cargo. The early endosomes play a significant role in determining the migration and eventual fate of class II molecules (42). The MHC class II vesicles (CIIV), a compartment rich in class II molecules, resembles the early endosomes but is distinct from those originating by endocytosis (43). CIIV is distinct from the MHC class II compartment (MIIC), which is a multivesicular structure composed of late endosomes and lysosomes (44). CIIV may therefore, represent an intermediary compartment for peptide loading and the subsequent egress of class II molecules to the cell surface (45). DCs, but not B cells contain CIIVs, suggesting different traffic routes for class II molecules to the cell surface in different cell types (10).

According to the third, which is considered the most significant pathway, class II-Ii complexes may be directly targeted to the MIIC for loading with

antigenic peptides. The MIICs play a greater role in loading class II molecules with antigenic peptides compared to the CIIVs (44). This difference is mostly due to the abundance of lysosomal enzymes and H-2DM in the MIICs and their near absence in the CIIVs. For a long time, it was unclear how peptide-loaded class II molecules exit MIICs and travel to the cell surface, i.e., whether via CIIV or by direct shuttling to the cell surface. In immature DCs, the majority of class II molecules are retained in the MIIC, and directed to the cell surface upon cytokine stimulation (46). When immature DCs are stimulated with lipopolysaccharide (LPS), or other stimuli, MIICs undergo morphological changes ultimately resulting in tubule formation. Tubules are transient in nature and only found in close proximity to the plasma membrane. Next, they fuse with the plasma membrane to allow class II display on the cell surface (44, 47).

Upon class II-Ii entry into the lysosomes, Ii degradation proceeds through a stepwise process regulated by various proteases. The first step is catalyzed by leupeptin-insensitive cysteine/aspartate proteases, followed by the action of the leupeptin-sensitive cysteine/aspartate proteases, cathepsin S and other cathepsins (48). Cathepsin L plays a significant role in Ii proteolysis specifically in cortical thymic epithelial cells (cTECs) as demonstrated by the defective thymic $CD4^+$ T cell selection in cathepsin L-deficient mice (49). On the other hand, cathepsin S has been shown to play a significant role in generating class II-restricted epitopes in B cells and DCs, since both cell types lack the enzymatic activity attributed to cathepsin L (50, 51).

H-2DM (HLA-DM in humans), a non-classical class II molecule, catalyses the enzymatic reaction that displaces CLIP with an antigenic peptide (52, 53). H-2DM is synthesized in the ER and then transported to the lysosomal compartment with the help of a tyrosine-based motif in the cytoplasmic tail of H-2DMβ. H-2DM α and β chains have homology with corresponding chains of the classical class II molecules; however the sides of the peptide binding groove of H-2DM are closely apposed to each other which leaves no space for peptide binding (54). Class II and H-2DM require direct contact for peptide exchange. H-2DM is suggested to keep empty and unstable class II molecules in a conformation permissive for peptide binding (55). In the absence of H-2DM, class II molecules are unstable, aggregate and, hence, are destined for degradation (56, 57).

Thus, class II-Ii migration from the ER, Ii proteolysis, H-2DM-mediated removal of CLIP and loading with the antigenic peptide are highly regulated processes. At the end of this series of events, a peptide MHC II complex travels to the cell surface carrying 12—20 amino acid residues long antigenic peptide (58). Such peptides are mainly generated within the lysosomes. Peptide (p)/class II complexes are presented on the cell surface for an appraisal by T_H cells.

Mechanisms governing class II processing and presentation in DCs

Professional APCs such as B cells, macrophages (Mϕ) and DCs differ in class II expression in their immature state. B cells and Mϕ constitutively express copious

amounts of class II, while DCs express significantly lower levels of these molecules (59).

DCs have a crucial role in regulating various aspects of both innate and adaptive immune responses. DCs were first described by Steinman and Cohn in 1973 (9). Since then, it was determined that DCs and to a lesser extent Mϕ participate in both antigen cross- and indirect presentation, thus raising questions regarding DC-specific rules governing class II loading with antigenic peptides. During DC maturation, cells are transformed from those that capture (phagocytosis, endocytosis, macropinocytosis) and process antigens, to those which present short processed peptides to T cells for an appraisal (60).

Immature DCs were first thought to be inefficient in class II-restricted antigen presentation. However, new evidence suggests that immature DCs can also load class II molecules with peptides and display them on the cell surface, suggesting that lysosome-resident class II are not retained, but instead p/class II complexes are transiently expressed at the cell surface (61). Such short lived class II molecules are rapidly endocytosed and degraded in the lysosomes (61).

The repertoire of class II-restricted peptides presented by immature and mature DCs do not differ as judged by the characteristics of naturally processed ligands eluted from class II molecules expressed by the differentiated states of the APCs. Nonetheless, differences are in the amount of CLIP-associated class II molecules, which is greater in the immature DCs (62).

In maturing DC, newly synthesized class II molecules originate from MIIC. Also, during maturation, there is a transient, ~18-hour long, increase in class II

synthesis. At the same time, there is a transient increase in proteolytic activity upon antigen capture (61). Exported class II molecules on the cell surface are long-lived, with cell surface half-time which is greater than 150 hours for immunodominant antigens, while immunorecessive antigens are presented with a half-life of ~10 hours. The half-life of peptide-class II complexes is a critical factor that dictates immunodominance and subsequent T_H cell responses (63). Strikingly, recent reports have suggested that even mature DCs retain the capacity to continue with antigen capture and processing (64, 65).

The cytoplasmic tail of the class II β-chain is ubiquitylated in murine immature DCs, which allows class II recycling from the cell surface. The number of ubiquitylated class II molecules in DCs decreases following maturation, resulting in the accumulation of MHC class II molecules at the cell surface (66).

In sum, emerging evidence indicates that immature DCs present class II-restricted antigens and participate in maintaining peripheral tolerance to self-antigens. This contrasts earlier reports, which suggested no class II molecules expressed by immature DCs. Upon antigen capture, class II molecules on the cell surface are replaced with newly synthesized molecules, and remain there for prolonged periods of time. Also, contrary to earlier reports, recent evidence indicates that mature DCs do indeed retain some capacity to capture new antigens and present them to T cells (64, 65).

There are several pathways that are utilized for presentation of exogenous and endogenous class II-restricted antigens. The following discusses the principles that governing antigen processing within class II pathway.

MHC class II-restricted processing of exogenous antigens in the lysosomes

Disulfide bonds maintain protein structural stability and aid in protein function. The reduction of disulfide bonds within antigens by γ-interferon-inducible lysosomal thiolreductase (GILT) is a critical initial step for protein unfolding and proteolysis (67). The enzymatic action of GILT requires an acidic pH. Many tumors down regulate GILT expression perhaps for evading host immunity (68).

Processing of antigens is accomplished with the help of various exopeptidases and endopeptidases, namely cathepsins (69). Here are summarized basic examples which govern cathepsin activity and their complex roles in antigen processing within the lysosomes:

- Specific cathepsins can be present at the mRNA as well as protein level in APC, but they do not necessarily display enzymatic activity. For example, DCs express cathepsin L, but it is not functional (49, 70).
- Cathepsins are greatly affected by the pH of their resident vesicle: low pH positively affects their activity (69).
- Another mechanism for regulating class II-restricted antigen processing is through low expression of lysosomal proteases in immature DC versus Mϕ. Upon DC activation, their expression rises to levels comparable to Mϕ (69).
- Individual cathepsins regulate activity of other proteases; e.g., cathepsin S in B cells lowers expression of cathepsin L, and also

affects GILT activity. Cathepsin L, on the other hand, affects cathepsins C and D in cTECs (71).

Therefore, class II Ag processing within the lysosomes includes a series of events, which are dependent on complex relationships between enzymes. Ag processing differs among professional APCs, thymic epithelium and activated epithelial cells.

Autophagy: a mechanism that provides cytoplasmic antigens for MHC class II-restricted presentation

Class II molecules are capable of binding and presenting peptides derived from endogenous proteins. Class II-restricted peptide elution and sequencing experiments have revealed that more than 20% of the naturally processed ligands originate from cytoplasmic proteins. A number of class II epitopes are also derived from cytosolic, plasma membrane and secretory vesicular proteins (72, 73).

Macroautophagy is constitutively active in cells of immune and non-immune system cells and tissues. The lysosomal compartment continuously receives input from the cytoplasm via macroautophagic mechanisms (74). Accordingly, the targeting of antigens to autophagosomes leads to enhanced class II-restricted antigen presentation, which in turn regulates T_H cell responses to cytoplasmic antigens (20). A related process, chaperone-mediated autophagy (CMA), mediates transfer of proteasomal substrates to the lysosomal compartment (21).

Autophagy involves stress-induced formation of double membrane structures, which non-specifically engulf cytoplasmic antigens, or damaged cellular organelles. During the initiation of this process, a damaged organelle, or a portion of the cytosol is encased in a structure termed the isolation membrane or phagophore. The phagophore then becomes enlarged during the elongation stages by the addition of new membrane — the origin of which is still unclear. The phagophore seals to form an autophagosome. Next, autophagosomes fuse with lysosomes where the cytoplasmic material is delivered for further processing (Figure 1) (75, 76).

Key players that regulate autophagy are the serine/threonine kinases, mammalian target of rapamycin as well as the class I and class III phosphoinositide 3-kinases. The initial steps of autophagosome formation include autophagy related 12 (Atg12) activation, which depends on Atg7 and Atg10. Ultimately, a complex of Atg12, Atg5 and Atg16 is formed. These molecules are accompanied by Atg4 activated Atg8 (also called light chain 3-II (LC3-II)), and the complex is present on the surface of newly formed autophagosomes. Next, LC3-II translocates to the centre of autophagosomes and is degraded following fusion with lysosomes to form autolysosomes (77).

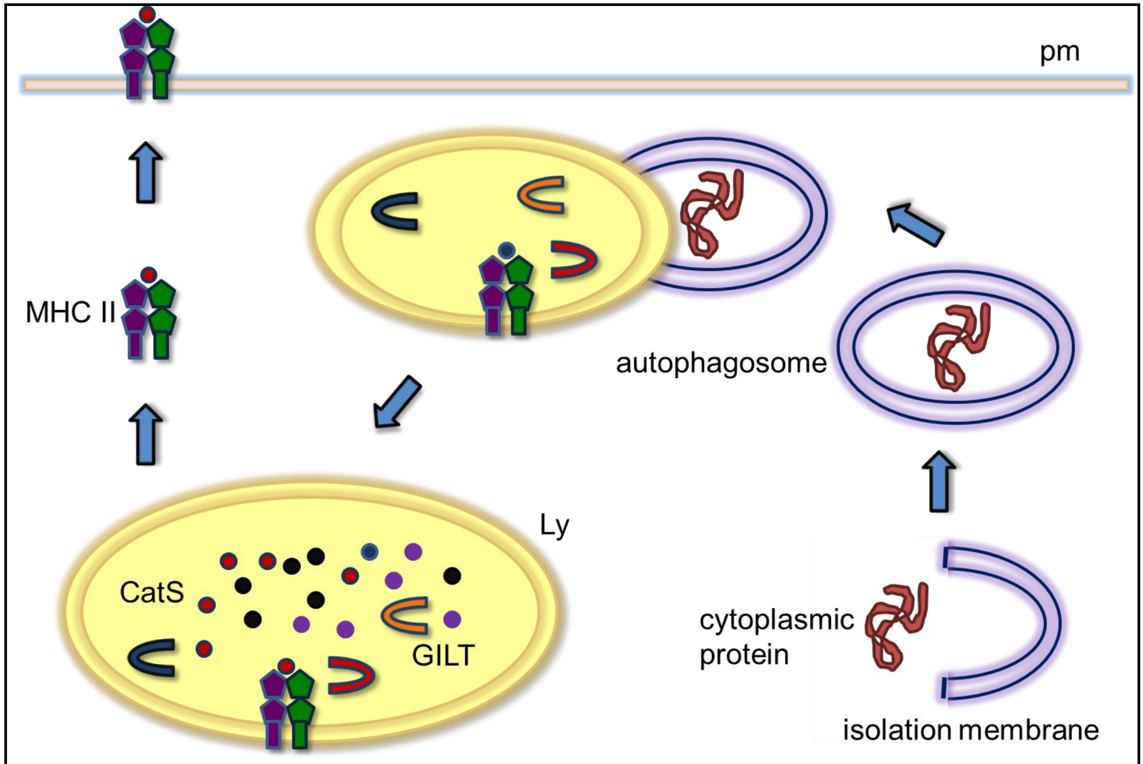


Figure 1. Macroautophagy donates cytoplasmic antigens to the lysosomal compartment for processing and loading MHC class II molecules with antigenic peptide. Cytoplasmic proteins are engulfed by isolation membrane which gives rise to autophagosomes. Autophagosomes fuse with lysosomes and deliver cytoplasmic proteins for further processing: GILT reduces disulphide bonds within proteins, while cathepsins S, as well as other cathepsins, further degrade protein to peptide fragments. Upon loading with antigenic peptide, p/class II complexes egress to the cell surface.

CatS, cathepsin S; GILT, γ-interferon-inducible lysosomal thiol reductase; Ly, lysosomes; MHC, major histocompatibility complex; pm, plasma membrane. Adapted from (75).

Emerging evidence suggests that numerous microbes utilize various mechanisms to affect autophagy while residing in the cytoplasm of infected cells.

One of the first reported autophagy-dependent class II antigens was derived from the Epstein-Barr virus nuclear antigen 1 (20, 78). Furthermore, HIV-1 inhibits autophagy in DCs.

Other pathogens—including *Shigella flexneri* and *Legionella pneumophila*, as well as Herpes simplex virus-1 (HSV-1)–have developed similar mechanisms to evade the immune system. They are able to evade their degradation in autolysosomes and, in some cases, even use autophagic vesicles for proliferation (79, 80).

Autophagy has been implicated in generating tumour-derived class II-restricted antigens. Class II presentation of peptides derived from a tumour antigen, Mucin gene 1, and neomycin phosphotransferase II was affected by blocking autophagy with the specific pharmacological inhibitor, 3-methyladenine (3-MA) (81).

CMA also contributes to the pool of cytoplasmic class II-restricted antigens. Proteasomal processing provides substrates for heat shock protein 70 complex (Hsc70), which stabilizes antigens and chaperones them to the lysosomal compartment (21).

Blum and coworkers found that ectopically expressed glutamate decarboxylase in a B cell line, which is normally expressed selectively within insulin-producing β cells in the islets of Langerhans, becomes a proteasomal substrate. The resulting proteasomal product is delivered to lysosomes upon

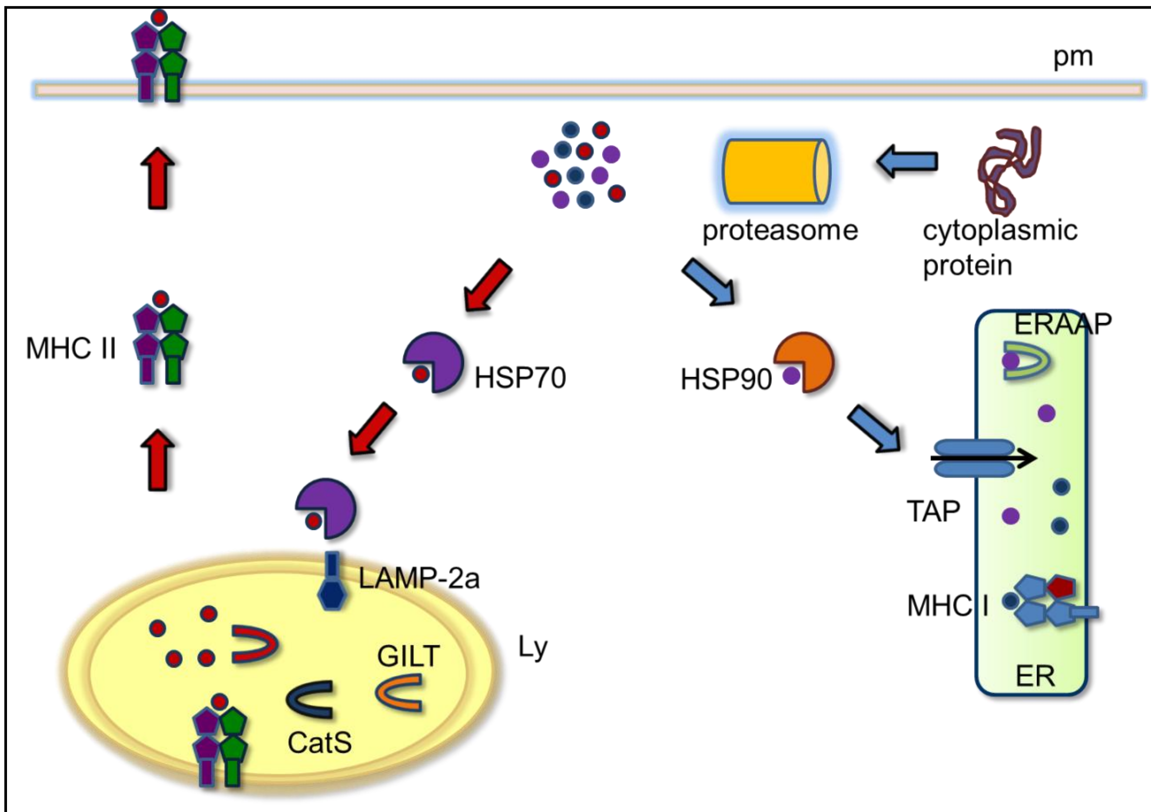


Figure 2: Chaperone mediated autophagy: HSP70 captures proteasomal products and transports them to LAMP-2a+ lysosomes for loading on MHC class II molecules. p/class II complexes egress to the cell surface. At the same time, proteasomal products are also the main source of antigens for the class I pathway, where HSP90 shields and delivers them to TAP which imports them to the ER lumen for processing by ERAAP.
CatS, cathepsin S; ER, endoplasmic reticulum; ERAAP, ER associated aminopeptidase associated with antigen processing; GILT, γ-interferon-inducible lysosomal thiol reductase; HSP- Heat shock protein; LAMP-2a, lysosomal associated membrane protein-2a; Ly, lysosomes; MHC, major histocompatibility complex; pm, plasma membrane; TAP, transporter associated with antigen processing. Adapted from (75).

Hsc70 binding to LAMP-2a positive lysosomes. LAMP-2a is a highly glycosylated lysosomal transmembrane protein. Interestingly, LAMP2a and Hsc70 complex do not affect the presentation of exogenous class II-restricted antigens (Figure 2) (82).

Thus, both macroautophagy and CMA are known to contribute to the pool of cytoplasmic, class II-restricted antigens and, hence, regulate presentation of cytoplasmic antigens of microbial origin.

Components of the MHC class I processing pathway affect presentation of cytoplasmic, class II-restricted antigens

Autophagy is not the sole source which supplies cytoplasmic antigens to lysosomes. A number of cytoplasmic class II-restricted antigens are independent of this process, which raises questions regarding factors that critically affect their presentation. Class II-restricted antigens derived from cytoplasmic bacteria, *Listeria monocytogenes* and *Francisella tularensis*, for example, critically depend on indirect presentation because they damage and destroy the host cells that they infect (83). Infected or dying cells are perhaps phagosytosed by APCs to allow indirect presentation of class II-restricted antigens. However, it is less well understood as to how cytoplasmic antigens donated by another cell become indirectly presented by class II. It is generally assumed that antigens donated by another cell are acquired and indirectly presented by APCs by a mechanism similar to direct presentation. This is because donated antigens are generally phagocytosed, endocytosed or pinocytosed and eventually enter the

endo/lysosomal compartment. As described above, these vesicles contain the processing enzymes and accessory proteins that facilitate indirect antigen presentation by class II molecules (32). Thus, indirect presentation is a variation on the direct presentation mechanism of extracellular microbes and their products. Nevertheless, many donated cellular antigens find their way into the cytoplasm. The mechanism by which such antigens are presented by class I molecules is incompletely understood.

Most studies thus far have only focused on examining processing properties of overexpressed cytoplasmic proteins in cell lines that leads to direct presentation by class II molecules (84). Such studies have revealed many class II-restricted cytoplasmic antigens. Examples include class II-restricted antigens derived from measles virus matrix and nucleocapsid proteins (85). Other examples include cytoplasmic tumour antigens such as human melanoma derived mutated forms of CDC27 and triosephosphate isomerase (24).

Recent studies have described a role for proteasomes in generating class II-restricted cytoplasmic epitopes. For example, a B cell line was transfected with a mutant form of Igκ (named SMA, which is the causative agent of light chain amyloidosis) and shown to depend on proteasomal processing for presentation of a SMA-derived class II-restricted epitope (Figure 2, top right). However, the presentation of exogenously added protein was not affected by proteasome inhibition with lactacystin, but was inhibited by lysosomal alkalinization, suggesting distinct, but possibly partially overlapping routes of antigen processing. Another independent study has demonstrated that ovalbumin (OVA)

introduced into the cytosol of APCs by osmotic shock, becomes a proteasomal substrate prior to being loaded onto class II molecules (86). This suggests that proteasomal processing is a prerequisite for efficient presentation of some cytosolic class II antigens, which are not dependent on autophagy or CMA.

However, the question still remains whether other components of the class I pathway—such as TAP and ERAAP (ER-associated aminopeptidase associated with antigen processing)—affect the pool of presentable class II-restricted antigens. Two T_H cell epitopes from two influenza virus glycoproteins, hemagglutinin and neuraminidase, were shown to depend on both the proteasomes and TAP for recognition by class II-restricted TH cells. TAP was required to transport both these class II-restricted epitopes into recycling endosomes where they were loaded on class II (87). Likewise, endogenously expressed hen egg lysozyme (HEL)-derived class II-restricted antigen required TAP function (88). Also, TAP is required for the loading of an overexpressed short cytosolic peptide derived from influenza nucleoprotein onto class II in a human B cell line (89). Thus, components of the class I-restricted antigen processing pathway, such as proteasomes and TAP, impact the presentation of cytoplasmic antigens by class II molecules. These studies have addressed the role of the class I-restricted antigen processing pathway in direct presentation of cytoplasmic, class II-restricted antigens. They, however, do not provide a mechanism(s) underlying indirect presentation of infected, tumour or allogeneic cells that are presented by recipient class II molecules.

Donor cell-derived cytoplasmic class II-restricted antigens acquired by recipient cells can seep into the cytoplasm where they may be exposed to the class I-restricted antigen processing pathway. In order to understand which factors affect indirect presentation of cytoplasmic antigens, a description of the currently purported mechanisms underlying class I-restricted antigen cross-presentation is discussed below. These mechanisms may shed light on factors that might significantly affect indirect presentation of cytoplasmic bacterial, tumour and allogeneic antigens.

MHC class I-restricted antigen processing pathway

All nucleated cells express class I molecules on the cell surface. Class I molecules present short, 8—10 amino acid residues long, peptides to CTLs for an appraisal. This pathway is referred to as the endogenous antigen processing and presentation pathway because of its role in presenting peptides derived from cytoplasmic proteins (10). However, exogenously derived proteins regularly gain access, especially within DCs and become substrates for its processing components through a process termed cross-presentation (32). Consequently, naturally processed class I-restricted peptides are derived from both cytoplasmic and exogenous sources.

The proteasome: structure and function

The class I processing pathway depends on several key components. Proteasomes serve as the main source of peptides for class I binding as

determined by selective pharmacological agents. This leads to a sharp decrease in cell surface class I expression (11).

Proteasomes are complex, multi-component particles that reside within the cytoplasm (90). They process proteins targeted for degradation. These are proteins with short half-life, or at the end of their life span (10). Proteasomal substrates also include freshly synthesized, but misfolded or misassembled protein complexes called “defective ribosomal products” (DRiPs) (91). Most proteins are degraded by a three-step process which starts with ATP-dependent transfer of multiple ubiquitin molecules to the ε-amino group of lysine residues. This process is dependent on various ubiquitin ligases (92). The resulting polyubiquitinylated substrates serve as a signal for rapid destruction within the proteasomes (93). However, not all proteins require this initial step: proteasomes readily degrade cytoplasmic proteins which contain repetitive, conserved peptide sequences rich in proline (P), glutamic acid (E), serine (S) and threonine (T), called the PEST motif (94).

The 26S proteasomes are complex structures of ~2000 kD. They are comprised of two 19S regulatory units, each of ~700 kD, which flank a 20S core subunit of ~650 kD. The 19S cap is displaced with 11S regulatory subunit upon cellular activation with interferon-γ (IFN-γ). This and changes in the composition of the 20S core give rise to immunoproteasomes (95, 96).

Under basal conditions, the 19S regulatory subunit is instrumental in recognizing and binding polyubiquitinylated proteins, as well as releasing free ubiquitin. The 19S subunit has ATPase activity, which is required to unfold

polypeptides and to direct them to the 20S core (97). The 20S core particle has a cylindrical structure. It is composed of 28 subunits arranged in four rings of 7 subunits each. The α subunits form the two outer rings which are arranged in such a manner that they form a central opening. This opening directs unfolded polypeptides toward the centre of the proteasome (98). Proteolytic activity is attributed to the two central rings, which are composed of β subunits (94, 99). Peptides emerging from the proteasomes range from 4—24 amino acid residues in length. Only about 10% of the peptides produced by the proteasomes are ~8 amino acid residues long; ~70% of the proteasomal products are of shorter length or are degraded to constituent amino acids, while ~20% are longer than those that typically bind to class I molecules (94).

The immunodominant ovalbumin-derived class I peptide SIINFEKL is generated from 6—8% of available molecules, suggesting that this class I immunodominant epitope is readily destroyed by proteasomal processing. Consistent with this finding, Yewdell and colleagues have suggested that in the case of DRiPs, one class I epitope is generated from 10^4 misfolded proteins, which suggest that high DRiP frequency does not result in high abundance of specific epitopes. Also, the low frequency of proteasomal products would suggest that TAP-mediated translocation and class I peptide loading are not saturating processes (91). There are conflicting reports regarding cleavage patterns for the central β subunits. The β1 subunit predominantly cleaves peptide bonds after acidic amino acids (caspase-like activity), β2 after basic amino acids (trypsin-like activity) and β5 after hydrophobic amino acids (chymotrypsin-like activity). Thus,

murine proteasomes preferentially create peptides which contain hydrophobic and basic amino acids at the carboxyl terminus. Human proteasomes in general follow this pattern to produce a diverse set of peptides (100). The N-terminus of potential class I-restricted peptides is not necessarily created by the proteasomes as they oftentimes create peptides that are N-terminally extended with regard to peptides that are presented by class I molecules (101).

Within the immunoproteasomes, 20S subunits β1, β5 and β2 are replaced with low molecular weight protein (LMP) 2 (iβ1), LMP7 (iβ5), and multicatalytic endopeptidase complex-like 1 (MECL-1, LMP10 or iβ2), respectively (102, 103). These three subunits are incorporated into newly assembled immunoproteasomes, while they have no effect on components of existing proteasomes. Cells of the immune system constitutively express immunoproteasomes, while other cells assemble immunoproteasomes in response to IFN-γ (94).

Immunoproteasomes also contain the 11S subunit, which is ~200 kD in molecular mass; this subunit is called the proteasome activator complex (PA28). It consists of PA28α and PA28β. Thus, immunoproteasomes represent a mixture of complexes comprised of the 20S catalytic core as well as 19S and 11S regulatory subunits. PA28 does not increase the rate of protein degradation, but impedes peptide degradation to constituent amino acids by the 20S subunit (104-106).

Immunoproteasomes have increased protein cleavage rates. Various studies have shown that the absence of the immunoproteasome subunits results

in altered class I-restricted peptide repertoire. Also, LMP2- and LMP7-deficiency in animal models revealed that these subunits lead to class I peptide creation with hydrophobic and to a lesser extent basic C-terminal amino acids. Both LMP2- and LMP7-deficiency leads to greatly reduced numbers of CTL (94).

Immunoproteasomes degrade a variety of proteins that are upregulated during inflammation in order to prevent formation of various, potentially harmful aggregates within the cytoplasm. Some of these proteins are damaged by the microbicidal reactive oxygen species (ROS) and require swift removal and clearance (107).

A recent study has revealed the presence of a thymus-specific proteasome. cTECs in mice express the novel β5t subunit. In humans, this subunit is expressed by ~80% of the cTECs and in a subset of cortical DCs. This novel proteasomal subunit is involved in the process of thymic positive selection as these thymoproteasomes affect the repertoire of class II-restricted peptides (108).

Proteasomes can also function as a polypeptide ligase. Within 20S complexes, novel class I epitopes are generated by excising a central peptide segment, followed by reconnecting the flanking regions to form a non-linear epitope. Furthermore, it was also demonstrated that proteasomal complexes can cleave nuclear protein and splice noncontiguous peptide segments in reverse order. Therefore, proteasomes not only cleave proteins into short segments, but also mix segments in direct or non-linear order followed by ligation of segments to produce novel noncontiguous class I epitopes (109).

The majority of peptides which exit proteasomes are immediately degraded by cytoplasmic proteases. Peptides that are shorter than 8 amino acid residues long are especially susceptible to such degradation. Cytoplasmic proteases which mediate this process are puromycin-sensitive aminopeptidase, bleomycin hydrolase, tri-peptidyl peptidase II, and thimet oligopeptidase (110-112). Thus, proteasomes cleave proteins into short segments, many of which are further degraded. Those that escape degradation in the cytosol become substrates for the TAP transporter.

TAP: structure and function

Proteasomal products that are greater than 8 amino acid residues long can be customized for class I binding by further proteolytic processing in either the cytoplasm or the ER lumen upon transfer by the TAP transporter.

Functional TAP molecules are heterodimers of TAP1 and TAP2 and are structurally classified as members of the ATP-binding cassette (ABC) transporter family (113). The role for TAP during antigen presentation has been established by creating TAP2- or TAP1-deficient cell lines and animal models, respectively, where most class I molecules are absent from the cell surface (114, 115).

Each TAP subunit consists of ten transmembrane domains. Each TAP heterodimer consists of three structural domains found within six C-terminal transmembrane segments: pore, peptide binding and nucleotide binding domains (NBD). The remaining four transmembrane segments allow contact with the large peptide loading complex (PLC) via tapasin (116).

The central hydrophobic pore domain results from TAP1 and TAP2 interaction. The peptide binding domain is located in the vicinity of the pore domain and is formed by cytoplasmic loop 2 and part of transmembrane segment 6 originating from both TAP1 and TAP2. The mechanism of peptide binding to this domain is beginning to be elucidated since it is essential for TAP function (116, 117).

NBD is located at the C-terminus of each subunit. ATP binds to specific Walker A and B motifs leading to triphosphate hydrolysis. The NBD is highly evolutionarily conserved and is present in various bacterial ABC transporters as well (118, 119). ATP hydrolysis causes substantial conformational changes to the transmembrane domains that form the pore. However, the exact nature of these changes is not yet understood (120). There are two hypothetical models for NBD function and peptide translocation along TAP heterodimers. According to the "processive clamp" model, a peptide and two ATP molecules bind specific sites on the TAP heterodimer. It is presumed that hydrolysis of both ATP molecules opens the pore domain to facilitate peptide import into the lumen of the ER. Once ADP is released, the two NBDs dimerize again and close the pore. Next, new ATP molecules bind to the NBD domain and the whole process is repeated (121).

The second proposed model is called "constant contact". In this model, NBD monomers never separate, but only one site binds ATP and allows NBDs to dimerize, while the other is open and empty. This way, one NBD dimer is always open, and closes only upon ATP hydrolysis of the other. Next, peptide drifts into

the lumen of the ER while ATP is not bound to the NBD. However, the exact mechanism is still not determined and is a matter of intense research (121).

The N-terminal and the C-terminal amino acid residues determine peptide binding to human TAP, while the central part of the peptide does not bind to TAP. The central peptide segment forms a bulge, thus permitting promiscuity in TAP specificity for a large number of peptides. This allows TAP to bind a wide array of different peptides with high affinity (122).

Murine and human TAP display high affinity for hydrophobic and basic C-terminal amino acids and very little affinity for acidic amino acid residues. Peptides between 9—16 amino acid residues have the greatest affinity for TAP, even though peptides up to 40 amino acid residues in length bind TAP and are translocated to the ER lumen (123).

Moreover, TAP is a target for viral evasion of CTL-mediated adaptive immunity. HSV-1 produces ICP47, and human cytomegalovirus (HCMV) product US6. Both bind to TAP and by steric hindrance prevent peptide import into the ER (124-127). Also, various metastatic tumours completely lack TAP, or express non-functional, mutated forms, suggesting a potential mechanism for evading tumour recognition by the immune system (128, 129).

Newly synthesized class I heavy chains interact with immunoglobulin binding protein (BiP), CNX and calreticulin (CRT) in the lumen of the ER. Subsequently, CNX disassociates to allow binding with β2-microglobulin (β2m), and an oxidoreductase, ERp57. The peptide loading complex is brought to close proximity of the TAP heterodimer via tapasin. Even though this peptide-loading

complex (PLC) is in indirect contact with TAP, class I molecules can be loaded with peptides in TAP-deficient cells. At the same time, the rate of peptide import into the ER by TAP is not affected in class I-deficient cells. Thus, peptide import into the ER and peptide loading on class I are two independent processes despite close association between TAP and PLC (130).

Tapasin plays a very significant role during class I assembly with peptides. It increases the efficiency of class I-restricted antigen presentation by facilitating class I loading with high-affinity peptides, thus stabilizing cell surface p/class I complexes. At the same time, tapasin does not influence peptide binding to TAP and their influx into the ER lumen. Nonetheless, tapasin can regulate ERp57 activity by preventing its dissociation from the PLC. Class I molecules loaded with low affinity peptides are retained in the ER. This process occurs through formation of a covalent bond between tapasin and ERp57, which is released only upon binding of optimal peptide cargo to class I molecules (131-133). A large number of peptides in the ER lumen require further customization prior to being loaded on class I, or need to be destroyed if they do not fit into the class I peptide binding groove.

ER-associated aminopeptidase associated with antigen processing

Proteasomes generate class I binding peptides that are N-terminally extended. The presentation of such longer peptides by class I molecules depends on the activity of additional peptidases both within the cytoplasm and within the ER. The ER-associated aminopeptidase associated with antigen processing (ERAAP)

plays a central role in customizing peptides to correct size for binding to class I molecules. Because ERAAP is indiscriminate, it can completely destroy potential epitopes. ERAAP functions as an N-terminal exopeptidase. Structurally, ERAAP belongs to a group of zinc-dependent metalloproteases. ERAAP is ubiquitously expressed in all nucleated cells and its expression is greatly increased in response to IFN-γ (134, 135).

ERAAP's role in customizing potential class I peptides was the subject of many studies. ERAAP deficiency leads to decreased cell surface expression of class I molecules by ~40—50%, depending on the class I allotype. Nonetheless, levels of class I expression between wild type and ERAAP-deficient cells became indistinguishable upon IFN-γ treatment (136). For example, the expression of H-2L^d molecules is lowered by ~80% in ERAAP-deficient cells compared to wild type cells. The reason for this lies in ERAAP's inability to cleave N-terminal X-Pro bond (X, any amino acid), a motif, which is found in most H-2L^d peptides. Interestingly, TAP also poorly transports peptides with this N-terminal motif, suggesting they are transported as N-terminally extended peptides, which are then customized by ERAAP (137). Furthermore, the presentation of *Toxoplasma gondii* derived HF10-H-2L^d epitope depends on proteolysis by ERAAP. Expansion of protective CTL populations was significantly impaired in infected ERAAP-deficient mice, which were more susceptible to toxoplasmosis compared to wild type counterparts (138).

In addition to affecting class I expression, the assembled peptide-class I complexes in ERAAP-deficient cells have reduced cell surface half-life, thus

suggesting reduced stability (136). The presented class I-restricted peptides in ERAAP-deficient cells may display lower affinity for class I molecules, an effect similar to tapasin deficiency. Nevertheless, the quality of presented class I epitopes remains quite different, as demonstrated by Shastri and colleagues when comparing sequences of eluted class I epitopes from wild type and ERAAP-deficient cells (139). Thus, ERAAP-deficient cells present profoundly distinct peptide repertoire on class I molecules, as demonstrated by the ability of wild type CTL to recognize knockout APCs with the same activation level seen in the case of MHC-mismatched allogeneic cells. Conversely, ERAAP-deficient CTLs are also strongly activated when challenged with wild type APCs. This leads to the question whether N-terminally extended class I peptides are presented with a central "bulge" which may lead to altered interaction with the T cell receptor (TCR) (140). Thus, ERAAP deficiency greatly affects the repertoire of peptides presented by class I molecules; this in turn leads to altered CTL responses.

Human cells ubiquitously express ERAAP1, which is almost identical in function to mouse ERAAP. A second aminopeptidase, ERAAP2, which is ~51% identical to ERAAP1, is expressed in a tissue restricted manner. The two enzymes can form homodimers or heterodimers, which suggests that they may have non-overlapping functions and need to act concertedly during antigen processing. Human ERAAP1 prefers amino termini preceding large aliphatic residues such as leucine, whereas ERAAP2 prefers basic residues such as

arginine or lysine (141). The differential substrate specificity suggests that they generate a wide range of class I-restricted epitopes.

ERAAP-deficient mouse DCs showed defective in vitro class I-restricted OVA peptide presentation by both direct and cross-presentation. ERAAP-deficient DCs have enhanced response to Lymphocytic Choriomeningitis Virus (LCMV) gp33-derived class I peptide in vitro, suggesting that ERAAP degrades this epitope (136). At the same time, ERAAP negatively affects the immunorecessive influenza virus $NP_{396-404}$ class I-restricted epitope, and has only a minor effect on gp33-derived class I epitope in in vivo studies (142). Van Endert and colleagues demonstrated that ERAAP1 and 2 in humans affect N-terminal trimming of human immunodeficiency virus type-1 (HIV-1) p17 and p24 derived class I-restricted precursor epitopes based on their length and internal peptide composition (143).

ERAAP affects presentation of peptides displayed by non-classical class I molecules as well. Qa-2 molecules present a diverse set of nonameric peptides with an invariant histidine residue at position 7 in a TAP-dependent manner (136). Qa-2 has 30% reduced cell surface expression in ERAAP-deficient cells. On the other hand, $Qa\text{-}1^b$ specific CTL clones demonstrated slightly decreased activity against ERAAP-deficient cells (136). Therefore, ERAAP affects processing of class Ib-restricted peptides.

ERAAP poorly trims peptides of 8—9 residues in length. Preferred substrates are 9—16 amino acid residues long peptides, while shorter or longer peptides are poorly trimmed. Interestingly, ERAAP's enzymatic activity is normal

in the absence of class I molecules, however, in that case, its action leads to preferential destruction of peptides found in the ER. Thus, ERAAP trims N-terminally extended peptides and its action is dictated by peptide binding motifs of specific class I molecules. In sum, ERAAP works in synergy with class I molecules to facilitate generation of optimal class I epitopes (144).

Class I-restricted peptides from wild type and ERAAP-deficient cells were compared in order to determine differences in their sequences. This comparison between wild type and knockout class I-associated self-peptide repertoire has revealed differences in peptide sequences presented by the two cell types, thus confirming ERAAP's contribution to shaping class I associated peptide repertoire (139). These studies along with analyses of the binding kinetics of ERAAP-specific class I epitopes may point to changes during thymic CTL selection.

In sum, ERAAP affects a large number of TAP-imported peptides in the ER either by destroying them, trimming them to the appropriate size for loading onto classical and non-classical class I molecules, or not affecting them. ERAAP does not interact with the PLC or TAP, yet plays a significant regulatory role by affecting the quality of the peptide repertoire presented by class I molecules (134).

Completely assembled p/class I complexes negotiate the Golgi apparatus on their way to the cell surface. From the cell surface, class I molecules are slowly internalized and shuttled to the early endosomal compartment for peptide exchange. Endosomal peptide exchange is now considered as a potential mechanism for cross-presentation of class I-restricted antigens.

Insulin-regulated aminopeptidase: a novel aminopeptidase involved in class I-restricted antigen processing

It has been postulated that there are additional aminopeptidases involved in class I-restricted peptide customization. Recently, Van Endert and colleagues described insulin-regulated aminopeptidase (IRAP), a $Rab14^+$ endosome-resident protease in human DCs as a necessary protease for antigen cross-presentation. $Rab14^+$ endosomes are devoid of class II molecules and other lysosomal proteases, but rich in class I molecules and TAP. IRAP is a ubiquitous zinc-dependent aminopeptidase that is closely related to ERAAP1 and ERAAP2. These two classes of aminopeptidases are strictly found in distinct cellular compartments. IRAP activity is IFN-γ dependent and has broader peptide specificity compared to its ER-resident counterpart. IRAP can trim N-terminally extended class I peptides within endosomes and thereby allow their loading onto recycling class I molecules. Furthermore, IRAP-deficient DCs have poor cross-presentation capacity, suggesting that there are two non-redundant cross-presentation pathways, one that requires ERAAP and the other requiring IRAP (145).

In summary, the class I processing pathway begins within complex, multisubunit structures called proteasomes. Next, processed peptides critically depend on TAP import into the ER, where some peptides can be directly loaded on class I molecules, while others are either customized or completely destroyed by ERAAP. Antigens presented by class I molecules are not only of endogenous origin, but also from exogenously-derived microbial, tumour or alloantigens

through the process of cross-presentation. This process is of great importance for establishing CTL-mediated immunity to pathogens and alloantigens, and eradicating tumours. How components of the class I pathway participate in cross-presentation is discussed in detail below.

Cross-presentation mechanisms and their role in establishing CTL immunity

In 1977 Bevan first reported that class I molecules present exogenous antigens to CTLs through a process termed cross-presentation (146). Initial observations led to the conclusion that foreign antigens readily gain access to components of the class I processing pathway. APCs survey all tissues for tumour, allogeneic and infected cells (12). Upon encountering them, or particulate antigens, APCs acquire such cells by phagocytosis or internalize antigens from the extracellular environment by endocytosis or pinocytosis, and travel to the local draining lymph node or the spleen to present antigens to CTL and T_H cells through the process called cross- and indirect presentation, respectively (147).

Cross-presentation is a process of great importance because it allows presentation of exogenous allogeneic, tumour and microbial antigens to CTL for an appraisal (12). Nevertheless, despite the progress in elucidating mechanisms of cross-presentation, there is still controversy regarding the details of the underlying mechanism(s) and which proposed mechanism(s) operate under physiological conditions.

Cross-presentation leads to extensive communication between class I and class II processing pathways, thus blurring previously established strict boundaries between them. Internalized antigens are destined to the lysosomes for processing and presentation by class II molecules or for terminal degradation (69, 148). Nonetheless, exogenous antigens derived from dead, or tumour cells can also seep into the cytoplasm and become substrates for the class I processing machinery (11). Under such conditions, components of the class I antigen processing pathway capture and process these antigens, thereby affecting the process of indirect presentation of these antigens by class II molecules to T_H cells for an appraisal. It is of significance to understand the progress made regarding cross-presentation mechanism(s) in order to gain insight into it's practical effects on the processing of class II-restricted, cytoplasmic antigens from dead cells for indirect presentation. The ultimate question addressed in this thesis is how components of the class I pathway modulate T_H cell responses.

The first mechanism proposes that cross-presentation of soluble and particulate exogenous antigens occurs entirely within the endocytic compartment (149). Endosomes can serve as the compartment where endogenously derived class I peptides are exchanged with those of exogenous origin. Such reported antigens are derived from influenza virus and other exogenous particulate antigens (150-153). This process was dependent on the activity of the lysosomal protease cathepsin S, and was entirely independent of proteasomes and TAP (Figure 3) (154, 155). Additionally, disulfide bond rich HSV-1 and influenza virus

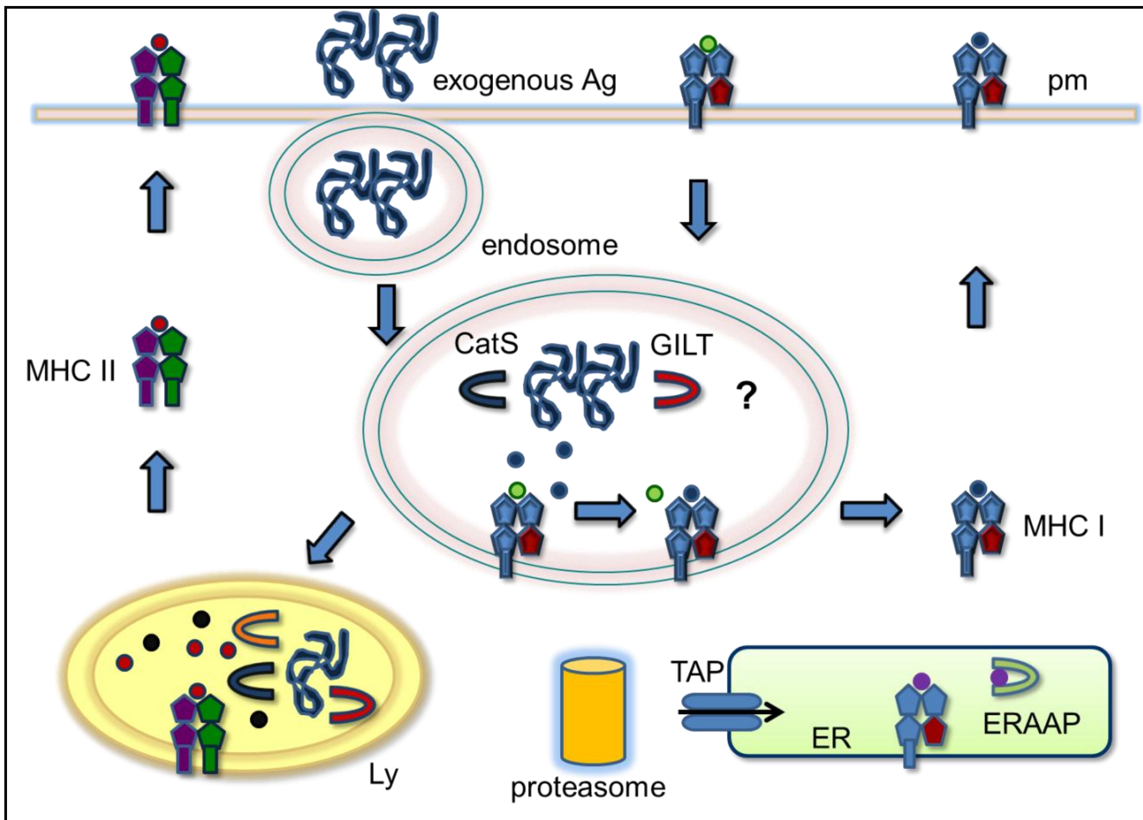


Figure 3. Mechanism of cross-presentation: components of the MHC class II pathway facilitate peptide exchange and the loading of MHC class I with antigenic peptide. Exogenous antigens are processed by GILT and cathepsin S within the endosomal compartment and resulting peptides are loaded onto recycling class I molecules and p/class I complexes egress to the cell surface. At the same time, maturing endosomes fuse with the lysosomes, where class II molecules are loaded with cognate peptides.

Ag, antigen; CatS, cathepsin S; ER, endoplasmic reticulum; ERAAP, ER-associated aminopeptidase associated with antigen processing; GILT, γ-interferon-inducible lysosomal thiol reductase; Ly, lysosomes; MHC, major histocompatibility complex; pm, plasma membrane; TAP, transporter associated with antigen processing. Adapted from (69).

derived antigens required GILT, a component of the class II processing pathway. GILT reduces disulfide bonds, which is a prerequisite for efficient cross-presentation (156, 157). It is not clear what the contribution of this pathway is to the total pool of cross-presented antigens and whether lysosomal proteases can precisely mimic the functions of various components of the class I antigen processing and presentation pathway.

The second proposed mechanism of cross-presentation suggests that macropinocytosed soluble antigens can escape from early endosomes in DCs, but not from those in Mϕ, thus gaining access to proteasomes and the TAP transporter (Figure 4) (158, 159). Furthermore, the fate of potential class I antigenic peptides may be influenced by ERAAP (142).

Thus, cross-presentation of exogenous antigens requires escape of the source antigen from endosomes to the cytoplasm and subsequent access to components of the class I-restricted antigen processing pathway (160, 161).

Cresswell and colleagues have further investigated this process and suggested a somewhat different mechanism for antigen trafficking and access to components of the class I processing pathway in the ER. Accordingly, soluble internalized antigens negotiate the Golgi apparatus and translocate by retrograde traffic to the lumen of the ER. Such antigens are then released into the cytosol by

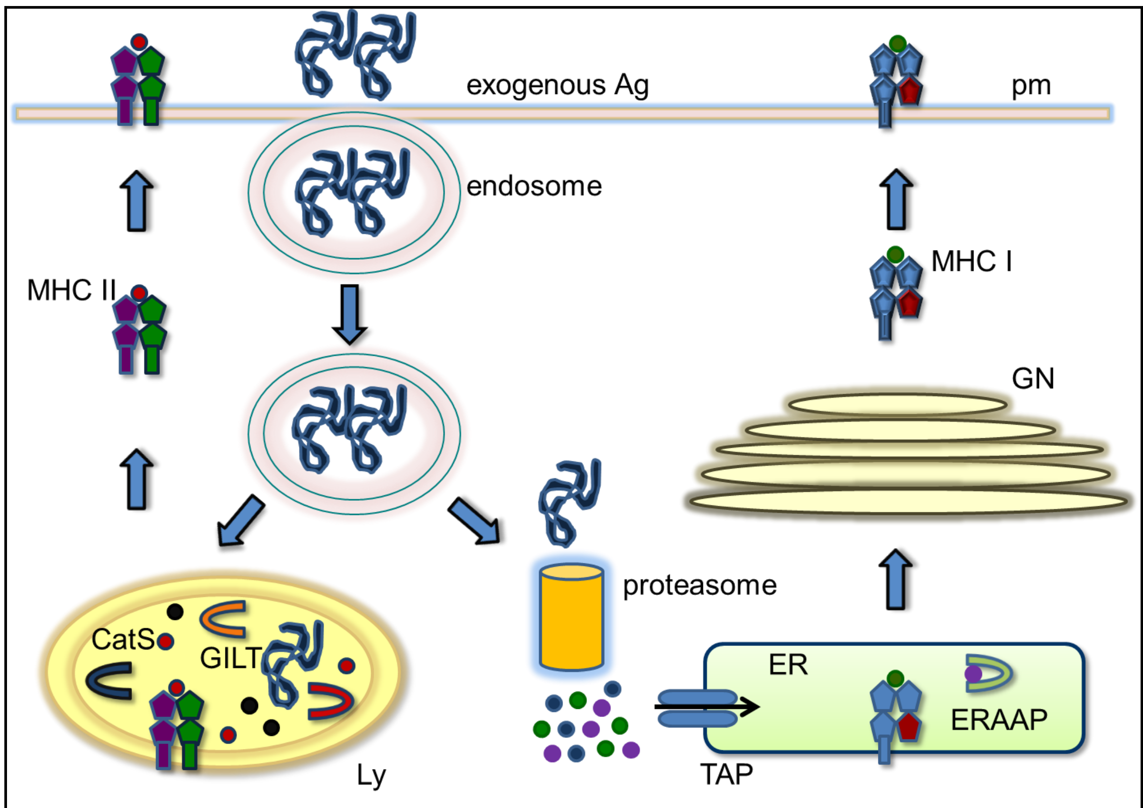


Figure 4. Mechanism of cross-presentation: exogenous antigens escape into the cytoplasm, where they are degraded within proteasomes to peptides which are transported via TAP to the ER. Exogenous antigens seep into the cytoplasm from the endosomes where they are degraded within proteasomes and the resulting peptides are transported to the ER via TAP where they are customized by ERAAP. At the same time, exogenous antigens are delivered to the lysosomes where cathepsins mediate their degradation in a GILT dependent manner.

Ag, antigen; CatS, cathepsin S; ER, endoplasmic reticulum; ERAAP, ER associated aminopeptidase associated with antigen processing; GILT, γ-interferon-inducible lysosomal thiol reductase; GN, Golgi network; Ly, lysosomes; MHC, major histocompatibility complex; pm, plasma membrane; TAP, transported associated with antigen processing. Adapted from (149).

sec61 where they become targets for proteasomes and TAP (32, 162). When soluble US6 is added to immature DC cultures, it is internalized by macropinocytosis and leaves these vesicles to translocate to the lumen of the ER. This then allows US6 to bind the ER luminal segment of TAP, and blocks peptide import from the cytoplasm. As a result, class I cell surface expression is decreased (32, 163). The same mechanism has been demonstrated for exogenously added β2m, as it increased cell surface class I expression in β2m-deficient DCs, but not Mϕ (164).

The drawback of this mechanism is that in DCs, macropinocytosed material is quickly targeted to the lysosomes for degradation. Therefore, antigen escape from endocytic vesicles remains somewhat controversial. However, how would endosomal cargo in DCs find its way into the cytoplasm? There are several proposed mechanisms, which will be discussed later.

The above studies nonetheless strongly point to the escape of exogenous cargo from the early endosomes suggesting the potential for class II-restricted antigens to be indiscriminately shunted into the class I-restricted antigen processing pathway and possibly lost to cytosolic degradation. Therefore, understanding how potential class II antigens are diverted to the class I processing pathway, as well as which components critically affect this process is missing. A better understanding of which factors affect indirect presentation will provide insights into T_H cell mediated immunity against microbes, tumours and allografts.

The third proposed mechanism of cross-presentation is the most controversial and has drawn substantial criticism and support. An intriguing line of evidence suggests close contact between the ER and newly formed phagosomes. The ER membrane directly contributes to the formation of phagosomes, thus shuttling components of the class I pathway to the newly formed organelle. This mechanism suggests that Sec61 from within the phagosomal membrane allows export of engulfed proteins to the cytoplasm for proteasomal degradation. The resulting peptides are then preferentially transported back to the phagosomes via TAP or directed to the ER for loading class I molecules (Figure 5) (165, 166).

This mechanism of cross-presentation has several drawbacks which include in vivo significance of this mechanism. Other concerns are regarding the purity of phagosomes isolated by ultracentrifugation. Such phagosomes were shown to contain components of class I processing machinery due to possible contamination with the ER membrane (167). Also, very frequently the endo-lysosomal membranes are disrupted due to the use of latex or similar beads owing to their large size (167).

Grinstein and colleagues found that the existing endosomes greatly contribute to phagosomal membrane formation, with little-to-no contribution from the ER to these membranes. They demonstrated that the major source of phagosomes is the plasma membrane. This finding also depended on the size of the particle used in the experiments: 1—3 μm particles do not involve ER membrane; however larger particles may disrupt the endosomes. However,

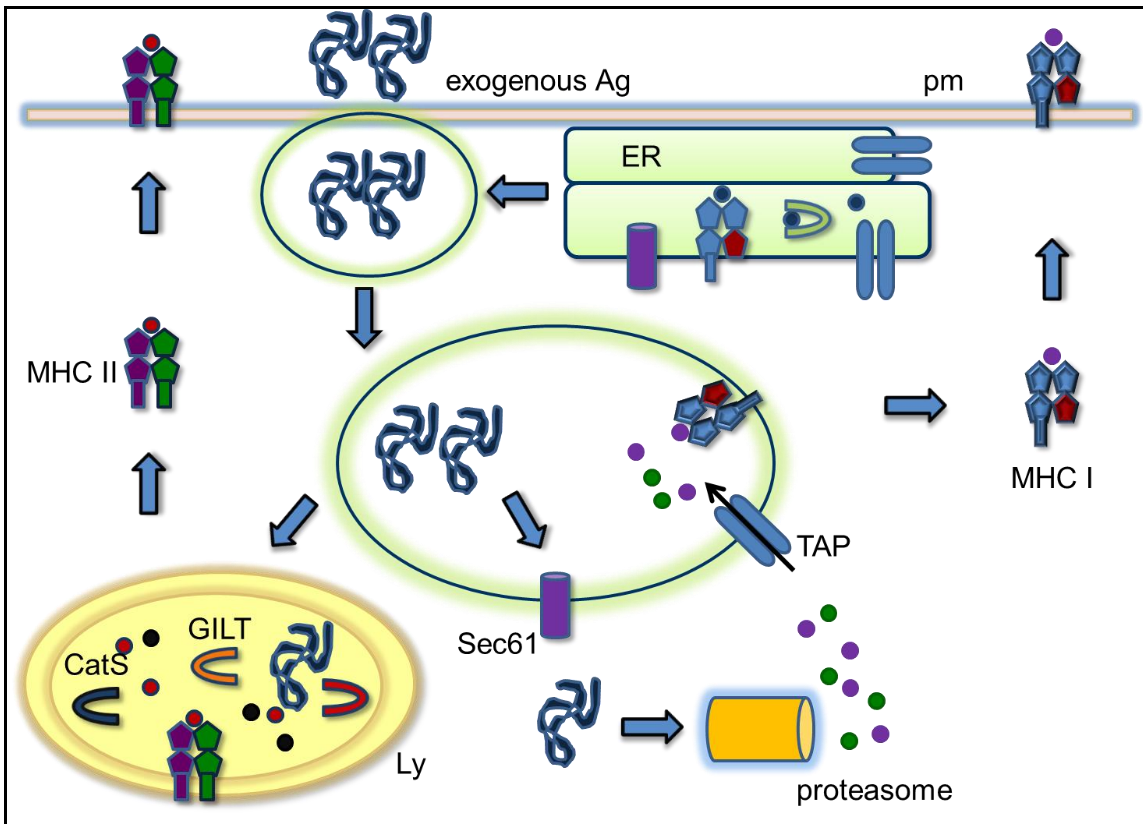


Figure 5. Mechanism of cross-presentation: Exogenous antigen is captured within endo/phagosomes containing ER membrane. Exogenous antigens are retrotransported via Sec61 to the cytoplasm where they become proteasomal substrates. Processed peptides are next imported via TAP to endo/phagosomes where class I molecules are loaded with antigenic peptide. p/class I complexes egress to the cell surface. At the same time, exogenous antigens may be shuttled to the lysosomal compartment for processing and loading onto class II molecules.

Ag, antigen; CatS, cathepsin S; ER, endoplasmic reticulum; ERAAP, ER associated aminopeptidase associated with antigen processing; GILT, γ-interferon-inducible lysosomal thiol reductase; Ly, lysosomes; MHC, major histocompatibility complex; pm, plasma membrane; TAP, transported associated with antigen processing. Adapted from (149).

Grinstein et al. have used non-antigen coated latex beads (inert particles), which are not substrates for cross-presentation and are not processed by the class I pathway (168).

However controversial, the above mechanism may play a role during phagocytosis of dead and tumor cells by DCs because of their size. Interestingly, the third mechanism strongly supports extensive antigen escape from maturing phagosomes. Large phagocytosed antigens may allow for extensive donation of the ER membrane to maturing phagosomal vesicles.

The fourth proposed mechanism of cross-presentation suggests that gap junctions between cells participate in antigen exchange between cells. Gap junctions represent channels which are normally present in multicellular organisms. They are composed of two hemichannels each consisting of six connexin molecules. Previous studies have demonstrated that this type of cellular interaction allows the exchange of ions, nutrients and second messengers. These channels are not energy dependent, and allow passive diffusion of molecules up to ~1800 Daltons. Connexin 43 (Cx43) is most broadly expressed in cells of the immune system, including certain types of DCs. Interestingly, tumour cells, as well as cells infected with HSV-2 or human papilloma virus 16 lose gap junctions, in part to evade the activation of the immune system. Cx43 overexpression in cell lines led to fluorescent peptide diffusion into the neighbouring cells (169-171).

Numerous cytosolic peptidases mediate effective clearance of the majority of proteasomal products, thus preventing uninterrupted peptide exchange

between cells. So, gap junction mediated cross-presentation may be effective for high copy number peptides. Also, peptides within class I and class II pathways regularly require protection by binding to chaperones, namely heat shock proteins (HSPs) and CRT (163, 172, 173).

Another proposed mechanism of cross-presentation suggests that cells release vesicles rich is class I and class II molecules called exosomes, which participate in antigen exchange between cells (174, 175). It is not clear how effective and predominant this process is in transferring antigens to other cells for CTL recognition and activation.

The majority of cross-presentation mechanisms suggest that exogenous antigens readily become available for class I processing components, namely proteasomes and TAP, before they are loaded onto class I molecules (159). It is increasingly evident that CTL-mediated immunity extensively depends on cross-presentation as a source of exogenous antigens. Thus, the question remains which intricate mechanism(s) facilitate antigen cross-presentation and which APCs predominantly and most efficiently mediate this process.

How do APCs mediate cross-presentation?

The nature of cross-presented antigens remains a critical unanswered question. The answer may shed light on factors that drive donation of antigen and may also pin point factors within recipient APCs that allow for antigen cross-presentation. Depending on the experimental model, various groups have arrived at different conclusions. This may not be surprising as there may be more than

one form of donated antigen. Earlier studies suggested that the whole protein is donated (176-178). However more recent studies have suggested that either peptides alone, or stabilized in complex with HSPs are cross-presented. Class I epitopes, or N-terminally extended proteasomal products frequently bind to HSP90 and CRT to both prevent peptide degradation, and facilitate donation/transfer to the ER (179-184). Shastri and colleagues found that HSP90 stabilizes peptides and prevents their degradation during cross-presentation (172). On the other hand, the majority of studies suggest that HSP70 facilitates indirect presentation of class II-restricted antigens (183). Thus, based on available data, HSP90, HSP70, CRT and possibly other chaperones prevent cytosolic peptidase-mediated degradation of antigens and serve as chaperones of such antigens.

Another question is whether necrotic cells, apoptotic cells, or cells under stress that causes autophagy (or autophagic type of cell death), or all three types, donate cross-presented antigens. Medzhitov and colleagues have reported that apoptotic cells/bodies do not serve as a source of cross-presented antigen. However, their results are perhaps influenced by the experimental model and its inherent limitation, especially since their conclusions were based only on in vitro studies. Also, they used apoptotic bodies, which were triggered to undergo transformation six hours prior to being used in experiments. However, apoptotic cells at earlier time points were not examined, because, at earlier time points, the donated antigens may have escaped complete destruction (185).

In separate studies, apoptotic or dying cells were directly injected into mice to investigate their cross-presentation potential (186, 187). Recent reports also indicate that apoptotic cells have modified cholesterol crystals present in the plasma membrane, as well as other protein aggregates which are recognized by cytosolic pattern recognition receptors (PRRs) of neighbouring cells, DCs or Mϕ which results in the activation of inflammasomes, the result of which is the secretion of proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 (188-190). Also, dying cells may release exclusive intracellular molecules, such as uric acid and fibrinogen, which in turn act as Toll-like receptor (TLR) agonists (191). Inflammasome activation is dependent on the adaptor molecule MyD88. Thus, signalling through MyD88 greatly facilitates innate immune responses (192). Apoptotic cells also release ATP and UTP, which are sensed by the nucleotide receptors on the target cells, P2Y2 and P2X7 (193, 194). Some of the released dead cell products are also sensed by cell surface and endosomal TLRs resulting in the release of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), IFN-γ and IL-12. Cross-presentation can be triggered by necrotic cells which are inducers of the inflammatory cytokine IL-1α (12, 195).

Necrotic cells also release urate and pyrophosphate crystals, which can trigger cytosolic PRRs called NOD-like receptors (NLRs) that are expressed by tissue Mϕ and dendritic cells. Activation of NLRs could potentially affect innate immune responses in the case of dying cells (188).

Both in vitro and in vivo studies suggest that $CD8\alpha^{+}CD11c^{high}$ DC are the major APCs, which mediate antigen cross-presentation through two potential

mechanisms: (1) Peripheral DCs, viz., skin Langerhans cells arrive in the lymph node carrying antigen and donate it to $CD8\alpha^+$ DCs. (2) $CD8\alpha^+$ DCs survey peripheral tissues, and upon acquisition of foreign antigen, travel to local draining lymph node and spleen to present it to CTL. Although this is an unresolved issue, it is possible that both mechanisms play a role during cross-presentation (9).

Other studies have implicated $M\phi$ in cross-presentation and showed that this heterogeneous tissue-specific cell type can also significantly contribute to cross-presentation. Recently, CD169+ subcapsular $M\phi$ from lymph nodes were demonstrated to participate in tumor-derived antigen presentation, suggesting that APCs other than DCs also have the potential to mediate antigen cross-presentation (196).

Many studies point to DCs as the most efficient APCs dedicated to cross-presentation. However, it remains unclear which factors direct ingested material to the class I processing pathway, and which steer them to the class II pathway. One explanation for activating both CTL and T_H cells may be that there are two distinct subtypes regulating differential T cell activation. Thus, splenic DCs may be divided into two main groups: (a) $CD8\alpha^+$ DCs, which express high levels of the C-type lectin DEC205; and (b) $CD8\alpha^-$ DCs, which stain positive for mouse dendritic cell inhibitory receptor-2 (DCIR-2).

$CD8\alpha^+DEC205^+$ DCs localize to the T cell zone, while $CD8\alpha^-DCIR\text{-}2^+$ DCs reside in the red pulp and marginal zone of the spleen. When $CD8\alpha^+DEC205^+$ DCs were pulsed with OVA, they were able to present the antigen to both peptide-specific CTL and T_H cells. Nonetheless, the T_H cell response was less

pronounced. On the other hand, CD8α^-DCIR-2$^+$ DCs preferentially presented antigen to T_H cells, but not to CTLs. In sum, distinct DC subsets are essential to ensure optimal CTL and T_H cell responses to antigen (197).

Another key issue regarding antigen cross-presentation pertains to the cellular factors that influence shuttling of the internalized cargo to specific cellular compartments. Recently, mannose-receptor (MR) containing endosomes were demonstrated to target exogenous antigens towards the class I processing pathway. CD8α^+ DCs and Mϕ specifically express MR on the cell surface. These DCs and Mϕ critically depend on MR for efficient cross-presentation of exogenous OVA to CTL. Nonetheless, MR-deficiency does not perturb T_H cell activation. MR positive endosomes were shown to be Rab5 and early endosome antigen 1 (EEA1) positive, but negative for the late endosomal and lysosomal markers, Rab7 and LAMP-1 (198, 199).

Other factors can also favour antigen shuttling toward the lysosomes in DCs and Mϕ. In the case of DCs, they mainly pinocytose antigens that are presented by class II molecules. On the other hand, Mϕ may utilize other cell surface molecules such as scavenger receptors (SR) to direct extracellular cargo to lysosomes, as seen by Rab7 and LAMP-1 co-staining with such receptors (200). Therefore, within Mϕ, a balance between MR and SR may affect the final destination of internalized antigen. These results suggest a requirement for two types of receptors, MR and SR, to control exogenous cargo and its compartmentalization.

Some of the previously described mechanisms of cross-presentation suggest that in DCs, phagocytosed antigens must escape fusion with lysosomes in order to access the cytoplasm for efficient cross-presentation. However, the exact mechanism(s) of antigen escape remains unclear, even though multiple studies have suggested that phagosomal cargo readily seeps into the cytoplasm. One proposed explanation is that immature DCs express lower levels of lysosomal enzymes compared to Mϕ, suggesting that DCs may be specialized for antigen cross-presentation (69).

The second possible explanation is that DC phagosomes maintain neutral pH for a longer time period (several hours) compared to the acidic pH of Mϕ phagosomes. Neutral pH is maintained by the assembly of NADPH oxidase complex 2 (NOX2). Phagosomal maturation involves activation of vacuolar ATPases, which pump H^+ ions into the lumen, thus lowering phagosomal pH and allowing fusion with the lysosomes. NOX2 complex in DCs leads to ROS formation, which consumes available H^+ ions, thus raising the pH value to neutral. Amigorena and colleagues have demonstrated that NOX2 plays a role in cross-presentation of soluble antigens in DC (201-203). Different phagosomes may have distinct routes of activation, as suggested for IRAP-containing endosomes which are devoid of class II and do not fuse with the lysosomes. Phagosomes of different professional APCs may contain different levels of vacuolar ATPases versus NOX2 components, thus allowing antigen shuttling toward the endolysosomal compartment for presentation by class II molecules, or antigen escape to the cytosol.

Thus, specific cell surface receptors (MR versus SR), or specific cell surface markers/receptors (DEC205 versus DCIR-2) might determine whether the antigen is presented by class I, class II, or both. Furthermore, within DCs, NOX2 activation aids in antigen escape to the class I pathway and limits antigen availability to the lysosomes. In sum, there are numerous factors which regulate how and whether endocytosed antigens will be directed to the class I pathway and cross-presented to CTL for an appraisal.

Minor histocompatibility antigens

The critical role of minor HAgs in transplant outcome has been known since the advent of human leukocyte antigen (HLA) typing and HLA-identical sibling bone marrow transplantation. Herein, graft-versus-host disease (GVHD) fatalities occur even among those who received grafts from an identical twin. Allograft rejection and GVHD caused by minor histoincompatibility are complex genetic diseases (204). Humans differ from each other by ~0.1%, which is about 3 million sites across the 3 billion-nucleotide genomic landscape, caused by single nucleotide polymorphisms, short tandem repeats and copy number variations (205, 206). This is also true for monozygotic twins who were once considered genetically identical (207-209). Several mechanisms underlie these genetic differences, including somatic mutations in utero during development, meiotic and mitotic recombination and biased gene conversion (207, 210). Thus, genes and

genomes are constantly evolving, which in part explains how minor histoincompatibility arises in HLA identical transplants.

MHC class I and class II-restricted murine minor HAgs

The ideal setting for solid organ transplantation as well as bone marrow transfusion is when donor and recipient are MHC matched, or at least partially matched. However, even under conditions of full MHC compatibility, GVHD ensues, and it often requires life-long immunosuppressive treatments (211, 212). Such treatment leaves the patient vulnerable to dire side effects of medication, and most importantly, to increased risk of infections and development of invasive cancers (213).

GVHD develops in MHC identical pairs as a result of subtle differences in self-proteins between donor and recipient. They stem from altered amino acid sequences, or as a result of gene silencing, thus lack of protein expression within the recipient. These donor-recipient differences often (but not always) result in MHC molecules presenting allogeneic peptides called minor HAgs. Class I- and class II-restricted minor HAgs result from subtle alterations of self-peptide sequences, or they are epitopes originating from proteins with differential expression, thus being recognized as non-self in the recipient (214).

A summary of the major murine class I-restricted minor HAgs, with the donor representing 129- or C.B10-$H2^b$-background (variant B) and the recipient C57BL/6-background (variant A) is presented in Table 1. The summary of murine class II-restricted minor HAgs reveals that very few have been described so far

(Table 2). The lack of information regarding class II-restricted minor HAgs precludes complete understanding regarding the ensuing T_H response and its role in GVHD.

Human HY minor HAgs and their impact on GVHD and organ transplantation

There are many HLA class I- and class II-restricted minor HAgs that are encoded by genes residing within the Y chromosome. The list of human HLA class I and class II-restricted Y chromosome-encoded mHAgs reveals that the majority are encoded by genes orthologous to the murine counterparts (Table 3). Relatively little is known about other HLA class II-restricted minor HAgs.

Recent studies have shown that HY minor HAgs have significant impact on the outcome of corneal tissue allografts. Minor HAg-specific CTL clones were isolated from the recipients. Recent studies on cornea transplant recipients indicate that mismatching for the minor HAg HLA-A1/HY promotes cornea rejection (204, 215).

In stem cell transplantation recipients, the most immunodominant non-HY minor HAg is HLA-HA-1, whereas HY minor HAgs promote lesser immune response (216).

Kidney transplants are very frequent in patients where haemodialysis becomes insufficient for toxin and waste metabolite removal. It was reported that there was reduced renal graft survival in the HLA-A2 group compared to other HLA class I recipients in gender-mismatched renal transplant pairs, indicating a

role for HLA-A2/HY minor HAg in the allograft outcome. These mismatches in minor HAgs might induce activation of CTL and T_H effector cells, which ultimately lead to the destruction of the kidney epithelium (204).

Table 1. Major histocompatibility complex class I-restricted murine minor histocompatibility alloantigens

Gene	Minor HAg/MHC restriction	Variant A	Variant B	Cause(s) of incompatibility	Tissue distribution	Protein function	Reference
Mouse (H2)							
H60	H60/K^b	/	LTFN**Y**RN**L**	gene silencing	leukocytes	non-classical class I molecule	(217)
Emp3	H4/K^b	**S**G**T**V***Y***IH***L***	pS**G**I**V*****Y***IH***L***	P1[2], P3 variation	leukocytes and epithelial cells	cell adhesion	(218)
H28	H28/K^b	*nd*	FILEN***F***PR***L***	induced	DCs and Mϕ	unknown function	(219)
Zfp106	H3a/K^b	**A**SP**C*****N***STV***L***	**T**SP**R*****N***STV***L***	P1, P4 variation	ubiquitous	nuclear protein	(220)
Stt3	H7/D^b	KAPD***N***R**E**T***L***	KAPD***N***R**D**T***L***	P7 variation	ubiquitous	glycosylation in the ER	(221)
H13	H13/D^b	SSV**V*****G***VWY***L***	SSV**I*****G***VWY***L***	P4 variation	leukocytes	unknown function	(222)
H47	H47/D^b	SCIL*L*Y**I**V***I***	SCIL*L*Y**F**V***I***[1]	P7 variation	ubiquitous	degradation of misfolded proteins	(223)
Uty	HY/D^b	WMHH***NM***DL***I***	WMHH***TV***DL***L***	male antigen	ubiquitous	histone demethylase	(224)
Smcy	HY/D^b	*nd*	KCSRNR***Q***Y***L***	male antigen	ubiquitous	lysine demethylase	(225)
Smcy	HY/D^k	*nd*	RRLRKTLL	male antigen	ubiquitous	lysine demethylase	(226)
Smcy	HY/K^k	G***E***GTGNMP	T***E***NSGKD***I***	male antigen	ubiquitous	lysine demethylase	(227)

minor HAgs, minor histocompatibility alloantigens; P, position in the amino acid sequence of the peptide; PΩ: carboxy-terminus of the peptide; nd: no described allele; ***bold italics***: anchor residues; **bold underlined**: amino acid variation between allelic variants; [1]unknown whether presented; [2]phosphorylated in variant B. Adapted from (3)

Table 2. Major histocompatibility complex class II-restricted murine minor histocompatibility alloantigens

Gene	Minor HAg/MHC restriction	Variant A	Variant B	Cause(s) of incompat-ibility	Tissue distribution	Protein function	Refer-ence
Mouse (H2)							
Il4i	H46/A^b	H**A**[***F***VEAIPEL QG]HV	H**M**[***F***VEAIPE LQG]HV	P-1 variation	IL-4 induced in B cells and DCs	flavin monoxidase	(228)
Rbp1	H3b/A^b	*np*	*np*	*np*	*np*	gene translation	/
DBY	HY/A^b	**NAG**[***F*N**S**N**RA NSS]RS**S**	**SSS**[**FS**S**S**RA SSS]RS**G**	P2, P4 variation	ubiquitous	RNA helicase	(229)
DBY	HY/E^k	REEA[LHQFR SG**RK**]PI	REEA[LHQFR SG**KS**]PI	P8, P9 variation	ubiquitous	RNA helicase	(229)

minor HAgs, minor histocompatibility alloantigens; P, position in the amino acid sequence of the peptide; PΩ: carboxy-terminus of the peptide; np: not published; ***bold italics***: anchor residues; **bold underlined**: amino acid variation between allelic variants; bracket: core epitope. Adapted from (3)

Table 3. Human major histocompatibility complex class I and class II-restricted HY minor histocompatibility alloantigens

Gene	Minor HAg/MHC restriction	Variant A	Variant B	Cause(s) of incompatibility	Tissue distribution	Protein function	Reference
Human (HLA) class I-restricted							
Smcy	B∗0702	S***P***SVDKA**R**AE***L***	S***P***SVDKA**Q**AE***L***	P8 variation	ubiquious	lysine demethylase	(230)
Smcy	A∗0201	*nd*	F***I***DSYICQ***V***	male antigen	ubiquious	lysine demethylase	(231)
Usp9y	A∗0101	I***V***D**C**LTEM***Y***	I***V***D**S**LTEM***Y***	P4 variation	ubiquious	ubiquitin specific peptidase 9	(232)
Uty	B∗0801	LP***H***N**H**TD***L***	LP***H***N**R**TD***L***	P5 variation	ubiquitous	histone demethylase	(233)
Uty	B∗6001	**R**ESEE**E**S**V**S***L***	**G**ESEE**A**S**P**S***L***	P7 variation	ubiquitous	histone demethylase	(234)
Rps4y1	B∗5201	TIRYPDP**V*****I***	TIRYPDP**L*****I***	P8 variation	ubiquitous	ribosomal protein S4	(235)
Tmsb4y	A∗3303	nd	E***V***LLRPGLHF***R***	male antigen	ubiquitous	thymosin beta 4	(236)
Human (HLA) class II-restricted							
Dby	DQ5	P[HIE**S**FSDI**VE**GE]	P[HIE**N**FSDI**DM**GE]	P4, P9, P10 variation	ubiquitous	RNA helicase	(237)
Dby	DRB1∗1501	**A**STASKGRYIPPHLRN**K**EA	**G**STASKGRYIPPHLRN**R**EA	not known	ubiquitous	RNA helicase	(238)
Rps4y1	DRB3∗0301	**L**IKVNDT**I**QI	**V**IKVNDT**V**QI	P1, P8 variation	ubiquitous	ribosomal protein S4	(239)

minor HAgs, minor histocompatibility alloantigens; P, position in the amino acid sequence of the peptide; PΩ: carboxy-terminus of the peptide; nd: no described allele; ***bold italics***: anchor residues; **bold underlined**: amino acid variation between allelic variants; bracket: core epitope. Adapted from (3)

Furthermore, HY-specific CTLs have been detected in a female recipient who received a kidney from her HLA-identical brother (204). Allograft survival was significantly reduced in female recipients transplanted with male organs. This increased graft loss was seen after one year and between two and ten years, implying that HY incompatibility plays a significant role in renal allograft outcome in both acute and chronic rejection (204).

Recent studies have suggested that beside T cell mediated responses in GVHD, there were antibody responses detected in male recipients when females were donors. These antibodies were directed against proteins encoded for by genes located in the Y chromosome that give rise to minor HAgs. These antibodies are associated with chronic GVHD (240). The human DBY protein causes both B and T cell responses (238, 241). Nonetheless, the presence of antibodies directed against RPS4Y1, another protein encoded by a gene located in the Y chromosome, and/or DBY are/is detected in female recipients transplanted with male allografts, which correlated with acute rejection (242, 243). However, it is still not known what role, if any the antibody responses to DBY play in GVHD.

Functional outcomes of T_H1 cell responses

T_H1 activity is exerted for the induction and maintenance of CTL in addition to other immune cells (244, 245). Moreover, T_H1 cells can also possess cytotoxic ability to directly kill infected and tumour cells (245). Taken together, the

evidence indicates that effective T_H cell help, or direct effector action is essential for anti-infection or anti-cancer immunity (245).

It is generally thought that the secondary, but not primary CTL responses depend on T_H cell help (246). However, there are reported examples that T_H cells exert their function in both cases, as discussed below. There are two major ways that T_H cells assist CTL responses: one, with the help of DCs, through the process described as “DC licensing”, and two, directly, through cytokine secretion (246, 247).

The DC licensing mechanism is considered more significant than the cytokine boost model. This model depends on CD40L expressed on T_H cells that interacts with CD40 on DCs (248). Upon interaction between the two cells, a series of intracellular events leads to an increase in DC-mediated IL-12 and IL-15 secretion that is followed by upregulation of costimulatory molecules. The resulting licensed DCs can effectively stimulate antigen-specific CTL expansion (247, 248).

This model also demonstrated that T_H cells and CTL do not need to be in direct contact with DC at the same time, because in vivo this type of random and complex interaction potentially could be difficult to achieve and maintain all the time during the induction of an adaptive immune response (249). The proposed model suggests that DCs capture antigen from necrotic or apoptotic cells, process the antigen for class II-restricted presentation to T_H cells. Upon activation, T_H cells upregulate CD40L and, through interaction with CD40, activate or license DCs to stimulate the response of naïve CTL that recognize

processed, class I-restricted antigen on the DCs (248). Thus, “educated” DCs efficiently provide costimulatory signals to CTL (249). According to this model, the encounter of naïve CTL with antigen on the surface of resting DCs in the absence of T_H cell help would not lead to priming, but to CTL tolerance. It is still not clear which factors favour CTL tolerance versus activation (250).

However, there is still some controversy why some primary CTL responses are entirely dependent on help, whereas others are not, and whether CTL responses may be independent of any provided help (250). In contrast to the clear requirement for CD4 T cell help in generating a measurable primary CTL response to non-inflammatory and allogeneic antigens, a strong primary CTL response against many infectious agents can be readily measured in animals that lack T_H cells. APCs are directly activated by the infectious agents that harbour or release TLR ligands. TLR activation then stimulates the APC to produce inflammatory signals, such as TNF-α or type 1 interferons (250, 251).

However, secondary CTL responses to microbes are either completely absent or severely diminished by the absence of CD4 T cell help during the primary response (252). The un-helped memory CTL divid poorly, made less IFN-γ and are unable to provide complete protection against the pathogen (253). Therefore, T_H cells are essential for generating long-lived, functional CTL memory responses, which can be easily triggered upon re-exposure to the same pathogen (254).

Independent studies have demonstrated that secondary CTL responses against tumour antigens also critically depend on T_H cell programming during

initial encounter with antigen. In the case of tumour and microbial antigens, T cell help is indispensable for effective memory CTL responses (254).

In contrast to agents that stimulate an inflammatory response, those that are generally non-infectious and, hence, non-inflammatory, require CD4 T cell help to generate a primary CTL response (255). Depending on the experimental model, the primary CTL response against soluble antigens as well as cellular non-infectious agents may be the best understood. For example, the primary in vivo CTL response to syngeneic spleen cells loaded with OVA requires T_H cell help (256).

An alternative model for T cell help predicates derived cytokines such as IL-2 and IFN-γ in CTL activation and differentiation. IL-2 is one of the few effector cytokines made by naive T_H cells, and is secreted for a relatively short period immediately after activation (252, 257). It is possible that IL-2 contributes to stabilization of CD40L expression in vivo, as recently suggested (258).

Summary and Unanswered Questions

Mechanisms of cross-presentation suggest extensive communication between class I and class II antigen processing pathways. During cross-presentation, exogenous antigens escape into the cytosol and become substrates for the class I processing pathway (12). However, some of these studies were limited only to model antigens and only examined presentation of overexpressed antigens (259).

The mechanisms of cross-presentation all favour shuttling of dead cell material or particulate antigens from phagosomes or endosomes to the cytoplasm where such antigens become exposed to components of the class I processing pathway (259). In this way, it may be proposed that dead cell or particulate antigens become limiting for lysosomal loading of antigenic peptides onto class II molecules.

Very few studies if any have defined the rules and factors governing indirect presentation of cytoplasmic antigens onto class II molecules (260). There are no data available that address the specific roles of the proteasomes, TAP and ERAAP on indirect presentation of cytoplasmic antigens. Furthermore, there is a significant lack of information regarding indirect presentation of cytoplasmic self, tumour or bacterial antigens.

Several cytoplasmic class II-restricted epitopes are dependent on proteasomes, and independent of autophagy (87, 260). Therefore, proteasomes could affect the resulting T_H cell TCR repertoire; however these changes were not addressed in previous research efforts.

It is not known whether TAP has any effect on indirect presentation of class II-restricted epitopes. Also, it is not known how TAP-deficiency may affect the T_H cell TCR repertoire. Current evidence based on serologic typing of select TCR beta-chains suggests that the TCR repertoire of TAP-null T_H cells is very similar to that of wild type T_H cells (261). However, it remains to be determined whether the CDR3 loops of the TCR alpha and beta chains are similar between the wild type and TAP-deficient T_H cells. Some class II epitopes are both

produced by proteasomes and transported by TAP into the endosomes (87). These distinct class II epitopes are presented by thymic epithelium and DCs and affect the process of thymic selection that would be predicted to result in changes in the T_H cell TCR repertoire. Therefore, it is of import to define the resulting T_H cell TCR repertoire in order to gain insight into the impact of the changes in the class I antigen processing pathway on peripheral T_H cell responses.

CHAPTER II

PROTEASOMES, TAP AND ERAAP CONTROL CD4$^+$ TH CELL RESPONSES BY REGULATING INDIRECT PRESENTATION OF MHC CLASS II-RESTRICTED CYTOPLASMIC ANTIGENS

Introduction

Cytoplasmic antigens 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 antigens, professional APCs acquire antigens during uptake of dead, class II-negative cells and present them via a process called indirect presentation.

It is generally assumed that the cytosolic, or class I-processing machinery—which supplies peptides for presentation by class I molecules—plays very little role in indirect presentation of class II-restricted, cytoplasmic antigens. Remarkably, upon testing this assumption, we found that proteasomes, TAP and ERAAP, but not tapasin, partially destroyed or removed cytoplasmic, class II-restricted antigens such that their inhibition or deficiency led to dramatically increased TH cell responses to cytosolic allograft (HY) and microbial (*Listeria monocytogenes*) antigens. In another set of experiments, we uncovered novel class II-restricted self-epitopes from TAP- and ERAAP-deficient APCs, which were absent from wild type cells. Such novel TAP- and ERAAP-knockout class II epitopes were found upon testing a panel of peptides derived from SV40 large T

antigen. From these findings, and the altered repertoire of class II-restricted self-peptides presented by wild type, TAP- and ERAAP-deficient cells, a novel model emerged in which the class I-processing machinery regulates the quantity, as well as quality of cytoplasmic peptides available for presentation by class II molecules, and hence modulates TH cell responses, as well as the T_H T cell receptor repertoire.

Results

Indirect presentation of HY alloantigen primes TH cells in vivo

In order to study the mechanism(s) underlying indirect presentation of cytosolic MHC class II-restricted antigens, we first determined how the male $H2A^b$-restricted HY minor HAg is presented to TH 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 (13, 229). No other $H2^b$-restricted T cell epitopes are derived from this helicase (262). Thus, female C57BL/6 (B6) and B6.129-$A^{b-/-}$ mice were immunized with $H2^b$-compatible but mHAg-incompatible (Table 5) male 129 donor splenocytes. After 7d, the ability of mHAg-reactive TH cells and CTL to produce IFN-γ was determined by ELISpot assay.

Immunisation of B6 mice resulted in IFN-γ-producing splenic TH cells to pHY but not to the control *Dbx*-encoded self HX peptide (pHX; Figure 6) expressed by both males and females. This response was specific because pHY did not elicit any IFN-γ response from immune B6.129-$A^{b-/-}$ mice (Figure 6).

Moreover, female B6 mice immunized with male 129-$A^{b-/-}$ splenocytes also primed pHY-reactive T_H cells (Figure 6). Therefore, we conclude that the $H2A^b$-restricted HY antigen 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 IFN-γ-producing CTL responses to the immunodominant $H2K^b$-restricted, H60 and $H4^b$ alloantigens but not to control $H2K^b$-restricted, SV40 T-Ag (TAg) epitope-IV (epi-IV) in B6 mice immunized with either male 129 or 129-$A^{b-/-}$ splenocytes (Figure 6). Nonetheless, B6.129-$A^{b-/-}$ recipients did not elicit CTL responses to class I-restricted pH60 and $pH4^b$ (Figure 7a). Furthermore, T_H and CTL responses were similar to those described above using Ii-deficient recipients (Figure 8). Recipient B6.129-$Ii^{-/-}$ mice immunized with 129 splenocytes did not elicit any T_H response to HY alloantigen. CTL response to the $H2K^b$-restricted, H60 and $H4^b$ alloantigens, akin to B6.129-$A^{b-/-}$ recipients, was lost (Figure 8). These data together suggest that the primary CTL response to mHAgs is entirely dependent on CD4 T cell help.

Indirect presentation of pHY requires CD8α^+ dendritic cells

Because *Dby* is broadly expressed (263), 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

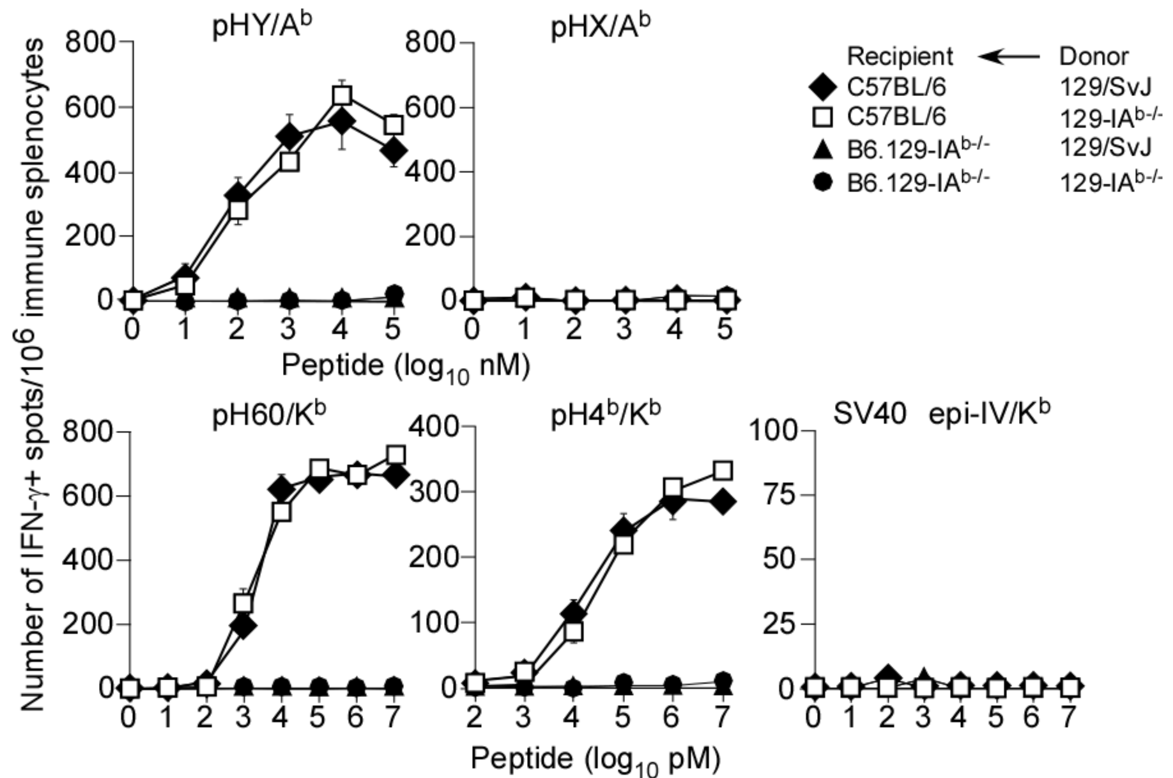


Figure 6. Indirect presentation of the male HY alloantigen. B6 and B6.129-A$^{b-/-}$ female mice were immunised with either male donor 129/SvJ or 129-A$^{b-/-}$ splenocytes. After 7d, IFN-γ response by TH cells to pHY/A^b or negative control pHX/A^b was assessed by ex vivo ELISpot assay. At the same time, IFN-γ 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).

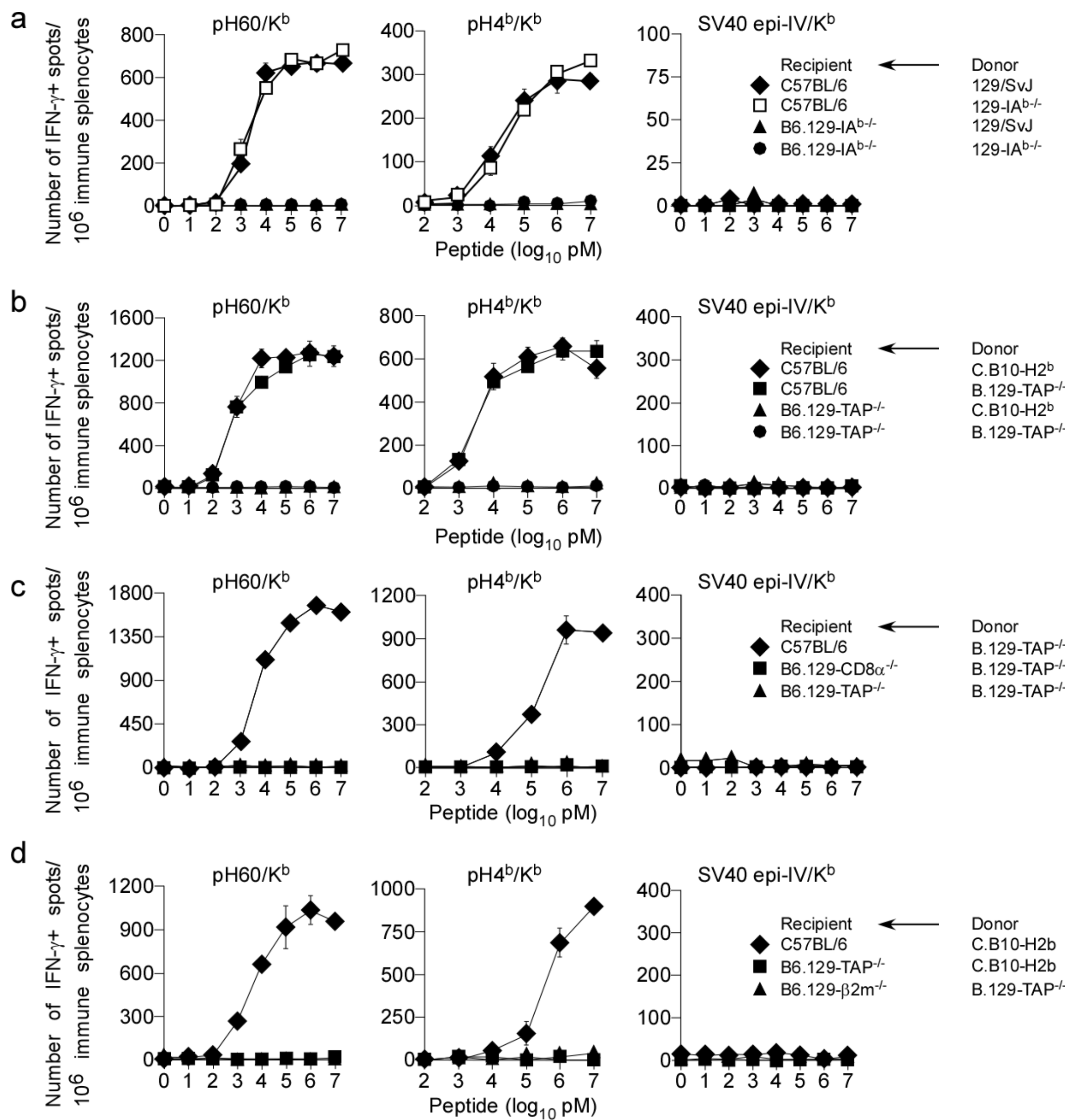

a
b
c
d
pH60/K^b
pH4^b/K^b
SV40 epi-IV/K^b
Number of IFN-γ+ spots/
10^6 immune splenocytes
Peptide (log10 pM)
Recipient
Donor
C57BL/6
129/SvJ
129-IA^b-/-
B6.129-IA^b-/-
C.B10-H2^b
B.129-TAP^-/-
B6.129-TAP^-/-
B6.129-CD8α^-/-
C.B10-H2b
B6.129-β2m^-/-

Figure 7. Cross-presented class I-restricted H60 and $H4^b$ alloantigens require $CD4^+$ T cell help. (a) B6 and B6.129-$A^{b-/-}$ female mice were immunised with either male donor 129/SvJ or 129-$A^{b-/-}$ splenocytes. After 7d, IFNγ response by CTL to pH60/K^b, pH4^b/K^b and negative control SV40 epi IV/K^b was assessed by ex vivo ELISpot assay. Data represent 6 similar experiments using ~4 recipient mice per group per experiment; ±sem (standard error of mean). **(b)** Female B6 and B6.129-$TAP^{-/-}$ female mice were immunized with male donor C.B10-$H2^b$ (BALB.B) or B.129-$TAP^{-/-}$ splenocytes. Seven days later, IFN-γ response by CTL to p60/K^b, pH4^b/K^b and SV40 epi-IV/K^b was determined by ex vivo ELIspot assay. Data represent 8 similar experiments using ~2—3 recipient mice per group per experiment; ± sem. **(c)** Female B6, B6.129 $CD8\alpha^{-/-}$ and B6.129-$TAP^{-/-}$ mice were immunized with male donor B.129-$TAP^{-/-}$ splenocytes. Seven days later, IFN-γ response by CTL to p60/K^b, pH4^b/K^b, and SV40 epi-IV/K^b was determined by ex vivo ELIspot assay. Data represents 4 similar experiments using ~3 recipient mice per group per experiment; ±sem. **(d)** Female B6 and B6.129-$TAP^{-/-}$ mice were immunized with male donor B.129-$TAP^{-/-}$ splenocytes, while B6.129-$\beta 2m^{-/-}$ female mice were immunized with male donor B.129-$TAP^{-/-}$ splenocytes. IFN-γ response by CTL to p60/K^b, pH4^b/K^b, and SV40 epi-IV/K^b was assessed 7 days later as above. Data represents 3 similar experiments using ~3 recipient mice per group per experiment; ±sem.

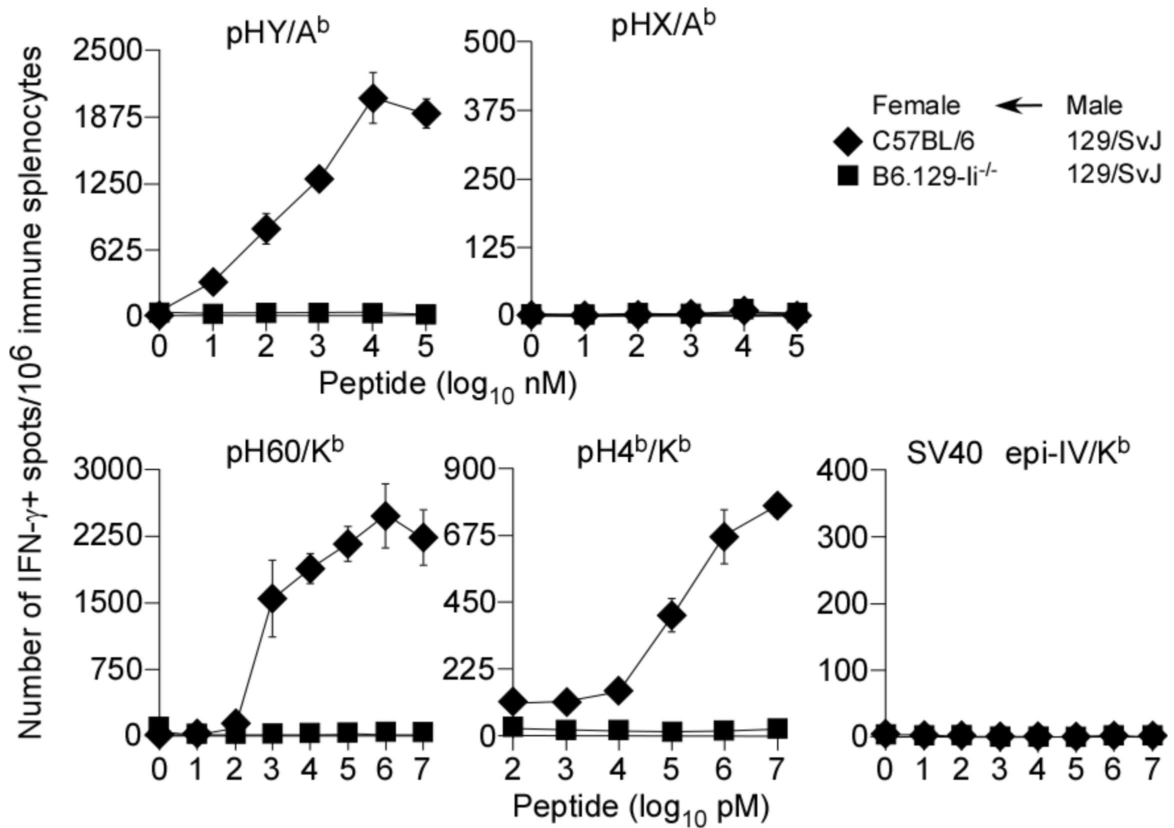


Figure 8. Cross-presented class I-restricted H60 and $H4^b$ alloantigens require $CD4^+$ T cell help. Female B6 and B6.129-$Ii^{-/-}$ female mice were immunized with male donor 129/SvJ splenocytes. Seven days later, IFN-γ response by T_H cells to pHY/A^b and to pHX/A^b and by CTL to p60/K^b, $pH4^b/K^b$, and SV40 epi-IV/K^b was determined as above. Data represent 3 similar experiments using ~3 recipient mice per group per experiment; ±sem

(264). Thus, DT administration renders hDTR^{tg} mice conditionally deficient in CD11c^{+} myeloid cells including DCs and splenic sub-capsular macrophages (264, 265). We previously reported that DT-treated B6.FVB-hDTR^{tg} mice became DC-deficient within ~18hrs and remained so for 72hrs (266).

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 TH cell responses were monitored. Depletion of recipients' CD11c^{+} cells dramatically tempered TH cell responses to pHY compared to that observed in mice containing CD11c^{+} cells (Figure 9a). Similarly, depletion of donor CD11c^{+} cells resulted in poor TH cell responses to pHY (Figure 9b) 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 TH cell response to pHY (Figure 9a).

Additional data revealed that both donor and recipient CD11c^{+} cells were required to mediate cross-presentation of class I-restricted pH60 and pH4^{b} in vivo (Figure 10). Depletion of recipient, and both recipient and donor CD11c^{+} cells greatly tempered CTL responses to class I-restricted H60 and H4^{b} (Figure 10a). Depletion of donor CD11c^{+} cells reduced the CTL response to half of the wild type response to H60 and H4^{b} (Figure 10b). 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.

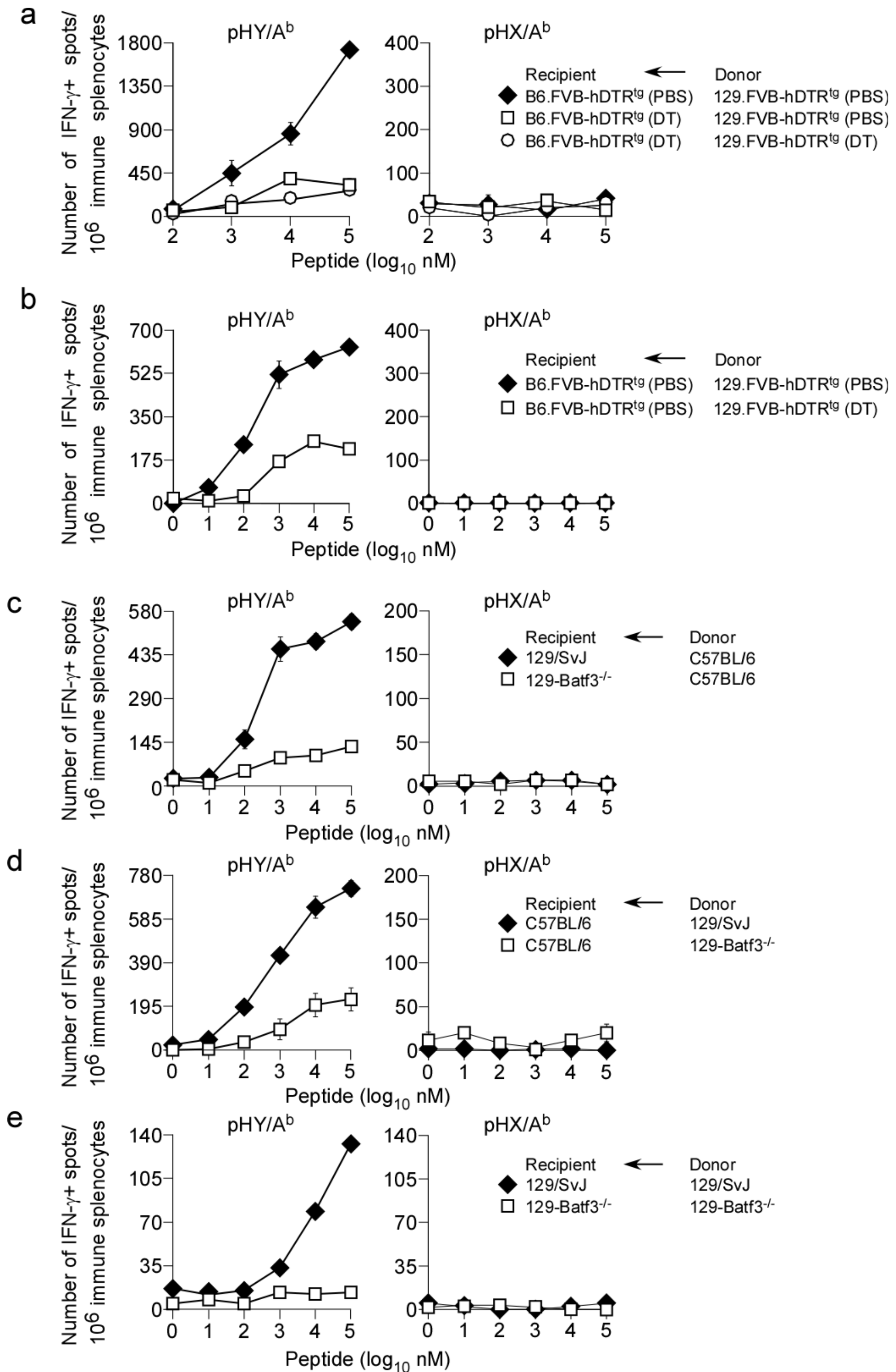

a
pHY/A^b
pHX/A^b
Number of IFN-γ+ spots/ 10^6 immune splenocytes
Recipient
Donor
B6.FVB-hDTR^tg (PBS)
129.FVB-hDTR^tg (PBS)
B6.FVB-hDTR^tg (DT)
129.FVB-hDTR^tg (PBS)
B6.FVB-hDTR^tg (DT)
129.FVB-hDTR^tg (DT)
Peptide (log10 nM)
b
pHY/A^b
pHX/A^b
Recipient
Donor
B6.FVB-hDTR^tg (PBS)
129.FVB-hDTR^tg (PBS)
B6.FVB-hDTR^tg (PBS)
129.FVB-hDTR^tg (DT)
Peptide (log10 nM)
c
pHY/A^b
pHX/A^b
Recipient
Donor
129/SvJ
C57BL/6
129-Batf3-/-
C57BL/6
Peptide (log10 nM)
d
pHY/A^b
pHX/A^b
Recipient
Donor
C57BL/6
129/SvJ
C57BL/6
129-Batf3-/-
Peptide (log10 nM)
e
pHY/A^b
pHX/A^b
Recipient
Donor
129/SvJ
129/SvJ
129-Batf3-/-
129-Batf3-/-
Peptide (log10 nM)

Figure 9. Indirect presentation of the class II-restricted HY alloantigen requires CD8α^+ 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, IFN-γ 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, IFN-γ 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$^{-/-}$ female recipients were immunized with male C57BL/6 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. **(d)** B6 female recipients were immunized with male 129/SvJ or 129-Batf3$^{-/-}$ 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. **(e)** 129/SvJ and 129-Batf3$^{-/-}$ female recipients were immunized with either male 129/SvJ or 129-Batf3$^{-/-}$ 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.

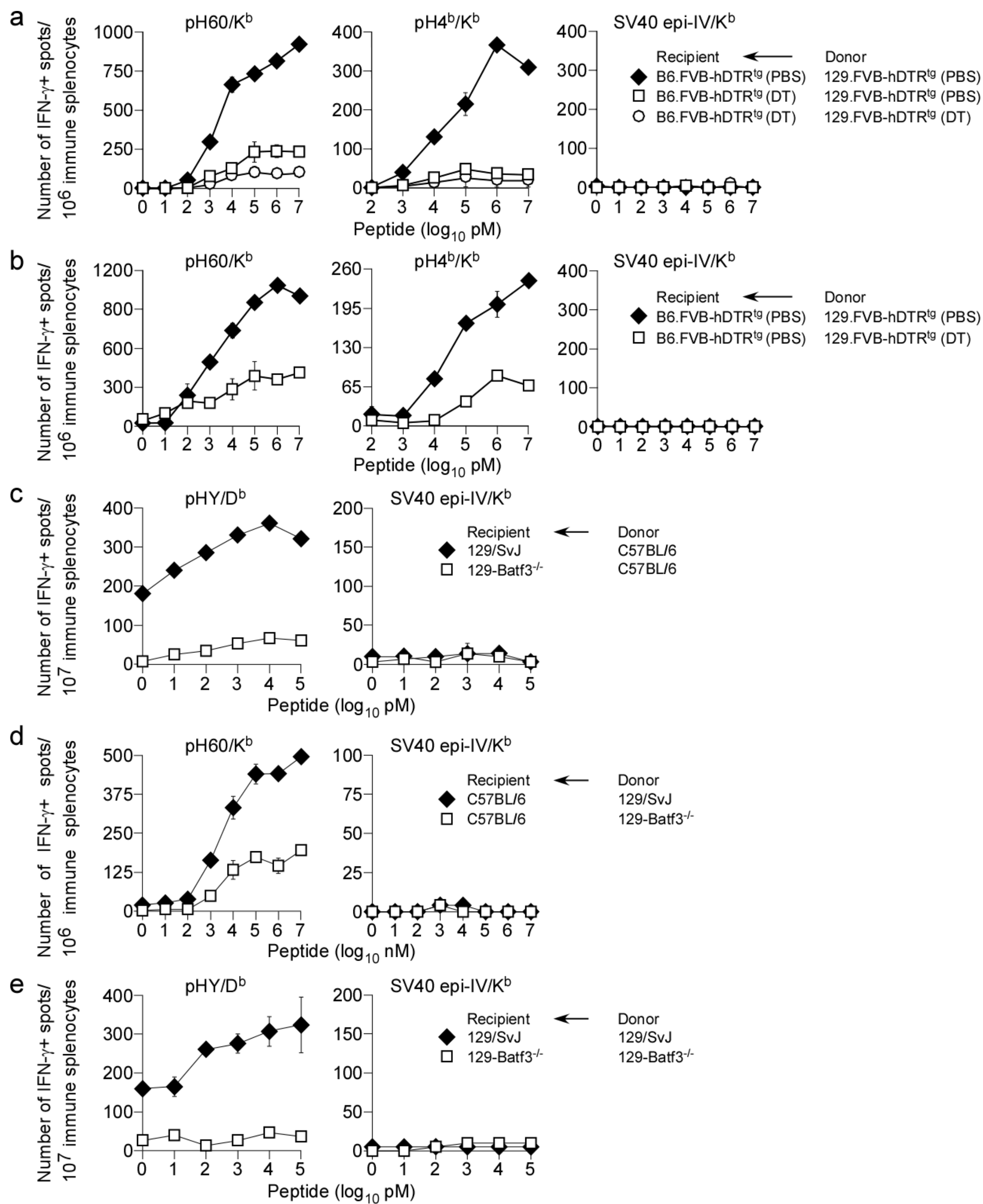

a
pH60/K^b
pH4^b/K^b
SV40 epi-IV/K^b
Number of IFN-γ+ spots/ 10^6 immune splenocytes
Peptide (log10 pM)
Recipient
Donor
B6.FVB-hDTR^tg (PBS)
129.FVB-hDTR^tg (PBS)
B6.FVB-hDTR^tg (DT)
129.FVB-hDTR^tg (PBS)
B6.FVB-hDTR^tg (DT)
129.FVB-hDTR^tg (DT)
b
pH60/K^b
pH4^b/K^b
SV40 epi-IV/K^b
Number of IFN-γ+ spots/ 10^6 immune splenocytes
Peptide (log10 pM)
Recipient
Donor
B6.FVB-hDTR^tg (PBS)
129.FVB-hDTR^tg (PBS)
B6.FVB-hDTR^tg (PBS)
129.FVB-hDTR^tg (DT)
c
pHY/D^b
SV40 epi-IV/K^b
Number of IFN-γ+ spots/ 10^7 immune splenocytes
Peptide (log10 pM)
Recipient
Donor
129/SvJ
C57BL/6
129-Batf3^-/-
C57BL/6
d
pH60/K^b
SV40 epi-IV/K^b
Number of IFN-γ+ spots/ 10^6 immune splenocytes
Peptide (log10 nM)
Recipient
Donor
C57BL/6
129/SvJ
C57BL/6
129-Batf3^-/-
e
pHY/D^b
SV40 epi-IV/K^b
Number of IFN-γ+ spots/ 10^7 immune splenocytes
Peptide (log10 pM)
Recipient
Donor
129/SvJ
129/SvJ
129-Batf3^-/-
129-Batf3^-/-

Figure 10. Cross-presentation of the class I-restricted H60 and $H4^b$ alloantigens depends on $CD8\alpha^+$ 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 7 days, IFNγ response by CTL cells to p60/K^b, $pH4^b$/K^b, and SV40 epi-IV/K^b was determined by ex vivo ELISpot assay. Data represent 6 similar experiments using ~2 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 7 days, IFNγ response by CTL cells to p60/K^b, $pH4^b$/K^b, and SV40 epi-IV/K^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-$Batf3^{-/-}$ female recipients were immunized with male C57BL/6 donor splenocytes. 7 days later, IFN-γ response by CTL to pHY/D^b, and SV40 epi-IV/K^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-$Batf3^{-/-}$ donor splenocytes. 7 days later, IFN-γ response by CTL to p60/K^b, and SV40 epi-IV/K^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-$Batf3^{-/-}$ female recipients were immunized with either male 129/SvJ or 129-$Batf3^{-/-}$ donor splenocytes. 7 days later, IFN-γ response by CTL to pHY/D^b, and SV40 epi-IV/K^b was determined by ex vivo ELIspot assay. Data represents 2 similar experiments using ~2—4 recipient per group per experiment; ± sem.

To firm the contribution of DCs in indirect presentation and to determine which DC subset is responsible, we used the recently reported 129-Batf3$^{-/-}$ mice which are deficient in splenic CD8α^+ DCs (267). Female 129 and 129-Batf3$^{-/-}$ mice were immunized with male B6, 129 or 129-Batf3$^{-/-}$ splenocytes and the HY-specific T_H cell response was monitored 7d later. The data revealed that the lack of splenic CD8α^+ DCs in the recipient dramatically reduced the T_H cell response to HY (Figure 9c). Similarly, the lack of donor CD8α^+ DCs resulted in much tempered T_H cell response to HY (Figure 9d), which was completely lost upon immunising CD8α^+ DC-deficient female recipients with male donor splenocytes lacking CD8α^+ DCs (Figure 9e). We also monitored pH60-specific CTL responses in the experiment described above. The data revealed a requirement for donor and recipient CD8α^+ DCs in cross-priming CTL responses to pH60, pH4^b and pHY (Figure 10c-e). Together, these data suggest that CD8α^+ DC play important roles in donating and indirectly presenting the HY alloantigen.

Upon DC activation in recipient mice, these cells secrete many cytokines and upregulate activation markers. DC mainly upregulate CD40, CD80 and CD86 as well as secrete IL-12, IL-2 and other cytokines. We sought to determine the role for IL-12 by immunizing B6 and B6.129-IL-12$^{-/-}$ mice with 129 donor splenocytes. The lack of IL-12 in recipients resulted in tempered T_H responses to HY (Figure 11). Therefore, this cytokine plays a role in establishing full T_H cell responses.

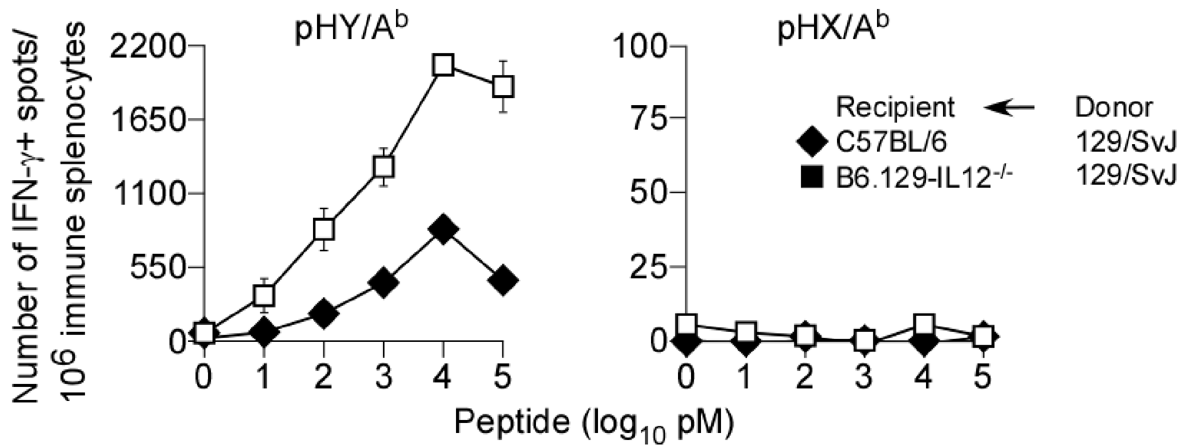


Figure 11. Recipient DC-derived IL-12 affects T_H cell response to class II-restricted HY alloantigen. Female B6 and B6.129-IL12$^{-/-}$ female mice were immunized with male donor 129/SvJ splenocytes. Seven days later, IFN-γ response by T_H cells to pHY/A^b and to pHX/A^b was determined as above. Data represent 3 similar experiments using ~3 recipient mice per group per experiment; ±sem

TAP and ERAAP regulate TH cell responses to pHY

Because pHY is derived from nucleo-cytoplasmic RNA helicase, we predicted that components of the class I processing 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 5) or B.129-$TAP^{-/-}$ (B stands for BALB.B) mice generated comparable pHY-specific TH cell responses (Figure 12a, b). Similarly, B6.129-$TAP^{-/-}$ female mice immunised with C.B10-$H2^b$ male splenocytes elicited comparable pHY-specific TH cell responses (Figure 12a, b). Surprisingly, however, when B6.129-$TAP^{-/-}$ female recipients were immunised with B.129-$TAP^{-/-}$ male donor splenocytes, 2—3-fold increased TH cell response against $H2A^b$-restricted pHY was observed (Figure 12a). Thus, TAP function in both donor and recipient cells had a detrimental effect on the indirect presentation of class II-restricted cytoplasmic antigen 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 unavailable for presentation. To test this possibility, B6, B6.129-$TAP^{-/-}$ and B6.129-$ERAAP^{-/-}$ female mice were immunised with C.B10-$H2^b$, B.129-$TAP^{-/-}$ or 129-$ERAAP^{-/-}$ male splenocytes. As with B6 and B6.129-$TAP^{-/-}$ mice, B6.129-$ERAAP^{-/-}$ female mice immunised with wild type male splenocytes elicited similar pHY-specific TH cell responses (Figure 12b—d). In striking contrast, immunisation of B6.129-

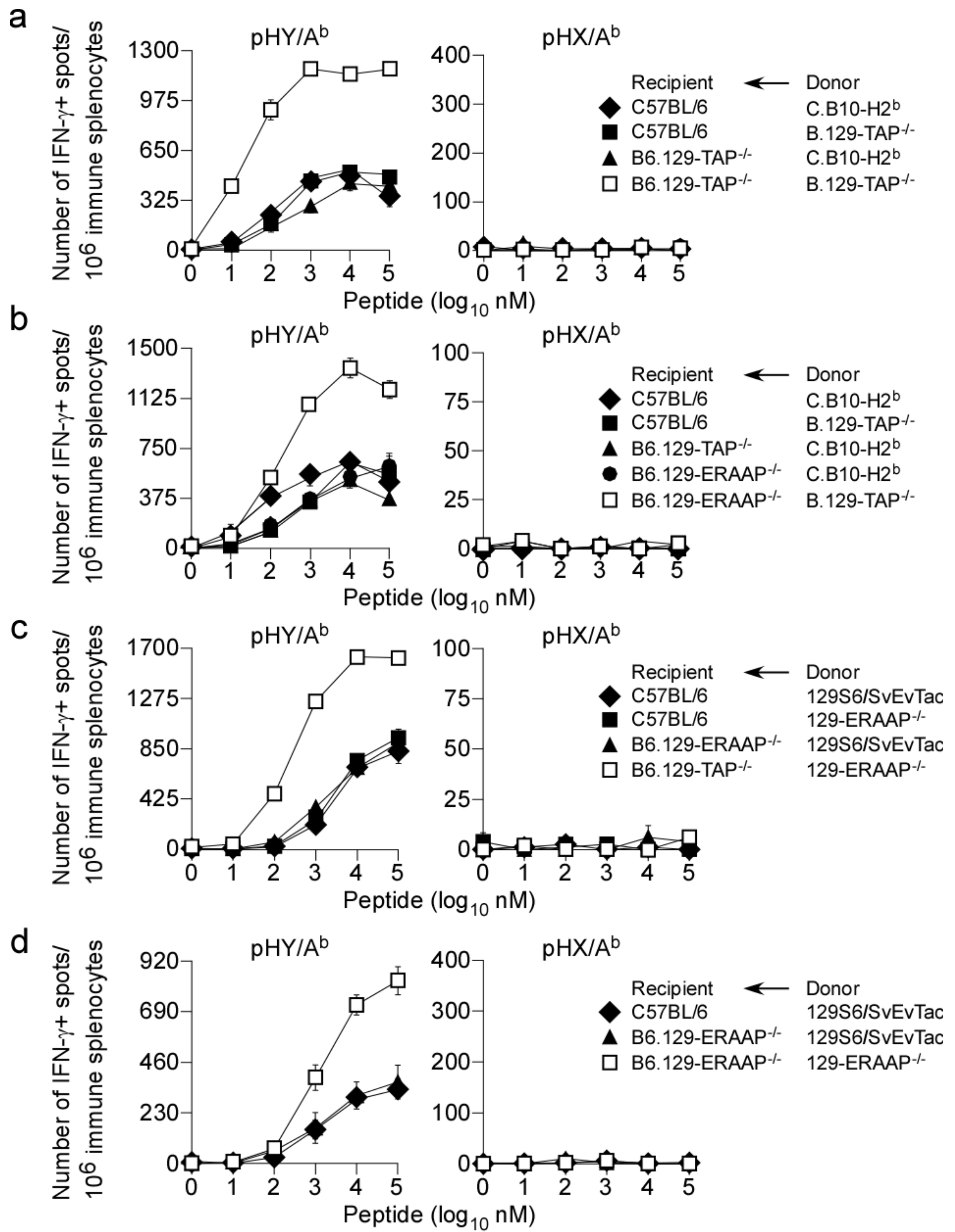

a
b
c
d
pHY/A^b
pHX/A^b
Number of IFN-γ+ spots/
10^6 immune splenocytes
Peptide (log10 nM)
Recipient
Donor
C57BL/6
B6.129-TAP-/-
B6.129-ERAAP-/-
C.B10-H2^b
B.129-TAP-/-
129S6/SvEvTac
129-ERAAP-/-

Figure 12. ERAAP and TAP impact indirect presentation of class II-restricted HY alloantigen. (a) B6 and B6.129-$TAP^{-/-}$ female mice were immunised with either male donor C.B10-$H2^b$ or B.129-$TAP^{-/-}$ splenocytes. IFN-γ 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-$TAP^{-/-}$ and B6.129-$ERAAP^{-/-}$ female mice were immunised with either male donor C.B10-$H2^b$ or B.129-$TAP^{-/-}$ splenocytes and IFN-γ 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-$TAP^{-/-}$ and B6.129-$ERAAP^{-/-}$ female mice were immunised with either male donor 129S6/SvEvTac or 129-$ERAAP^{-/-}$ splenocytes. IFN-γ 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-$ERAAP^{-/-}$ female mice were immunized with male donor 129S6/SvEvTac or 129-$ERAAP^{-/-}$ 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 represent 3 similar experiments using ~2—3 recipient mice per group per experiment; ±sem.

ERAAP$^{-/-}$ female mice with B.129-TAP$^{-/-}$ male splenocytes resulted in two-fold increases of pHY-specific T_H cell responses (Figure 12b). Similarly, immunisation of B6.129-TAP$^{-/-}$ or B6.129-ERAAP$^{-/-}$ mice with 129-ERAAP$^{-/-}$ male splenocytes resulted in a 2—3-fold increase in pHY-specific T_H cell response (Figure 12c, d).

As a control for the above experiments, monitoring of CTL response in wild type mice immunised with male wild type 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 (Figure 7b). As expected, TAP-deficient recipient mice did not respond to class I-restricted mHAgs as they are devoid of CD8$^+$ T cells (Figure 7b). We, therefore, conclude that a pool of cytoplasmic class II-restricted antigens is pumped into the ER by TAP, thence destroyed by ERAAP in both donor and recipient cells.

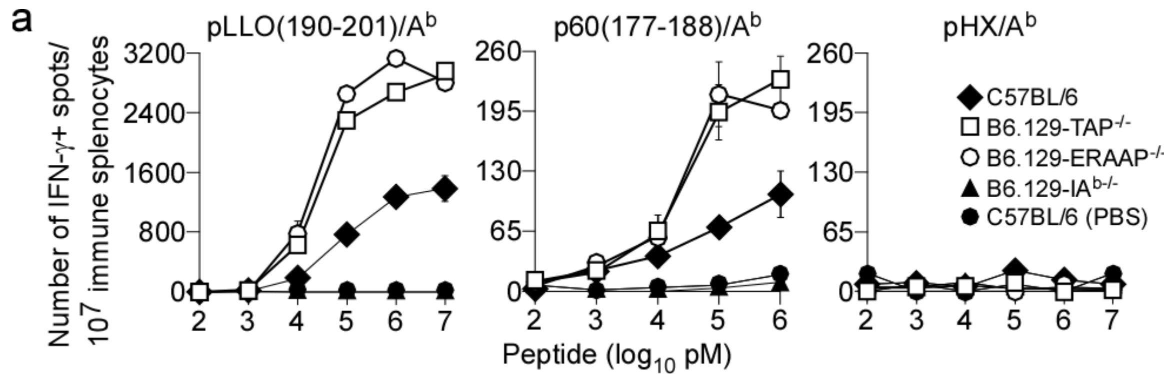
TAP and ERAAP regulate indirect presentation of class II-restricted bacterial antigens in vivo

To determine the generality of TAP's and ERAAP's role in indirect antigen presentation, we tested whether the class I pathway impacts indirect presentation of *L. monocytogenes*-derived class II-restricted antigens. *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 antigens requires indirect presentation (83, 147, 268). Thus, B6, B6.129-TAP$^{-/-}$, B6.129-ERAAP$^{-/-}$ and B6.129-A$^{b-/-}$ as well as 129S6/SvEvTac, 129-ERAAP$^{-/-}$, B6.129-A$^{b-/-}$ and 129-A$^{b-/-}$ mice were inoculated

i.p. with bacteria, boosted 14d later and secondary TH cell responses to known $H2A^b$-restricted epitopes were monitored after an additional 14d. PBS-treated B6 and 129 mice served as negative controls.

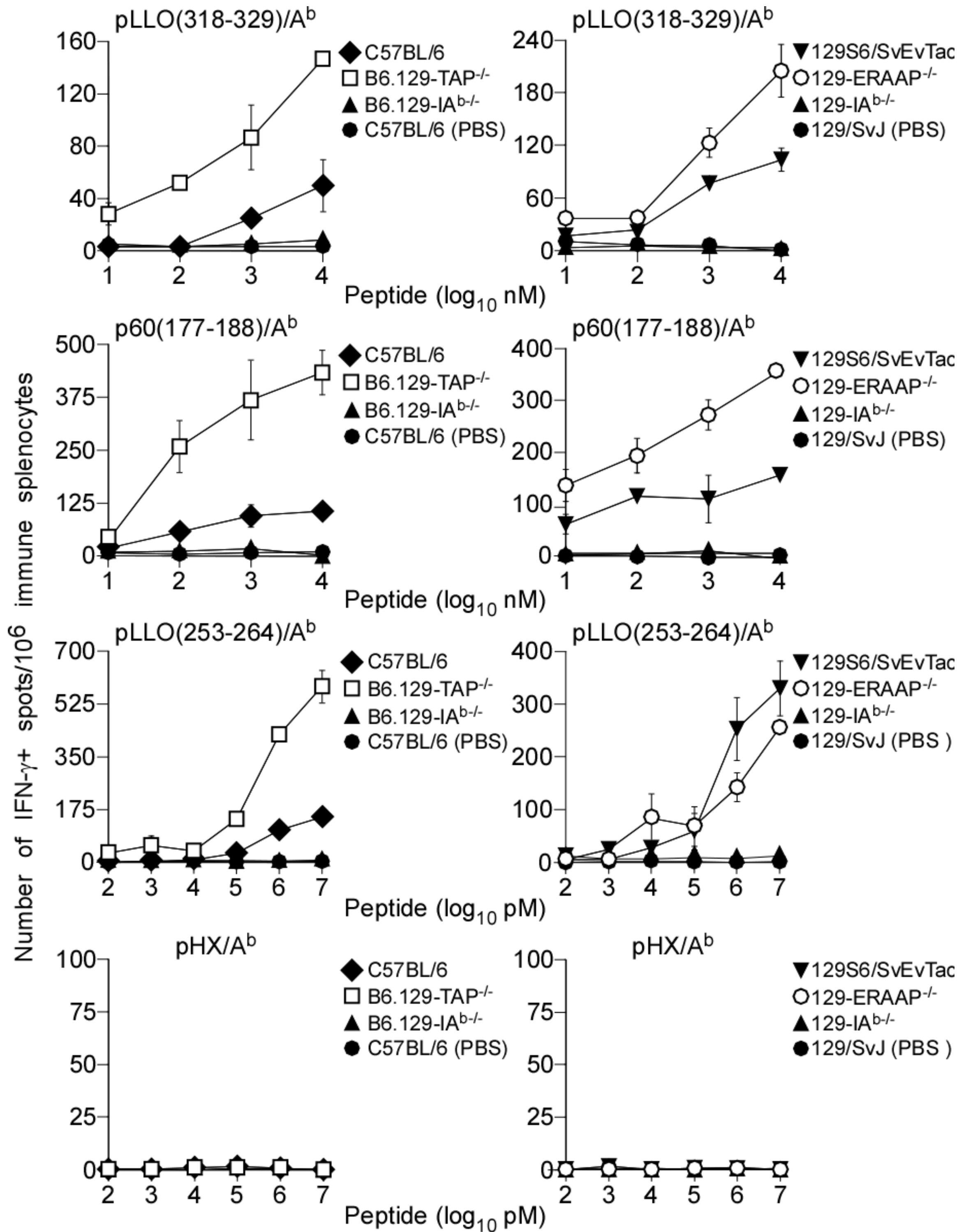
We observed a 2—5-fold increase in the secondary TH cell response to $H2A^b$-restricted pLLO(190-201), p60(177-188), pLLO(318-329), and pLLO(253-264) in B6.129-$TAP^{-/-}$ mice compared to B6 mice (Figure 13a, b). A similar pattern of increased TH cell reactivity to pLLO(190-201), p60(177-188) and pLLO(318-329) was observed in ERAAP-deficient mice compared to B6 mice (Figure 13a, b).

In contrast, the response to pLLO(253-264) was indistinguishable between wild type and ERAAP-deficient mice (Figure 13b). As expected, neither *Listeria*-inoculated $H2A^{b-/-}$ nor PBS-treated wild type mice responded to the four listerial peptides; none of the mice responded to irrelevant peptides (Figure 13). In additional experiments, we also found that the primary TH response to *L. monocytogenes* antigens—elicited by retro-orbital bacterial inoculation—yielded similar results as above (Figure 14). The primary TH cell response to $H2A^b$-restricted pLLO(318-329), p60(177-188), and pLLO(253-264) in B.129-$TAP^{-/-}$ mice was greater in comparison to wild type mice (Figure 14). Also, the T_H cell response to $H2A^b$-restricted pLLO(318-329), p60(177-188) in ERAAP-deficient mice was increased compared to wild type mice, except for pLLO(253-264) where, once again, there was no difference between 129 and 129-$ERAAP^{-/-}$ mice (Figure 14).


a
pLLO(190-201)/A^b
p60(177-188)/A^b
pHX/A^b
Number of IFN-γ+ spots/ 10^7 immune splenocytes
3200
2400
1600
800
0
260
195
130
65
0
2 3 4 5 6 7
2 3 4 5 6
Peptide (log10 pM)
C57BL/6
B6.129-TAP-/-
B6.129-ERAAP-/-
B6.129-IAb-/-
C57BL/6 (PBS)

b
pLLO(318-329)/A^b
C57BL/6
B6.129-TAP-/-
B6.129-IAb-/-
C57BL/6 (PBS)
pLLO(318-329)/A^b
129S6/SvEvTac
129-ERAAP-/-
129-IAb-/-
129/SvJ (PBS)
400
300
200
100
0
1600
1200
800
400
0
1 2 3 4
Peptide (log10 nM)
pLLO(253-264)/A^b
480
360
240
120
0
pLLO(253-264)/A^b
300
225
150
75
0
2 3 4 5 6 7
Peptide (log10 pM)
pHX/A^b
pHX/A^b
Number of IFN-γ+ spots/10^7 immune splenocytes
Peptide (log10 pM)

Figure 13. TAP and ERAAP regulate class II-restricted listerial antigen presentation. (a) B6, B6.129-TAP$^{-/-}$, B6.129-ERAAP$^{-/-}$ or B6-129-A$^{b-/-}$ 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$^{-/-}$, 129-ERAAP$^{-/-}$ or 129-A$^{b-/-}$ 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.



pLLO(318-329)/A^b
C57BL/6
B6.129-TAP-/-
B6.129-IAb-/-
C57BL/6 (PBS)
129S6/SvEvTac
129-ERAAP-/-
129-IAb-/-
129/SvJ (PBS)
Peptide (log10 nM)
p60(177-188)/A^b
pLLO(253-264)/A^b
Peptide (log10 pM)
pHX/A^b
Number of IFN-γ+ spots/10^6 immune splenocytes

Figure 14. TAP and ERAAP impact quantitative aspects of primary TH cell response to *L. monocytogenes*-derived class II-restricted epitopes. B6, B6.129-TAP$^{-/-}$ and B6.129-A$^{b-/-}$ or 129S6/SvEvTac, 129-ERAAP$^{-/-}$, and 129-A$^{b-/-}$ mice were inoculated i.v. with ~5x10^{4} cfu *Lm* or administered PBS. On d14, IFN-γ response by TH cells to pLLO(318-329), p60(177-188), pLLO(253-264) and pHX was assessed by ELIspot assay. Data represents 2 similar experiments using 3—4 mice/group; ± sem.

Thus, the TAP and ERAAP effect on indirect presentation of cytoplasmic class II-restricted antigens appears to be a general principle as they impact TH cell responses to mHAgs and bacterial antigens 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 antigen; (b) competition between $CD4^+$ and $CD8^+$ T cells; (c) enhanced autophagy and/or enhanced ER-associated degradation (ERAD); and (d) quantitative differences in the antigen(s) presented.

To test whether competition for antigen played a role, the TH 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-$TAP^{-/-}$ splenocytes. All three recipients elicited similar pHY-specific TH cell responses (Figure 15a), suggesting that simply lacking class I does not 'free up' more cytoplasmic antigens for presentation by class II molecules. In conjunction with the fact that no known CTL epitopes are derived from *Dby*-encoded helicase (262), competition for antigen is a less likely explanation for our finding.

To ascertain whether the increased TH 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-$TAP^{-/-}$ male splenocytes. The resulting TH cell response to pHY was comparable in both wild type and $CD8^+$ T cell-deficient mice (Figure 15b).

As expected, female B6.129-β2m$^{-/-}$ and B6.129-CD8α$^{-/-}$ recipients did not mount any IFN-γ response to class I-restricted mHAgs (Figure 7c, d). 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 and β2m deficiency enhances ERAD (269). ERAD can enhance autophagy (270), which is required for class II-restricted cytoplasmic antigen presentation (18, 20, 21, 25, 271). 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 the T_H response to pHY whilst DMSO-treated donor cells responded as expected (Figure 15c). Similarly, induction of autophagy—by maintaining donor male splenocytes in nutrition-free conditions prior to immunisation of female B6 mice—did not enhance, but instead abrogated the T_H response to pHY (Figure 15d). Additionally, constitutive autophagy was not enhanced in TAP$^{-/-}$ (TAPTAg) or β2m$^{-/-}$ (β2mTAg) TAg-SV40 large T antigen transformed fibroblast lines compared to similarly transformed wild type fibroblasts (wtTAg; (272) as similar levels of LC3-I and LC3-II were detected in immunoblots of proteins extracted from wild type and mutant lines (Figure 15e). Next, autophagy was inhibited in donor cells with the help of the pharmacological inhibitor 3-MA for ~4 hours. Absence of autophagy in donor cells increased the T_H cell response to HY alloantigen in recipients (Figure 16). Interestingly, the T_H response was slightly increased compared to vehicle-treated donor cells, suggesting that reduced

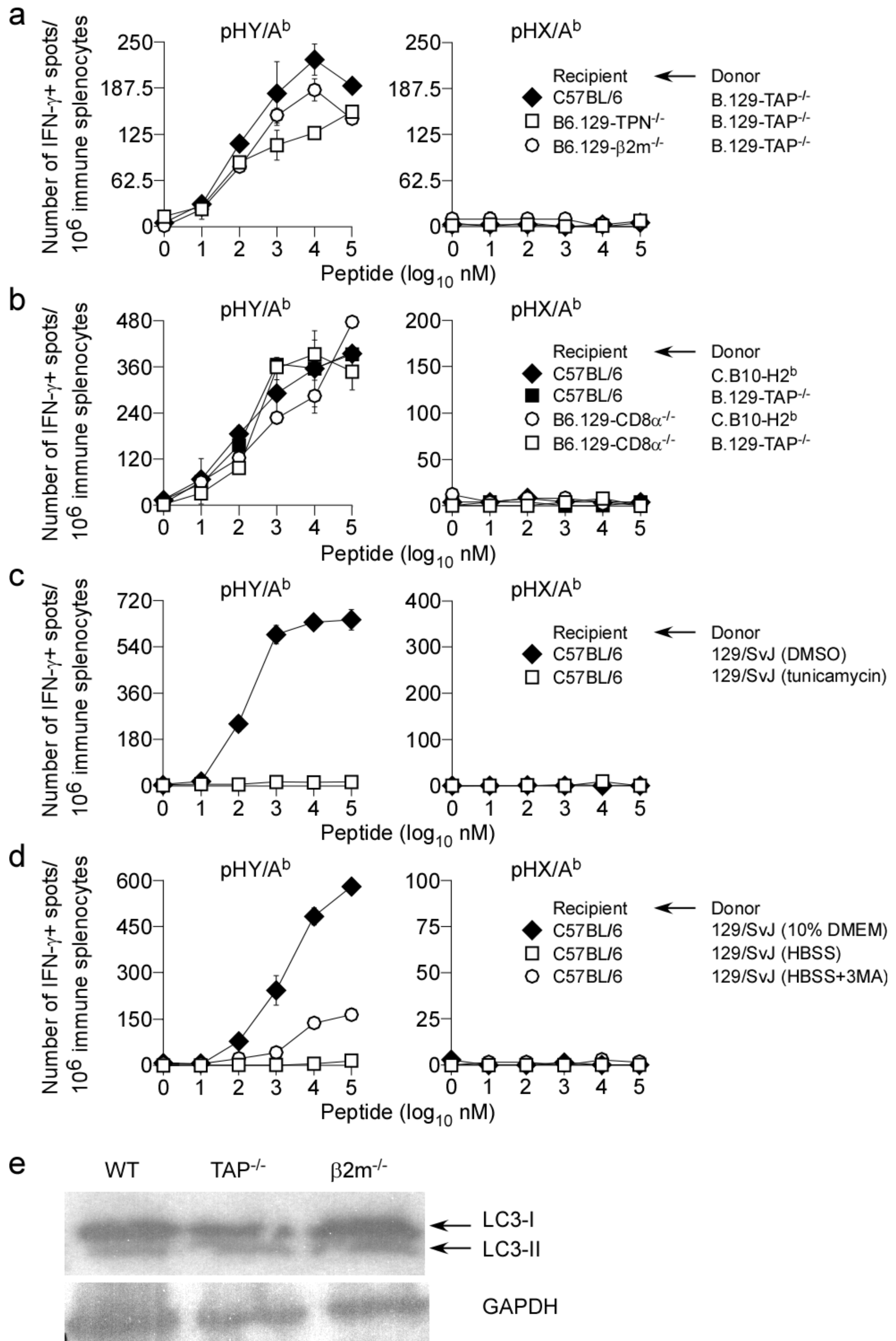

a
pHY/A^b
pHX/A^b
Number of IFN-γ+ spots/
10^6 immune splenocytes
Recipient ← Donor
C57BL/6 B.129-TAP-/-
B6.129-TPN-/- B.129-TAP-/-
B6.129-β2m-/- B.129-TAP-/-
Peptide (log10 nM)
b
C57BL/6 C.B10-H2b
C57BL/6 B.129-TAP-/-
B6.129-CD8α-/- C.B10-H2b
B6.129-CD8α-/- B.129-TAP-/-
c
C57BL/6 129/SvJ (DMSO)
C57BL/6 129/SvJ (tunicamycin)
d
C57BL/6 129/SvJ (10% DMEM)
C57BL/6 129/SvJ (HBSS)
C57BL/6 129/SvJ (HBSS+3MA)
e
WT
TAP-/-
β2m-/-
LC3-I
LC3-II
GAPDH

Figure 15. Neither antigen competition, T cell competition, ERAD nor enhanced autophagy explain increased TH cell response to pHY in $TAP^{-/-}$ mice. (a) B6, B6.129-$TPN^{-/-}$ and B6.129-$\beta2m^{-/-}$ female mice were immunised with male donor B.129-$TAP^{-/-}$ 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-$CD8\alpha^{-/-}$ female mice were immunised with either male donor C.B10-$H2^b$, or B.129-$TAP^{-/-}$ 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 TH 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 TH 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 wild type, $TAP^{-/-}$ and $\beta2m^{-/-}$ kidney fibroblast lines were separated by SDS-PAGE, transferred onto PVDF membrane and probed with LC3- and GADPH-specific mAbs.

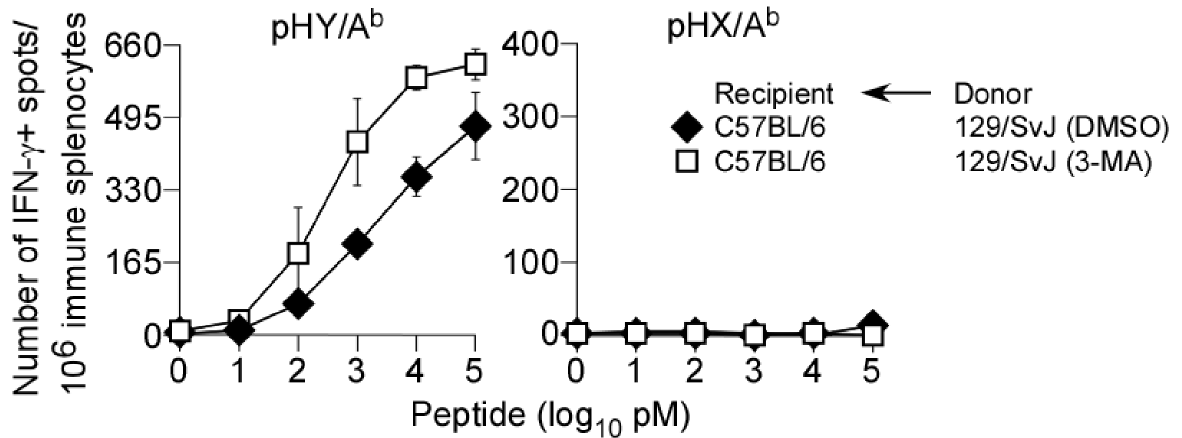


Figure 16. Inhibition of macroautophagy in donor does not negatively affect indirect presentation of class II-restricted HY alloantigen. Female B6 mice were immunised with male donor 129 splenocytes incubated in either with 3-MA or vehicle (DMSO) for 4hrs and IFN-γ response by TH cells to pHY/A^b and pHX/A^b was assessed 7d later as above. Data represent 1 experiment using 6 recipient mice; ±sem.

autophagy led to a slightly increased T_H cell response to indirect presentation of class II HY. Together, these data discount a role for enhanced ERAD and autophagy in explaining the impact of TAP and ERAAP on indirect presentation of cytosolic antigens. If anything, the data argue that, if autophagy is enhanced by TAP or ERAAP deficiency, it would destroy and not protect cytoplasmic antigens for indirect presentation by class II molecules.

As proteasomal degradation is enhanced by ERAD (270), we tested whether proteolysis within the cytosol of donor cells impacted indirect antigen presentation. If enhanced ERAD was the cause for the phenotype then proteasome inhibition should abrogate T_H cell responses 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 (273, 274) 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 (Figure 17a).

Thus, proteasomes negatively impact indirect presentation and the intact form of the HY alloantigen is perhaps donated to recipient $CD8\alpha^+$ DCs for indirect presentation. Contrary to this, proteasomal inhibition of donor cells negatively affected CTL responses to class I-restricted H60 and $H4^b$, suggesting that

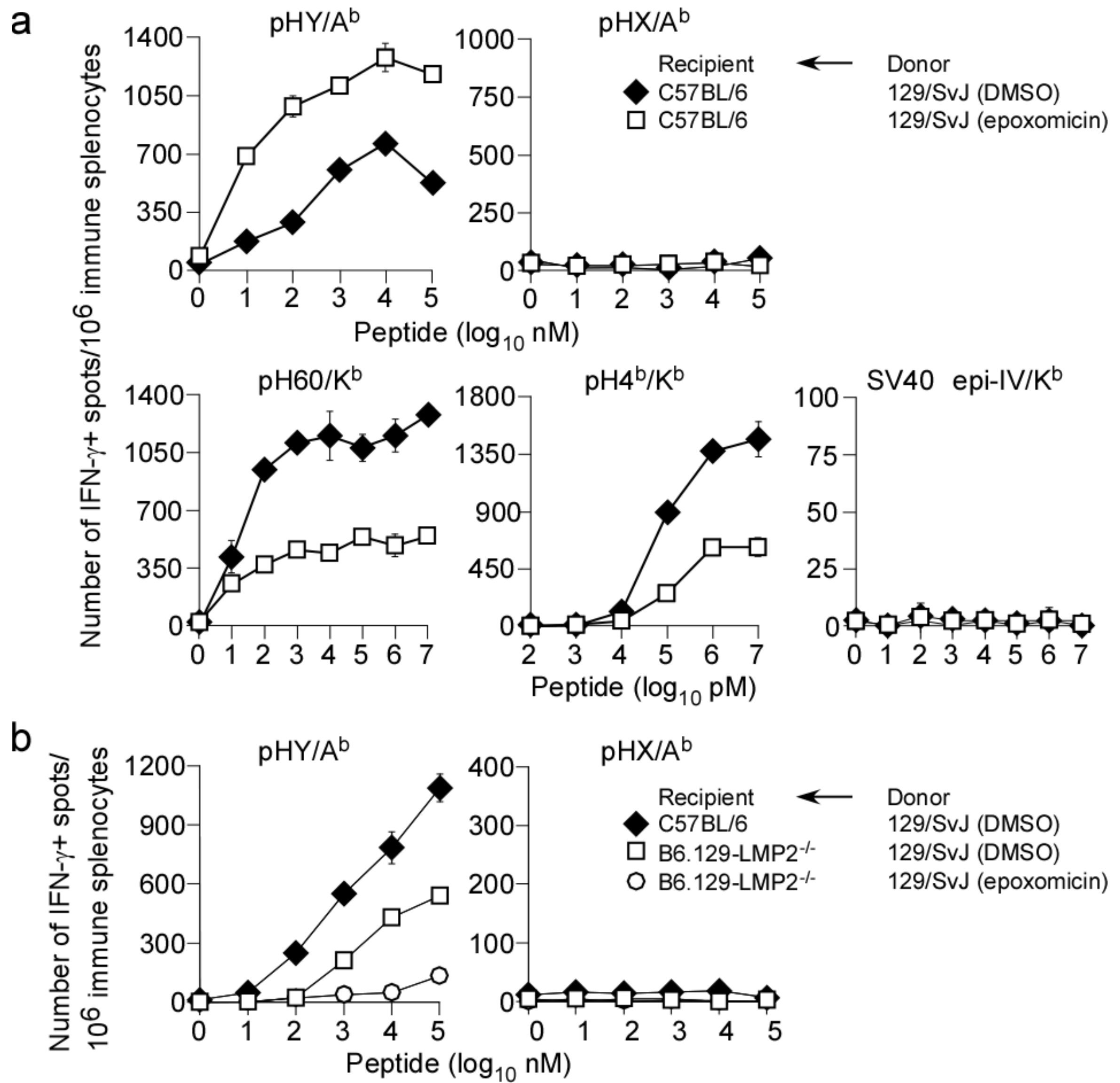

a
pHY/A^b
pHX/A^b
Recipient
Donor
C57BL/6
129/SvJ (DMSO)
C57BL/6
129/SvJ (epoxomicin)
Number of IFN-γ+ spots/10^6 immune splenocytes
Peptide (log10 nM)
pH60/K^b
pH4^b/K^b
SV40 epi-IV/K^b
Peptide (log10 pM)
b
pHY/A^b
pHX/A^b
Recipient
Donor
C57BL/6
129/SvJ (DMSO)
B6.129-LMP2-/-
129/SvJ (DMSO)
B6.129-LMP2-/-
129/SvJ (epoxomicin)
Number of IFN-γ+ spots/ 10^6 immune splenocytes
Peptide (log10 nM)

Figure 17. 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, IFN-γ 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-$LMP2^{-/-}$ female mice were immunised with extensively washed male donor 129/SvJ splenocytes. After 7d, IFN-γ 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.

perhaps proteasomal product is donated to recipient CD8α^+ DCs for cross-presentation (Figure 17a).

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 a tempered T_H response to pHY/A^b (Figure 17b). 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 animals (Figure 17b).

We wanted to test whether antigen in donor cells is affected by lysosomal processing and whether donated intact antigen exists in the cytoplasm of recipient cells using DCs deficient in the NOX2 component gp91^{PHOX}. NOX2 activation slows the kinetics of pH lowering during the course of endo/phagosomal maturation. Altered pH balance of the phagolysosome caused by a deficiency in donor and/or recipient gp91^{PHOX} did not affect T_H cell responses to pHY/A^b (Figure 18). These data suggest that the increased donation of intact HY antigen upon proteasomal inhibition of donor cells requires cytosolic processing within the recipient DCs.

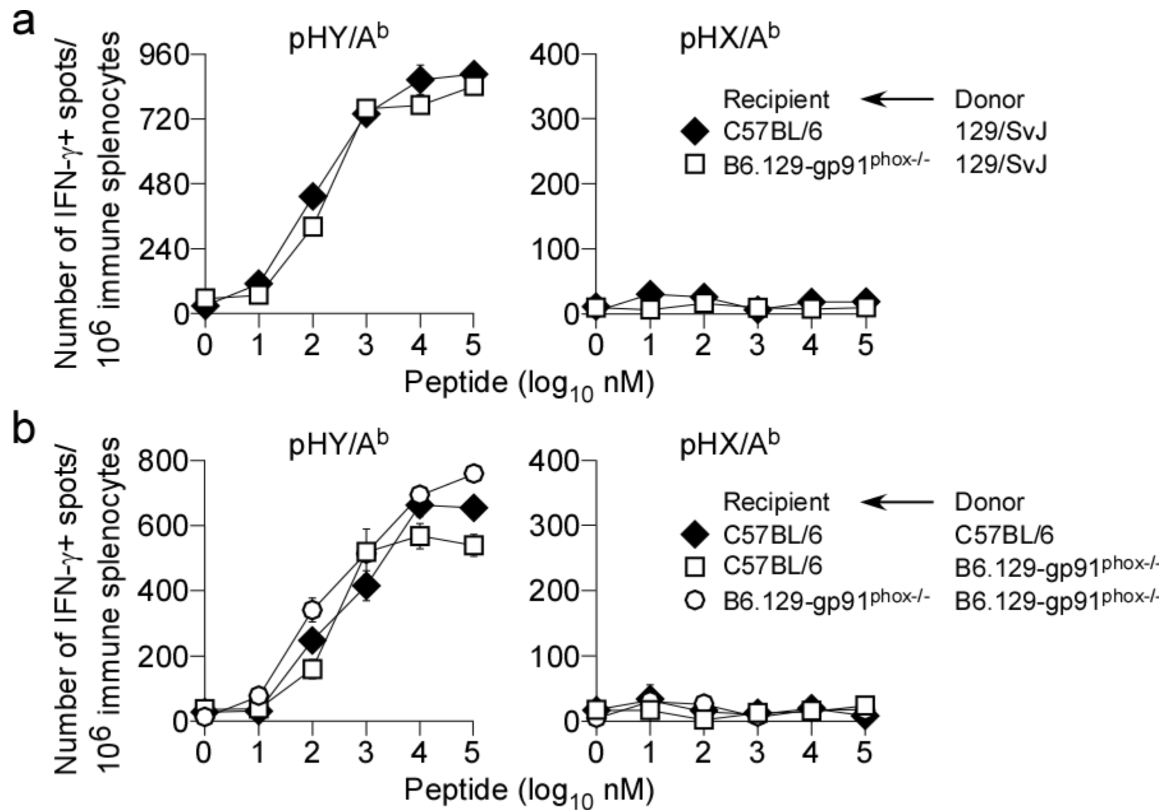


Figure 18. Donor lysosomes and recipient phagosomes do not participate in indirect presentation of the class II-restricted pHY alloantigen. (a) Female B6 and B6.129-gp91$^{PHOX-/-}$ mice were immunized with male 129/SvJ splenocytes. After 7d, IFN-γ response T_H cells to pHY/A^b and pHX/A^b was assessed by ex vivo ELISpot assay. Data represent 2 similar experiments using 3 recipient mice per group per experiment; ±sem. **(b)** Female B6 and B6.129-gp91$^{PHOX-/-}$ mice were immunized with either male B6 or B6.129-gp91$^{PHOX-/-}$ splenocytes. After 7d, IFN-γ 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.

Role for chaperones in indirect presentation of HY alloantigen

Cross-presentation of class I-restricted antigens requires heat shock proteins (82, 275). Because the HY alloantigen is a nucleo-cytoplasmic protein that is degraded by donor proteasomes (Figure 17), 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 2hrs and used to immunise B6 mice. Inhibition of HSP90 with either geldanamycin or KNK437 tempered T_H cell responses against pHY (Figure 19a). This result suggested a role for HSP90 in donor cells for 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 (276). We first generated $HY^{+}Hsf1^{-/-}$ and wild type MEF by *Dby* cDNA transfer because these cells do not otherwise express HY mHAg (Figure 19b). Immunisation of B6 mice with $HY^{+}Hsf1^{-/-}$ MEF resulted in tempered T_H cell responses to HY compared to mice immunized with HY^{+} wild type MEF (Figure 19b). These results imply a critical role for HSP90 in efficient indirect presentation of the HY alloantigen.

Calreticulin (CRT), an ER-resident chaperone, has been implicated in cross-presentation of class I-restricted antigens (277). Therefore, we determined

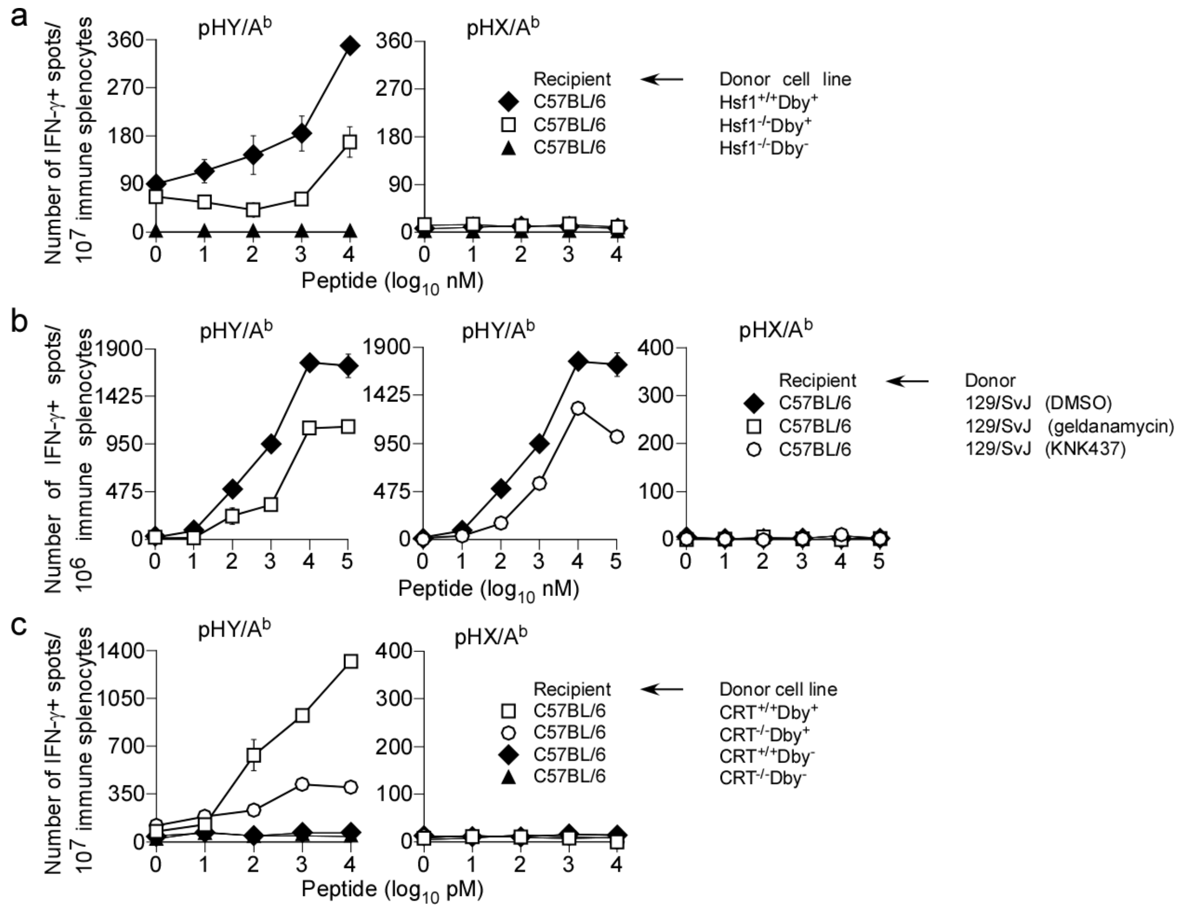

a
pHY/A^b
pHX/A^b
Number of IFN-γ+ spots/ 10^7 immune splenocytes
Recipient ← Donor cell line
C57BL/6 Hsf1+/+Dby+
C57BL/6 Hsf1-/-Dby+
C57BL/6 Hsf1-/-Dby-
Peptide (log10 nM)
b
pHY/A^b
pHY/A^b
pHX/A^b
Number of IFN-γ+ spots/ 10^6 immune splenocytes
Recipient ← Donor
C57BL/6 129/SvJ (DMSO)
C57BL/6 129/SvJ (geldanamycin)
C57BL/6 129/SvJ (KNK437)
Peptide (log10 nM)
c
pHY/A^b
pHX/A^b
Number of IFN-γ+ spots/ 10^7 immune splenocytes
Recipient ← Donor cell line
C57BL/6 CRT+/+Dby+
C57BL/6 CRT-/-Dby+
C57BL/6 CRT+/+Dby-
C57BL/6 CRT-/-Dby-
Peptide (log10 pM)

Figure 19. Donor HSP90, HSP70 and calreticulin facilitate indirect presentation of class II-restricted HY alloantigen. (a) B6 female mice were immunised with $Hsf1^{-/-}Dby^{+}$, $Hsf1^{+}Dby^{+}$ or $Hsf1^{-/-}Dby^{-}$ MEF. After 7d, IFN-γ 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, IFN-γ 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 $CRT^{+}Dby^{+}$, $CRT^{-/-}Dby^{+}$, $CRT^{+}Dby^{-}$ or $CRT^{-/-}Dby^{-}$ MEF. After 7d, IFN-γ response by TH 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.

whether CRT expression by donor APC was essential for indirect presentation of HY alloantigen.

For this, we first generated $HY^{+}CRT^{-/-}$ and $HY^{+}CRT^{+}$ MEF by *Dby* cDNA transfer. Immunisation of B6 mice with $HY^{+}CRT^{-/-}$ MEF resulted in tempered TH cell responses to HY compared to mice immunized with $HY^{+}CRT^{+}$ MEF (Figure 19c). These results imply a critical role for CRT in efficient indirect presentation of the HY alloantigen.

TAP regulates the quantity of class II-restricted antigens displayed

To test the idea that TAP and ERAAP regulate the quantitative aspects of class II-restricted antigen presentation, we determined the response of two distinct $H3b^{a}$ mHAg-specific TH 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, GJ Christianson and DC Roopenian, 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 antigen 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-$TAP^{-/-}$, C.B10-$H2^{b}$, B.129-$TAP^{-/-}$ or 129/SvJ splenocytes for 48 hrs and the number of IFN-γ^{+} spots determined. The data revealed that B6.129-$TAP^{-/-}$ splenocytes, compared to B6 splenocytes, induced 7—8-fold greater number of IFN-γ^{+} spots from the two $H3b^{a}$-specific TH clones (Figure 20).

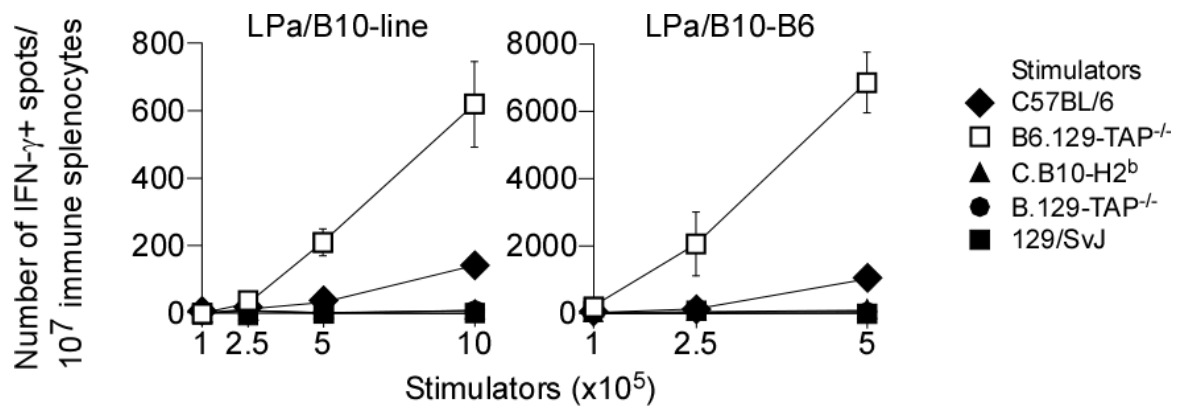


Figure 20. TAP affects the quantitative aspects of the direct presentation of class II-restricted H3b^{a} mHAg. H3b^{a}-specific T helper clones LPa/B10-line and LPa/B10-B6 were directly stimulated with the indicated numbers of B6, B6.129-TAP$^{-/-}$, C.B10-H2^{b}, B.129-TAP$^{-/-}$ or 129/SvJ splenocytes. After 48hrs, IFN-γ response by the TH clones was determined by ELISpot assay. Data represent 2 similar experiments; ±sem.

This response was antigen-specific because the two T_H cell clones did not respond to negative control C.B10-H2^b, B.129-TAP$^{-/-}$ and 129/SvJ splenocytes (Figure 20). Thus, TAP and ERAAP regulate the quantity of class II-restricted antigen presentation.

TAP- and ERAAP-deficiency uncovers novel class II-restricted self and tumour antigens

The data so far indicate that TAP and ERAAP impact the quantity of class II-restricted antigens. Therefore, I reasoned that TAP may affect the quality of class II-restricted epitopes, i.e., TAP-deficiency might reveal novel class II epitopes, which are absent in wild type cells. In order to address this question, we tested SV40 large T-antigen derived peptide library consisting of ~140 15-mer peptides with 10 amino acid overlap between them. Immune T_H cells from wild type and TAP-deficient recipients were stimulated with the library of overlapping peptides. Results revealed a distinct pattern of T_H cell reactivity to wild type and TAP-deficient APC (Figure 21). This suggests that distinct peptides were recognized by T_H cells, implying that TAP can completely destroy potential class II-restricted epitopes.

We next sought to determine the extent of potential changes in class II-restricted peptide repertoire presented by wild type and TAP-deficient professional APCs. These changes were determined by the characteristics of naturally processed ligands eluted from class II molecules.

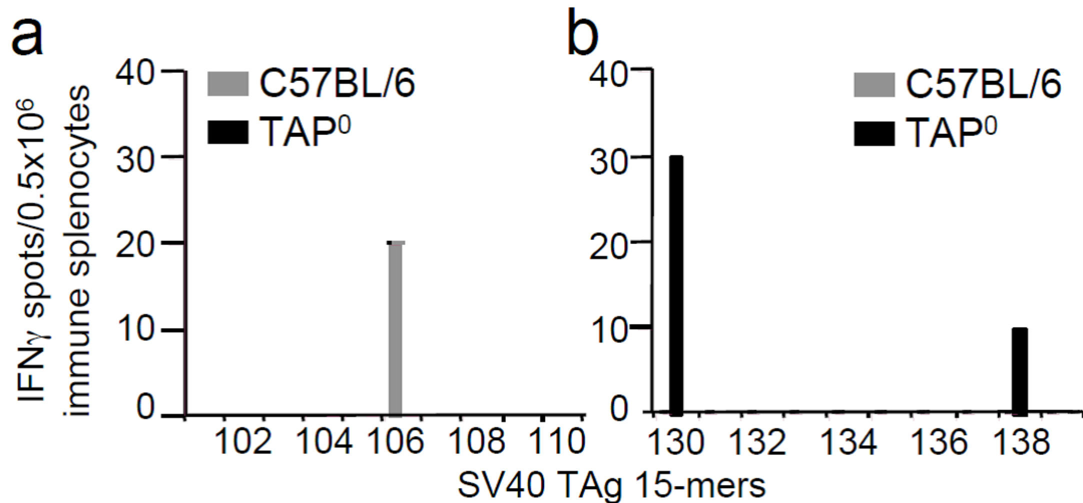


Figure 21. TAP and ERAAP deficiency unravels novel class II-restricted TH cell epitopes derived from SV40 T-antigen. (a) B6 and B6.129-TAP$^{-/-}$ mice were immunised with TAP-deficient SV40 TAg-transformed cell line TAP$^{-/-}$/WT/19. After 14 days, immune splenocytes were stimulated ex vivo for ~48 hours with B6-129-β2m$^{-/-}$ or B6-129-A$^{b-/-}$ APC pulsed with a panel of 140 15-mer peptides that overlap by 10 amino acid residues and span TAg. The resulting IFN-γ response was monitored by ELIspot assay. Data represents 5 similar experiments using 150 recipient mice; ± sem (standard error of mean). **(b)** B6 and B6.129-TAP$^{-/-}$ derived immune splenocytes resulting from immunisation with TAP$^{-/-}$/WT/19 were stimulated with B6-129-β2m$^{-/-}$ or B6-129-A$^{b-/-}$ APC pulsed with the indicated doses of peptides that activated TAg-reactive TH cells described above. The resulting IFN-γ response was monitored by ELIspot assay. Data represents 2 similar experiments using 55 recipient mice; ± sem (standard error of mean).

The eluted peptides were separated by reversed-phase HPLC. Each resulting fraction was subjected to mass spectrometric analysis in order to obtain peptide sequences. Next, peptide sequences were compared between those associated with $H2A^b$ molecules expressed by wild type, TAP- and ERAAP-deficient cells to look for potential similarities and differences. Preliminary results revealed distinct and overlapping peptides from the three samples, suggesting that TAP and ERAAP affect quality of class II-restricted peptide repertoire (data not shown). Thus, TAP and ERAAP-specific class II-restricted peptides could be recognized as non-self by TH cells derived from wild type mice.

TAP and ERAAP impact the T_H cell TCR repertoire

The differences in the self-peptide repertoire between wild type and TAP- and ERAAP-specific class II-restricted epitopes led us to ask the question whether such changes could potentially result in altered T_H TCR repertoire. Potential alterations in the T_H TCR repertoire could be a result of changes during the process of thymic positive and negative selection as these processes are controlled by the quality and quantity of self-peptides presented by the MHC molecules (278, 279).

Thus, CDR3β region diversity of purified $CD4^+$ T cells was determined by spectratyping and compared between wild type, TAP- and ERAAP-deficient $CD4^+$ T cells. CDR3 regions in the Vβ-Jβ rearrangements were either identical between the three comparators, demonstrated altered length distribution (loss or

addition of unique sizes), or showed alterations only in the ratio of individual peak heights (Figures 22 and 23).

These changes in CDR3 TCR regions indicate that TAP and ERAAP have profound effect on the T_H cell TCR repertoire. The impact of such changes in response to challenge to pathogens, tumour cells and alloantigens remains to be determined.

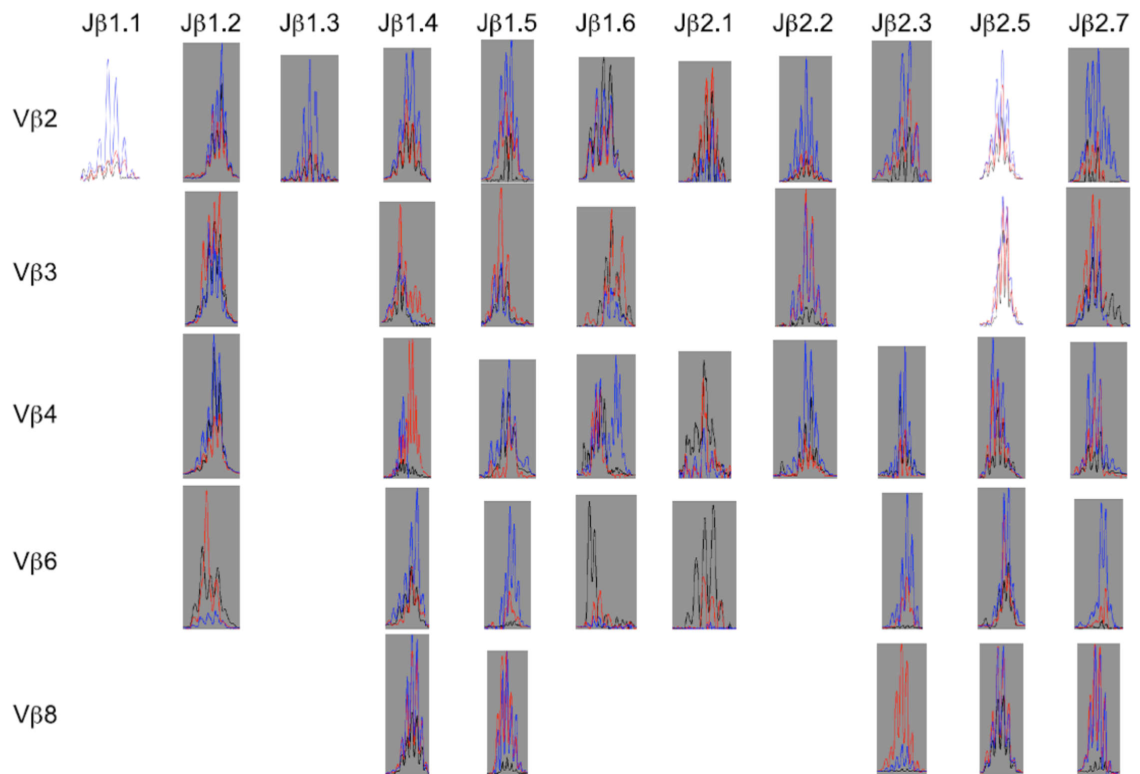


Figure 22. TAP and ERAAP deficiency alters CD4^{+} T cell repertoire. CDR3β region diversity of the indicated, randomly selected TCRs expressed by highly (MACS enriched followed by FACS purification) purified CD4^{+} T cells was analysed by spectratyping and compared between wild type, TAP- and ERAAP-deficient T cells. Electrophoretograms from one such experiment are shown. Black, wild type CD4^{+} T cells; blue, TAP$^{-/-}$ CD4^{+} T cells; red, ERAAP$^{-/-}$ CD4^{+} T cells. These electrophoretograms were used to generate the schematic representation of the different patterns of CDR3β diversity. FACS purified CD4^{+} T cells were pooled from several sorts and used for spectratyping experiment.

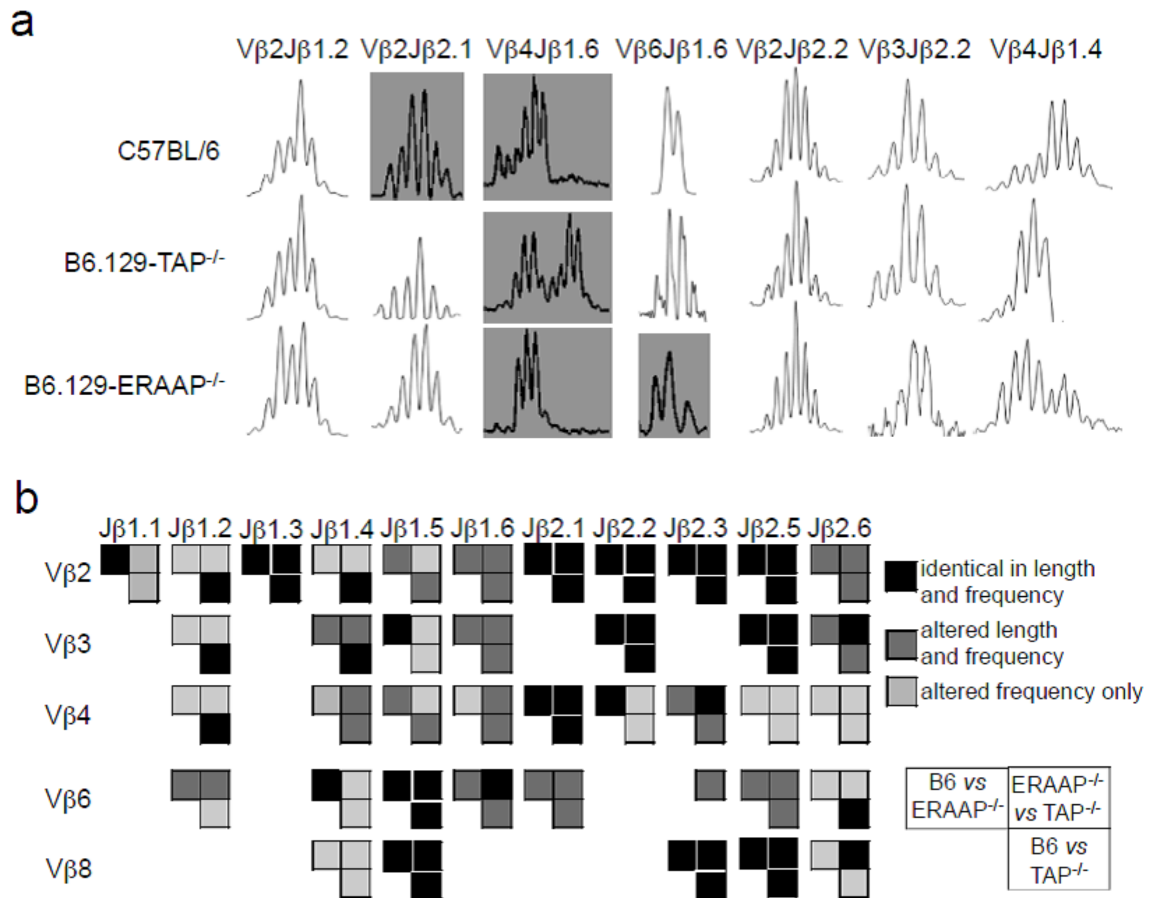


Figure 23. CDR3β regions of CD4⁺ T cell repertoire greatly differs due to TAP and ERAAP deficiency. **(a)** CDR3β region diversity of the indicated, randomly selected TCRs expressed by highly purified (MACS enriched followed by FACS purification) $CD4^+$ T cells was analysed and compared between wild type, TAP- and ERAAP-deficient T cells. Electrophoretograms representing different patterns identified in **(b)** are shown. **(b)** CDR3 regions in the indicated Vβ-Jβ rearrangements were either identical between the three comparators, demonstrated altered length distribution (loss or addition of unique sizes), or showed alterations only in the ratio of individual peak heights. These patterns were characterized for each of the Vβ-Jβ pairings analysed and represented schematically; based on data represented in Figure 22.

Discussion

We show that indirect presentation of class II-restricted antigens is mediated by $CD8\alpha^+$ donor and recipient DCs. Within these cells, proteasomes, TAP, and ERAAP, but not tapasin, significantly impact indirect presentation of class II-restricted antigens, which results in an increased T_H cell response to cytoplasmic alloantigens (HY and $H3b^a$) and bacterial (*L. monocytogenes*) antigens. Based on this, the class I-processing machinery may affect indirect presentation of cytoplasmic antigens in other cases as well- further investigation could produce new examples for such an effect. Therefore, the impact of the class I-processing pathway on indirect presentation of cytoplasmic antigens might be a general regulatory process.

Both the donor and recipient $CD8\alpha^+$ DCs need to be deficient in TAP and ERAAP in order to measure their effect on indirect presentation. Donated antigens need to escape to the cytoplasm of the recipient $CD8\alpha^+$ DC upon ingestion of allogeneic, tumour or infected cells. The resulting T_H cell response revealed not only quantitative differences in response to reported class II antigens, but our studies revealed novel SV40 large T antigen derived and self-antigens in TAP- and ERAAP-deficient professional APCs. TAP and ERAAP could lead to complete destruction of self-class II epitopes, thus leading to altered $CD4^+$ T cell selection. Such altered class II epitopes could potentially affect T_H cell responses to microbial and tumour antigens, which may be absent in wild type mice. In agreement with this, novel SV40 large T antigen class II epitopes were identified. Therefore, novel T_H cell responses could be elicited

against such antigens which may lead to increased microbial clearance and effective removal of tumour cells. Therefore, TAP and ERAAP regulate the quality and quantity of the class II-associated self (mHAgs) and non-self (listerial and TAg) peptide repertoire.

Based on changes in class II-restricted peptide repertoire, we observed altered CDR3β TCR regions in TAP- and ERAAP-deficient $CD4^+$ T cells, suggesting that TAP and ERAAP may have an effect on the T_H cell TCR repertoire. The impact of such changes in response to challenge to pathogens, tumour cells and alloantigens remains to be determined.

Inhibition of HSP90, as well as the absence of calreticulin negatively affected indirect presentation of class II-restricted antigens. Both HSP90 and calreticulin are implicated as chaperonins for the donation of cross-presented antigens to presenting APC. Therefore, HSP90 and calreticulin may work together to chaperone antigens for indirect presentation of class II-restricted antigens as well.

Material and Methods

Mice: All mouse strains, their histocompatibility genotype and sources are described in Table 4. 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 (184) as well as Hsf1-null MEF (276) were maintained in RPMI-1640 (Cellgro) supplemented with 5% foetal

calf serum (FCS; Hyclone), L-glutamine, HEPES and antibiotics (Cellgro). These MEFs were transfected with *Dby* cDNA (229) 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 (GGTCTGGAAAACTGCTGC) and reverse (TTGGTGGCATTGTGTCCTGC) primers (229).

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 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 ~2x10^{8} cells/ml and used for immunisation. In order to inhibit autophagy, donor cells were treated with 3-MA and proteasomal inhibitors for 2 hours, washed and resuspended accordingly.

Peptides: All peptides used in this study (Table 5) 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 (280). SV40 large T antigen

Table 4: List of mouse strains used in this study and their immune phenotype

Mouse strain	Immune phenotype	Source/ Reference
Recipient strains		
C57BL/6	Wild type $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	Jackson Lab
B6.129-TAP$^{-/-}$	TAP KO introgressed into B6 background; lacks cell surface class I expression; does not directly or cross present class I-restricted extracellular antigens; do not develop CD8 T lymphocytes; $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	Jackson Lab
B6.129-ERAAP$^{-/-}$	ERAAP KO introgressed into B6 background; lacks aminopeptidase that trims long peptides to 8—12 amino acid residues long ligands that bind class I molecules; $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	This study
B6.129-β2m$^{-/-}$	β2m KO introgressed into B6 background; lacks functional H2^b class I molecules; $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	Jackson Lab
B6.129-TPN$^{-/-}$	Tapasin KO introgressed into B6 background; lacks functional H2^b class I molecules; $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	(281)
B6.129-A$^{b-/-}$	H2A^b KO introgressed into B6 background; lacks H2^b class II molecules; deficient in direct and indirect presentation of class II-restricted antigens; do not develop conventional CD4 T lymphocytes; $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	Jackson Lab
B6.FVB-hDTR^{tg}	*Cd11c*-driven human diphtheria toxin receptor (hDTR) transgene introgressed into B6 background; expresses the transgene within CD11c$^+$ myeloid cells; DT treatment renders them deficient in CD11c$^+$ myeloid cells within ~18 hrs and maintains this state for ~72 hrs; $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	(266)
B6.129-CD8$\alpha^{-/-}$	Lacks CD8 T lymphocytes; $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	Jackson Lab
B6.129-IL12$^{-/-}$	No IL-12 production; $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	Jackson Lab
B6.129-gp91$^{PHOX-/-}$	NOX2 activation absent in DC; $H2^b$ $H60^{null}$ $H4^a$ $H3b^a$	Jackson Lab
129-Batf3$^{-/-}$	they lack CD8α^+ DCs; $H2^b$ $H60^b$ $H4^b$ $H3b^b$	(267)
Donor strains		
129/SvJ	Wild type $H2^b$ $H60^b$ $H4^b$ $H3b^b$	Jackson Lab
129S6/SvEvTac	Wild type $H2^b$ $H60^b$ $H4^b$ $H3b^b$	Taconic
C.B10-H2^b	Wild type BALB.B; $H2^b$ $H60^b$ $H4^b$ $H3b^b$	Jackson Lab
BALB.B.129-TAP$^{-/-}$	TAP KO introgressed into BALB.B background; lacks H2^b class I molecules; deficient in direct presentation of class I-restricted mHAg; $H2^b$ $H60^b$ $H4^b$ $H3b^b$	This study
129-ERAAP$^{-/-}$	ERAAP KO introgressed into 129/SvEvTac background; $H2^b$ $H60^b$ $H4^b$ $H3b^b$	(136)
129-A$^{b-/-}$	H2A^b KO introgressed into 129/SvJ background; deficient in direct presentation of class II-restricted mHAg; $H2^b$ $H60^b$ $H4^b$ $H3b^b$	This study
129.FVB-hDTR^{tg}	hDTR transgene introgressed into 129/SvJ background; $H2^b$ $H60^b$ $H4^b$ $H3b^b$	This study
Histocompatibility genotypes are indicated in italics		

peptide library of ~150 peptides was generated using solid-phase technology with estimated peptide purity of 70% (Mimotopes, Pepscan Systems, Australia). Each peptide is 15 amino acid residues long and there is 10 amino acid overlap between subsequent peptides (Table 6).

Immunisation and ELISpot assay: Recipient mice were immunised i.p. with $2x10^7$ 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 IFN-γ capture monoclonal antibody (mAb; AN18; eBiosciences) overnight. Excess mAb was washed and blocked with 10% FCS in RPMI-1640. Meanwhile, $2.5—3x10^5$ red blood cell-free immune splenocytes were stimulated with the indicated concentrations of peptides (see Table 6) 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 IFN-γ-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 $\sim10^5$ cells with increasing numbers of splenocytes isolated from the indicated mouse strains at 1:1; 1:2.5; 1:5; and 1:10

Table 5: List of peptides used in this study

	Antigen	H2 restriction	Epitope sequence	Reference
1	HY	A^b	NAGFNSNRANSSRSS	(229)
2	HX	A^b	SSSFSSSRASSSRSG	
3	H60	K^b	LTFNYRNL	(282)
4	$H4^a$	K^b	SGTVYIHL	(218, 283)
5	$H4^b$	K^b	^{P}SGIVYIHL	
6	SV40 epi-IV	K^b	VVYDFLKL	(272)
7	LLO(190-201)	A^b	NEKYAQAYPNVS	(268)
8	LLO(318 329)	A^b	AFDAAVSGKSVS	
9	LLO(253–264)	A^b	QIYYNVNVNEPT	
10	p60(177–188)	A^b	TTQATTPAPKVA	

ratio of responder to stimulator. After 48hrs, IFN-γ-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 $\geq$90% splenic $CD11c^+$ cells within 18hrs and remained in this state for ~72hrs (266).

***L. monocytogenes* infection:** To elicit primary $CD4^+$ T cell responses, mice were inoculated retro-orbitally with ~$5x10^4$ cfu *L. monocytogenes*. After 12—14d, the response of 0.5—$1x10^6$ 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—$1x10^6$ 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 5).

Peptide isolation and fractionation: Each group, consisting of splenocytes harvested from ~70 mice, were used as a source of class II-restricted peptides. C57BL/6, B6.129-$TAP^{-/-}$, B6.129-$ERAAP^{-/-}$ and mixed group of B6.129-$A^{b-/-}$ and 129-$A^{b-/-}$ spleens were isolated and grinded in PBS in order to prepare single-cell

suspension. Next, cells were washed and kept in Ack lysis buffer to remove RBC. Upon removal of buffer, cell pellets were lysed in buffer (50 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA, pH8) containing 2% Igepal (NP-40, Sigma), 50 mM iodoacetamide (Acros Organics), and mammalian cell protease inhibitor cocktail (Sigma) (284). In order to remove cellular and tissue debris, lysates were ultracentrifuged at 90K on 4°C for 2 hours. Lysates were next precleared overnight on 4°C with normal mouse serum (NMS) and protein A Sepharose beads (RepliGen, Waltham, MA). The following day lysates were subjected to sequential MHC class II affinity purification using separate columns packed with beads bound to: W6/32-irrelevant antibody, and class II-specific antibody (Cedarlane, Burlington, NC). Lysates were passed two times over each column. The eluates were made to 2.5 M acetic acid and incubated for 20 minutes ina boiling bath, and immediately placed on ice (285). Next, samples were centrifuged using Centricon 5 ultrafilters (Millipore). Frozen filtrates were dried to powder under vacuum and stored at -80°C. Concentrated peptides, approximately in 100-200 μL, were subjected to RP HPLC fractionation. Each collected fraction was next analysed using LC/MS/MS in order to obtain high quality spectra, which are indicative of peptide sequences.

Table 6: List of SV40 large T antigen derived peptides used in this study

Name	Amino acid sequence
Tag-1	MDKVLNREESLQLMD
Tag-2	NREESLQLMDLLGLE
Tag-3	LQLMDLLGLERSAWG
Tag-4	LLGLERSAWGNIPLM
Tag-5	RSAWGNIPLMRKAYL
Tag-6	NIPLMRKAYLKKCKE
Tag-7	RKAYLKKCKEFHPDK
Tag-8	KKCKEFHPDKGGDEE
Tag-9	FHPDKGGDEEKMKKM
Tag-10	GGDEEKMKKMNTLYK
Tag-11	KMKKMNTLYKKMEDG
Tag-12	NTLYKKMEDGVKYAH
Tag-13	KMEDGVKYAHQPDFG
Tag-14	VKYAHQPDFGGFWDA
Tag-15	QPDFGGFWDATEIPT
Tag-16	GFWDATEIPTYGTDE
Tag-17	TEIPTYGTDEWEQWW
Tag-18	YGTDEWEQWWNAFNE
Tag-19	WEQWWNAFNEENLFC
Tag-20	NAFNEENLFCSEEMP
Tag-21	ENLFCSEEMPSSDDE
Tag-22	SEEMPSSDDEATADS
Tag-23	SSDDEATADSQHSTP
Tag-24	ATADSQHSTPPKKKR
Tag-25	QHSTPPKKKRKVEDP
Tag-26	PKKKRKVEDPKDFPS
Tag-27	KVEDPKDFPSELLSF
Tag-28	KDFPSELLSFLSHAV
Tag-29	ELLSFLSHAVFSNRT
Tag-30	LSHAVFSNRTLACFA
Tag-31	FSNRTLACFAIYTTK
Tag-32	LACFAIYTTKEKAAL
Tag-33	IYTTKEKAALLYKKI
Tag-34	EKAALLYKKIMEKYS
Tag-35	LYKKIMEKYSVTFIS
Tag-36	MEKYSVTFISRHNSY
Tag-37	VTFISRHNSYNHNIL
Tag-38	RHNSYNHNILFFLTP
Tag-39	NHNILFFLTPHRHRV
Tag-40	FFLTPHRHRVSAINN
Tag-41	HRHRVSAINNYAQKL
Tag-42	SAINNYAQKLCTFSF
Tag-43	YAQKLCTFSFLICKG
Tag-44	CTFSFLICKGVNKEY
Tag-45	LICKGVNKEYLMYSA

Tag-46	VNKEYLMYSALTRDP
Tag-47	LMYSALTRDPFSVIE
Tag-48	LTRDPFSVIEESLPG
Tag-49	FSVIEESLPGGLKEH
Tag-50	ESLPGGLKEHDFNPE
Tag-51	GLKEHDFNPEEAEET
Tag-52	DFNPEEAEETKQVSW
Tag-53	EAEETKQVSWKLVTE
Tag-54	KQVSWKLVTEYAMET
Tag-55	KLVTEYAMETKCDDV
Tag-56	YAMETKCDDVLLLLG
Tag-57	KCDDVLLLLGMYLEF
Tag-58	LLLLGMYLEFQYSFE
Tag-59	MYLEFQYSFEMCLKC
Tag-60	QYSFEMCLKCIKKEQ
Tag-61	MCLKCIKKEQPSHYK
Tag-62	IKKEQPSHYKYHEKH
Tag-63	PSHYKYHEKHYANAA
Tag-64	YHEKHYANAAIFADS
Tag-65	YANAAIFADSKNQKT
Tag-66	IFADSKNQKTICQQA
Tag-67	KNQKTICQQAVDTVL
Tag-68	ICQQAVDTVLAKKRV
Tag-69	VDTVLAKKRVDSLQL
Tag-70	AKKRVDSLQLTREQM
Tag-71	DSLQLTREQMLTNRF
Tag-72	TREQMLTNRFNDLLD
Tag-73	LTNRFNDLLDRMDIM
Tag-74	NDLLDRMDIMFGSTG
Tag-75	RMDIMFGSTGSADIE
Tag-76	FGSTGSADIEEWMAG
Tag-77	SADIEEWMAGVAWLH
Tag-78	EWMAGVAWLHCLLPK
Tag-79	VAWLHCLLPKMDSVV
Tag-80	CLLPKMDSVVYDFLK
Tag-81	MDSVVYDFLKCMVYN
Tag-82	YDFLKCMVYNIPKKR
Tag-83	CMVYNIPKKRYWLFK
Tag-84	IPKKRYWLFKGPIDS
Tag-85	YWLFKGPIDSGKTTL
Tag-86	GPIDSGKTTLAAALL
Tag-87	GKTTLAAALLELCGG
Tag-88	AAALLELCGGKALNV
Tag-89	ELCGGKALNVNLPLD
Tag-90	KALNVNLPLDRLNFE
Tag-91	NLPLDRLNFELGVAI
Tag-92	RLNFELGVAIDQFLV
Tag-93	LGVAIDQFLVVFEDV
Tag-94	DQFLVVFEDVKGTGG

Tag-95	VFEDVKGTGGESRDL
Tag-96	KGTGGESRDLPSGQG
Tag-97	ESRDLPSGQGINNLD
Tag-98	PSGQGINNLDNLRDY
Tag-99	INNLDNLRDYLDGSV
Tag-100	NLRDYLDGSVKVNLE
Tag-101	LDGSVKVNLEKKHLN
Tag-102	KVNLEKKHLNKRTQI
Tag-103	KKHLNKRTQIFPPGI
Tag-104	KRTQIFPPGIVTMNE
Tag-105	FPPGIVTMNEYSVPK
Tag-106	VTMNEYSVPKTLQAR
Tag-107	YSVPKTLQARFVKQI
Tag-108	TLQARFVKQIDFRPK
Tag-109	FVKQIDFRPKDYLKH
Tag-110	DFRPKDYLKHCLERS
Tag-111	DYLKHCLERSEFLLE
Tag-112	CLERSEFLLEKRIIQ
Tag-113	EFLLEKRIIQSGIAL
Tag-114	KRIIQSGIALLLMLI
Tag-115	SGIALLLMLIWYRPV
Tag-116	LLMLIWYRPVAEFAQ
Tag-117	WYRPVAEFAQSIQSR
Tag-118	AEFAQSIQSRIVEWK
Tag-119	SIQSRIVEWKERLDK
Tag-120	IVEWKERLDKEFSLS
Tag-121	ERLDKEFSLSVYQKM
Tag-122	EFSLSVYQKMKFNVA
Tag-123	VYQKMKFNVAMGIGV
Tag-124	KFNVAMGIGVLDWLR
Tag-125	MGIGVLDWLRNSDDD
Tag-126	LDWLRNSDDDDEDSQ
Tag-127	NSDDDDEDSQENADK
Tag-128	DEDSQENADKNEDGG
Tag-129	ENADKNEDGGEKNME
Tag-130	NEDGGEKNMEDSGHE
Tag-131	EKNMEDSGHETGIDS
Tag-132	DSGHETGIDSQSQGS
Tag-133	TGIDSQSQGSFQAPQ
Tag-134	QSQGSFQAPQSSQSV
Tag-135	FQAPQSSQSVHDHNQ
Tag-136	SSQSVHDHNQPYHIC
Tag-137	HDHNQPYHICRGFTC
Tag-138	PYHICRGFTCFKKPP
Tag-139	RGFTCFKKPPTPPPE
Tag-140	FKKPPTPPPEPET
All peptides were synthesised by F-moc chemistry; the SV40 T-antigen peptides (Tag) were synthesised by Chiron Mimotopes	

CD4 T cell spectratyping: CDR3β region diversity of the indicated, randomly selected TCRs expressed by highly purified (MACS enriched followed by FACS purification) $CD4^+$ T cells was analysed and compared between wild type, TAP- and ERAAP-deficient T cells. Electrophoretograms represent different patterns which were identified during the analysis. CDR3 regions in the indicated Vβ-Jβ rearrangements were detected as patterns for each of the Vβ-Jβ pairings, analysed and represented schematically. CDR3 regions in the indicated Vβ-Jβ pairings were analysed using specific primers used in this experiment (Table 7).

Table 7: Forward, reverse and run-off primers used for CDR3β spectratyping

Forward Vβ primers	
Vβ2	TAC AGA CCC CAC AGT GAC TTT GC
Vβ3	CTT CTA GGA ATT TTG AAT TCA AAG TCA
Vβ4	GCA GGT CCA GTC GAC CCG AAA AT
Vβ5	ATT CTG GGT TGT CCA GTC TCC AA
Vβ6	CCC TCC AAA CTA TGA ACA AGT GG
Vβ8	CAA AAC ACA TGG AGG CTG CAG TCA
Vβ9	CAG CCA CTT TTG TGG ATA CTA CGG
Vβ14	TTC TTG GGT GTT AGT GCT CAG ACT A
Reverse Cβ primer	
Cβ	GGG TGG AGT CAC ATT TCT CAG ATC
Run-off Jβ primers	
Jβ1.1	ACT GTG AGT CTG GTT CCT TTA CC
Jβ1.2	AAA GCC TGG TCC CTG AGC CGA AG
Jβ1.3	CTT CCT TCT CCA AAA TAG AGC
Jβ1.4	GAC AGC TTG GTT CCA TGA CCG
Jβ1.5	GAG TCC CCT CTC CAA AAA GCG
Jβ1.6	TCA CAG TGA GCC GGG TGC CTG C
Jβ2.1	GTG AGT CGT GTT CCT GGT CCG AAG
Jβ2.2	CCA GAG TGA GCC GGG TGC CTG C
Jβ2.3	GTT CCT GAG CCA AAA TAC AGC G
Jβ2.4	GTG CCC GCA CCA AAG TAC AAG
Jβ2.5	GTG CCT GGC CCA AAG TAC TGG
Jβ2.7	CTA AAA CCG TGA GCC TGG TGC

CHAPTER III

CONCLUSIONS AND FUTURE DIRECTIONS

MHC class II processing pathway displays antigens through direct and indirect presentation mechanisms. Direct antigen presentation is dependent on several mechanisms which facilitate cytoplasmic antigen supply to the lysosomal compartment. Such mechanisms include macro-autophagy, chaperone-mediated autophagy (CMA) and, for few reported antigens, by a proteasome and/or TAP-dependent pathway (20, 21, 76, 84, 86, 87, 260, 286). On the other hand, very little, if anything, is known about the mechanism(s) underlying indirect presentation by class II molecules. According to the assessment from the available literature, exogenous antigens- microbial, tumour and allogeneic- that are captured through endocytosis or phagocytosis, are shuttled to the lysosomes where resident reductases and proteases mediate antigen processing; such processed antigens are subsequently presented by class II molecules (44, 48, 67, 69). The findings presented here provide new evidence that indirect presentation of cytosolic class II-restricted antigens is negatively affected by components of class I pathway- proteasomes, TAP and ERAAP, but not tapasin. Such new information reveals a previously unappreciated role for class I processing machinery in regulating the availability of cytoplasmic antigens for indirect presentation by class II molecules. As a result, such process affects p/class II presentation and alters the outcome of T_H cell responses. Thus, the

cellular and biochemical mechanism(s) that underlie indirect presentation of cytoplasmic, class II-restricted antigens need to be reexamined and elucidated.

One of the first questions was which professional APC removes dead or dying class II-negative cells and uses derived antigens (microbial, tumour or allogeneic) for indirect presentation by class II molecules. Data presented here suggest that CD8α^+ DCs have the capacity for indirect presentation of allogeneic HY antigen. Furthermore, donor-derived CD8α^+ DCs serve as the predominant source of *DBY*-encoded cytoplasmic antigen.

Interestingly, previous studies have revealed that CD8α^+ DCs, a minute cell population within the immune system, are highly specialized in exogenous antigen supply for cross-presentation of class I-restricted antigens and contain components of the class I processing pathway (160, 259). Cross-presentation, in many cases, requires that foreign antigens escape from the endosomes/phagosomes into the cytosol where such antigens become substrates for proteasomes and downstream components of the class I pathway (287). Therefore, in CD8α^+ DCs, donated cytoplasmic antigens readily become available to components of the class I pathway, raising questions whether such process limits/affects antigen supply for the class II processing pathway. The answer to this question was not available from previous research efforts.

My data suggest that TAP, both in donor and recipient CD8α^+ DCs, negatively affects indirect presentation of cytoplasmic HY alloantigen. Interestingly, only when TAP was absent from both the donor and recipient did we see the 2-3 fold increased T_H cell response, suggesting that TAP may

transport class II-restricted peptide into the ER lumen, thus limiting its availability to the lysosomal compartment (Figure 24 and 25). Such role for TAP transporter was not previously described.

TAP has been demonstrated to most efficiently transport peptides with hydrophobic and basic amino acid residues at the carboxyl terminus. Central amino acids do not affect affinity for the transporter (288, 289). Therefore, TAP has very broad specificity for a large number of proteasomal products, so it is not surprising that the TAP heterodimer may bind and transport peptides that do not fit within the class I peptide binding groove, but may be more suitable for binding to class II molecules instead.

L. monocytogenes secretes LLO to disrupt the phagosomal membrane and allows bacterial escape into the cytosol for proliferation. LLO also ruptures plasma membrane to allow bacterial escape to the neighbouring cells (290, 291). APCs phagocytose infected, dead or dying class II-negative cells for indirect class II antigen presentation (83). Based on my experimental data, TAP was suggested to affect indirect presentation of *L. monocytogenes* LLO and p60 derived class II-restricted epitopes- pLLO(190–201), pLLO(318–329), p60(177–188) and pLLO(253–264)- again by having negative effect on indirect presentation of cytoplasmic antigens derived from the two virulence factors, LLO and p60.

ERAAP acts downstream of TAP by customizing the amino terminus of TAP imported peptides either by trimming them to size, or facilitating their complete degradation (136, 137, 142, 292). Therefore, the next question was

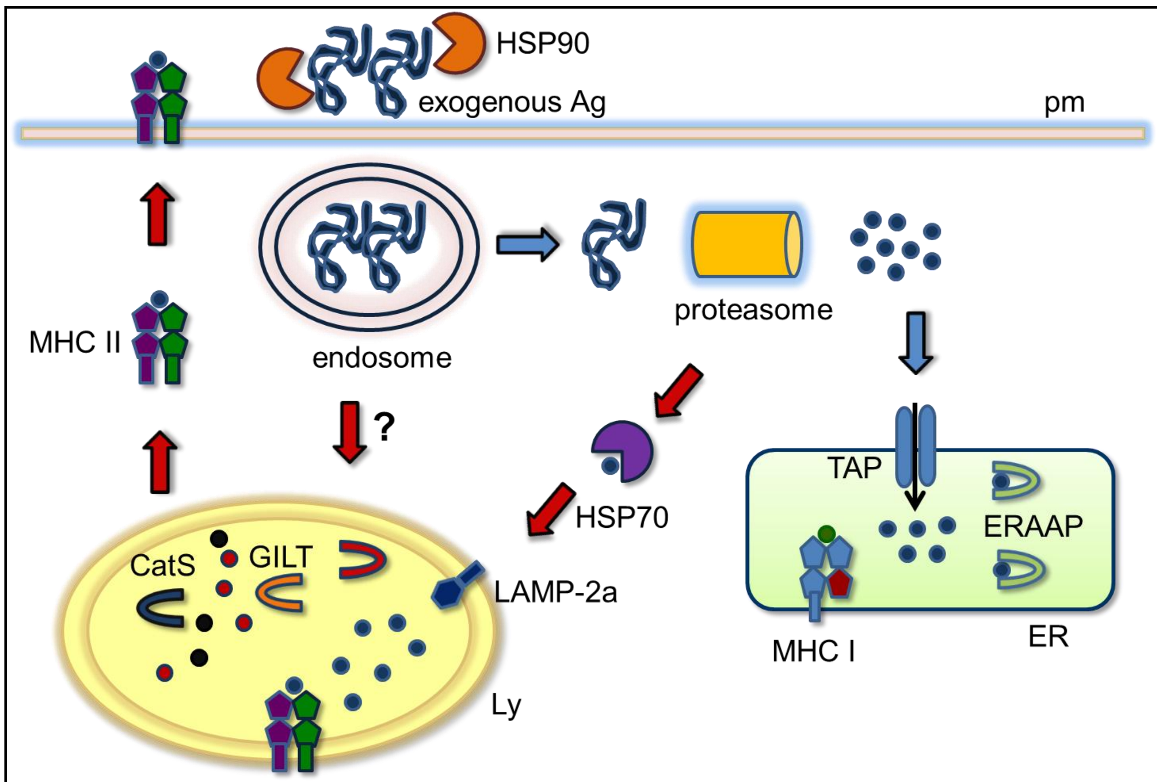


Figure 24. A proposed mechanism of indirect presentation of cytoplasmic, class II-restricted antigens. Donated antigens, stabilized in the complex with HSP90 (or CRT) are endocytosed. Such antigens seep into the cytoplasm of recipient APC where they are processed within the proteasome and resulting peptides are transported via TAP transporter and degraded by ERAAP. Alternatively, processed peptides are captured upon entry into the cytoplasm and stabilized by HSP70 and transported to the LAMP-2a$^+$ lysosomes through CMA. Upon release into the lysosomes, peptides are loaded on class II molecules and shipped to the cell surface. Another alternative is that part of the endocytosed antigens is directly released into the lysosomes.
Ag, antigen; CatS, cathepsin S; CMA, chaperone mediated autophagy; ER, endoplasmic reticulum; ERAAP, ER-associated aminopeptidase associated with antigen processing; GILT- γ-inducible lysosomal thiol reductase; HSP, heat shock protein; LAMP-2a, lysosomal associated membrane protein-2a; Ly, lysosome; MHC, major histocompatibility complex; pm, plasma membrane; TAP, transporter associated with antigen processing.

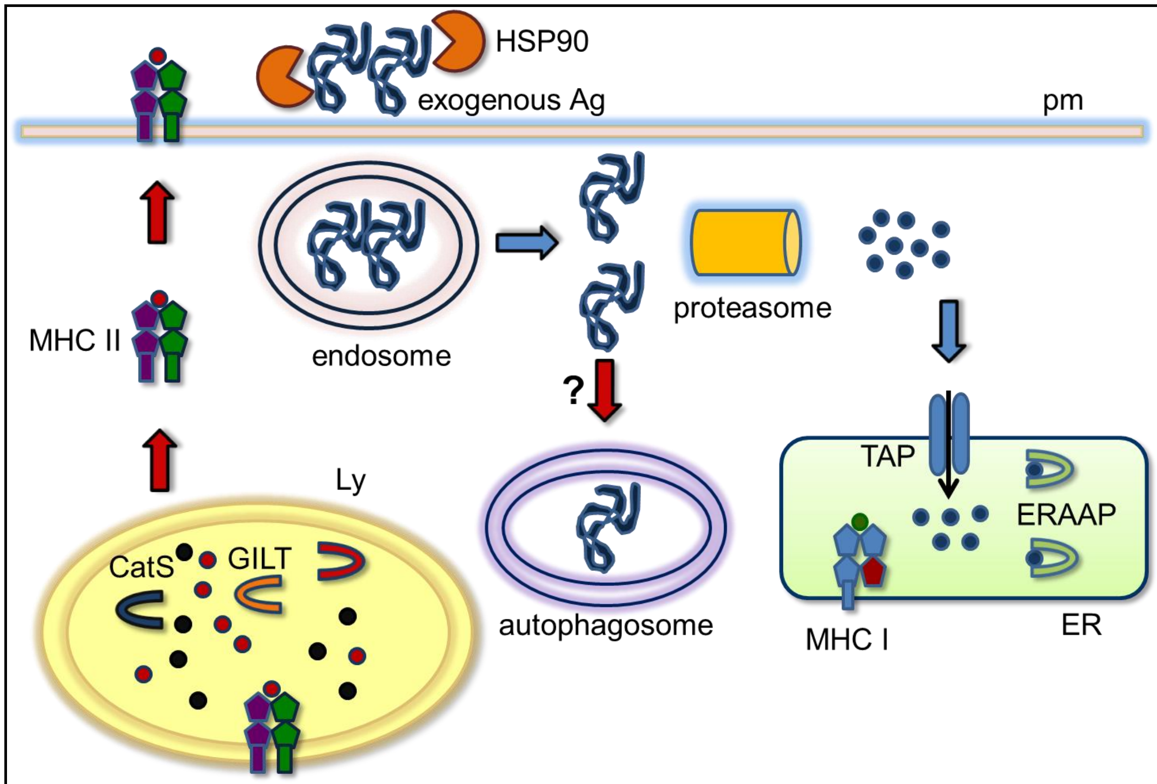


Figure 25. An alternative mechanism for indirect presentation of cytoplasmic, class II-restricted antigens. Donated antigens, stabilized in the complex with HSP90 (or CRT) are endocytosed. Such antigens seep into the cytoplasm of recipient APC where they are processed within the proteasome and resulting peptides are transported via TAP transporter and degraded by ERAAP. Alternatively, exogenous antigens are in part trapped by autophagosomes and donated to the lysosomes for processing and loading on class II molecules for presentation on the cell surface.
Ag, antigen; CatS, cathepsin S; ER, endoplasmic reticulum; ERAAP, ER-associated aminopeptidase associated with antigen processing; HSP, heat shock protein; Ly, lysosome; MHC, major histocompatibility complex; pm, plasma membrane; TAP, transporter associated with antigen processing.

whether ERAAP potentially affects cytoplasmic allogeneic HY as well as listerial LLO and p60 derived class II antigens by possibly degrading them. My data suggest that ERAAP in both donor and recipient exerts a negative effect on allogeneic HY antigen presentation leading to greatly reduced T_H cell responses. Likewise, ERAAP also had a negative effect on indirect presentation of cytoplasmic listerial LLO and p60 derived class II antigens- pLLO(190–201), pLLO(318–329) and p60(177–188). Interestingly, pLLO(253–264) was not affected by ERAAP, suggesting that it could follow a different processing route that is independent of ERAAP.

Despite the fact that TAP and ERAAP negatively affect T_H cell responses, it is still unclear how this effect is exerted and a mechanism is lacking. It should be investigated whether the absence of precursors to veto CTL in TAP-mutants may affect T_H cell responses to bacterial and alloantigens (293).

Another question is whether the absence of the recipient $CD8^+$ T cell population in TAP-deficient mice could affect spleen architecture akin to B cell deficiency (294). This remains to be determined in future research.

$CD8^+$ T cell-deficiency alters the number of $CD4^+$ T cells. Therefore, another question is whether the lack of the $CD8^+$ T cell population is responsible for increased T_H cell responses to HY alloantigen. In order to address this question, immune splenocytes were stimulated with pHY and activated T_H cells were fluorescently stained for intracellular IFN-γ. This experiment confirmed an increased T_H cell response in TAP-knockouts compared to wild type.

In another experiment, the T_H cell response between $TAP^{-/-}$, $\beta 2m^{-/-}$ and $CD8\alpha^{-/-}$ recipients— all of whom lack $CD8^+$ T cells— was compared and no change was observed in the latter two; that is, $\beta 2m^{-/-}$ and $CD8\alpha^{-/-}$ recipients responded similarly to wild type recipients. Such result suggests that CTL deficiency may not be the reason for an increased T_H cell response, because an increased response was not found in other class I-deficient strains.

The next question was whether altered natural killer (NK) cell activity in TAP-deficient mice could have caused an increased T_H cell response to HY alloantigen. Previous studies in TAP-deficient mice have reported a population of hyporesponsive NK cells which do not lyse autologous class I-deficient cells (295). At the same time, such NK cells indirectly and negatively controlled T_H cell responses to *Toxoplasma gondii* (296). Nonetheless, I found that IL-15-deficient recipient mice- which lack NK and mature NKT cells- displayed similar T_H cell response to the alloantogen HY akin to wild type recipients, suggesting that NK cells did not cause the increased T_H cell response (data not shown).

Both TAP and ERAAP play a significant role during cross-presentation. Cross-presented exogenous antigens readily exit from the endosomal/phagosomal compartment to gain access to proteasomes and downstream components of the class I pathway (149, 164). On the other hand, indirect presentation includes shuttling of exogenous antigens from the endosomal/phagosomal compartment to the lysosomes where they are loaded on class II molecules (13, 83). Therefore, cross-presentation and indirect presentation proceed through distinct, yet partially overlapping routes for antigen

supply at the level of endosomes. Thus, internalized exogenous antigens from dead or dying cells could be exposed to proteasomes, and TAP- and ERAAP-mediated processing upon which they become degraded and lost. Such a mechanism then explains the observed loss of cytoplasmic allogenic HY and listerial antigens in wild type cells.

The role of donor and recipient proteasomes was investigated using pharmacological inhibition and LMP-2 deficient recipient immunoproteasomes. Inhibition of donor proteasomes revealed an increased T_H cell response in recipients, suggesting that *DBY* encoded RNA helicase is a potential proteasomal substrate, and that intact helicase, instead of proteasomal product, is predominantly donated for indirect presentation. If the whole protein enters the recipients' cytoplasm, the question is whether such antigen requires processing within immunoproteasomes.

Supporting evidence that indirectly presented cytoplasmic class II-restricted antigens enter the cytoplasm in recipient CD8α^+ DCs stems from experimental results with LMP-2-deficient immunoproteasomes. When T_H cell responses to indirectly presented HY alloantigen were compared between wild type and LMP-2 deficient recipients, the wild type response was significantly greater compared to knockouts, suggesting that proteasomal processing within recipient CD8α^+ DCs is a prerequisite for an optimal T_H cell response. Further support for such conclusion came from experiments with pharmacological inhibition of donor and LMP-2 deficiency of recipient proteasomes which resulted in virtual absence of T_H cell responses to HY alloantigen.

Notwithstanding, LMP-2 deficiency also causes impairment in T cell activation, prevention of T-cell mediated autoimmunity and reduced T cell apoptosis upon TNF-α exposure (297, 298). LMP-2 deficient T_H cells also provided poor help to activated B cells. In sum, these results suggest that lower T_H cell response to HY alloantigen may be caused by such intrinsic defects within LMP-2 deficient TH cells. However, direct comparison of T_H cell response between LMP-2-null recipients given vehicle (DMSO) or proteasomal inhibitor treated donor cells revealed that immunoproteasomes play a significant processing role for indirectly presented cytoplasmic antigens. This finding further suggests that the donor antigen appears in the recipient cytoplasm and, hence, is accessed by the class I antigen processing machinery thereby leading to the loss of class II-restricted antigens of cytoplasmic origin.

The next question we asked was which factors mediate antigen chaperoning from donor to recipient $CD8\alpha^+$ DC during indirect presentation. Previous research had shown that HSP90 shields proteasomal products from destruction by cytosolic peptidases. Furthermore, HSP90 stabilizes donor antigens in order to facilitate cross-presentation by $CD8\alpha^+$ DCs (172, 181). At the same time, CRT plays a similar chaperone role during cross-presentation, although CRT may have other roles, including relaying apoptosis signals to other cells of the immune system (299, 300). Such role may explain poor recipient T_H cell responses in the absence of CRT. In sum, my data suggest an unexpected role for the two prominent chaperones—HSP90 and CRT that facilitate cross

presentation by class I molecules—for indirect presentation of the class II-restricted alloantigen, HY (Figures 24 and 25).

Additionally, HSP70 is known to facilitate indirect presentation of class II-restricted antigens (183). Data presented herein confirm such a role for HSP70 in partially shielding allogeneic HY antigen (Figure 24). Therefore, indirect presentation of class II-restricted HY alloantigen unexpectedly follows the established cross-presentation route for class I-restricted antigens by employing HSP90 and CRT, but in part also uses HSP70 to facilitate antigen transfer to recipient $CD8\alpha^+$ DCs (Figure 24 and 25).

Emerging evidence suggests that macroautophagy and CMA are the main sources of cytoplasmic antigens which are fed to the lysosomal compartment for processing and loading onto class II molecules (286, 301). Therefore, I addressed whether macroautophagy and CMA were mechanistically involved in indirect presentation. Surprisingly, induction of macroautophagy in donor cells entirely abolished indirect presentation of cytoplasmic HY alloantigen as judged by complete lack of T_H cell responses in mice that received donor cells in which macroautophagy was induced by a 2-hour nutrient starvation. Conversely, inhibition of macroautophagy with a pharmacological inhibitor, or with the use of *conditional* Atg5 deficient spleen cells as the donor ((302) and data not shown), did not affect class II-restricted HY-specific T_H cell responses. On the contrary, the measured response was greater in *conditional* Atg5 deficient compared to wild type donors and recipients (see explanation below). Therefore, macroautophagy, in the case of class II HY alloantigen, has a detrimental effect

on the indirect presentation, suggesting that this cellular process limits the pool of available class II-restricted antigens.

The latter process that has been shown to provide cytosolic antigens for class II-restricted presentation is CMA. CMA starts with proteasomal processing and requires HSP70 to transfer antigens to LAMP-2a$^+$ lysosomes for loading onto class II molecules (21). We suggest that proteasomes process class II-restricted HY alloantigen, while HSP70 in part facilitates antigen transfer to recipient CD8α^+ DCs. Furthermore, inhibition of macroautophagy is in turn compensated by an increase in CMA (303, 304). Therefore, when donor cells are treated with pharmacological inhibitors of autophagy, or, possibly, when donor DCs lack Atg5, a compensatory mechanism within DCs greatly upregulates CMA (303, 304). As a result, the T_H cell response in recipients is enhanced with 3-MA treated or Atg5-deficient APCs compared to recipients receiving vehicle treated donor cells. Therefore, one hypothesis is that, following donation of intact protein, it is submitted to proteasomal processing. Next, allogeneic HY alloantigen may be sequestered both by HSP70 shuttling antigens to LAMP2a$^+$ lysosomes, or by HSP90, which diverts the antigen to the TAP transporter and ERAAP (Figure 24).

The next question was whether TAP and ERAAP deficiency could uncover novel class II-restricted epitopes, which are otherwise completely destroyed by aminopeptidase(s). With the use of an overlapping SV40 large T antigen peptide library, this hypothesis was put to the test. Data revealed two novel class II-restricted epitopes in the absence of TAP, while a distinct class II epitope was found in wild type but not in TAP-knockout mice. Such experiment revealed novel

class II-restricted epitopes recognized only by TAP-deficient T_H cells. This finding is especially important since cancerous cells often accumulate mutations in TAP, rendering the complex ineffective for peptide transport into the ER and subsequent reduction or inhibition of class I-restricted CTL response to tumour antigens (305-307).

We next sought to compare the naturally occurring peptides eluted from class II molecules expressed by wild type, TAP and ERAAP-deficient professional APCs. To determine if there were qualitative differences, in a preliminary experiment, $H2A^b$ molecules were affinity purified from wild type, TAP- and ERAAP knockout mice and the associated peptides eluted, RP-HPLC fractionated and subjected to ESI-MS and MSMS analyses. Preliminary results suggest that the resulting peptide sequences revealed that $H2A^b$ molecules assemble with an altered class II-restricted peptide repertoire in TAP and ERAAP deficient splenocytes as compared to those displayed by wild type cells. Further studies will examine these peptides and test the potential role of these peptides by comparing T_H cell responses among wild type, TAP- and ERAAP-deficient animals.

Another line of experiments investigated differences in T_H cell TCR repertoire among the three groups—wild type, TAP and ERAAP deficient–by spectratyping. These experiments revealed substantial differences, both quantitative (as judged by differences in peak height) and qualitative (as judged by differences in the number of peaks and in elution time of the different peaks) of some of the TCR variable regions of the β-chain. A previous study based on

serologic typing of T cells reported that the TCR repertoire of T_H cells was unaltered in TAP deficient mice (261). Our finding does not refute the previous report as we found differences within the CDR3β loops and not Vβ usage. Hence, we conclude that the T_H cell TCR repertoire is different in TAP and ERAAP deficient mice as compared to the TCR repertoire of wild type $CD4^+$ T cells. This altered repertoire could partly explain the qualitative and quantitative differences in the T_H cell response to cytosolic antigens that are indirectly presented by class II molecules.

In sum, proteasomes, TAP and ERAAP regulate the quantity of class II-restricted allogeneic and listerial peptide repertoire. Furthermore, TAP also impacts the quality of the presented class II-restricted peptides, as revealed by the presentation of novel SV40 large T antigen and the eluted self-class II-restricted peptides expressed by TAP and ERAAP mutant cells. These differences lead to altered T_H cell TCR repertoire in TAP- and ERAAP-deficient animals and consequently impact T_H cell responses to cytoplasmic class II-restricted microbial, tumour and allograft antigens.

Future directions

The research presented in this thesis raises a number of new questions that could be addressed in future studies. The first; it will be interesting to determine the molecular mechanisms regulating trafficking properties of proteins which give rise to class II epitopes- DBY (encodes alloantigen HY), RBP1 (encodes alloantigen $H3b^a$) and *L. monocytogenes* derived p60 (encodes p60(177-188)

and p60(253-264)). It is of interest to determine whether these proteins can traffic to lysosomes or proteasomes for degradation, to the nucleus (DBY and RBP1), or can be shuttled via autophagosomes to lysosomes. The purpose of this series of proposed experiments is to provide direct evidence regarding the trafficking properties of proteins- DBY, RBP1 and *L. monocytogenes* p60 in bone marrow derived DCs, or alternatively, in the murine Mϕ cell lines RAW 264.7, J774 or DC-like line KG1.

The above proteins, either radio-labelled or labelled with His- or Flag-tag, could be added to DCs. Such DCs could be used to test for proteasomal targeting through polyubiquitilation. Other organelles could be isolated through gradient density differential ultracentrifugation and tested for the presence of proteins of interest. Therefore, the presence of the added proteins in specific fractions could be detected. This approach may help in determining the change in the levels of protein of interest in organelles over a period of time. An alternative approach could be to transduce target cells with constructs containing the above mentioned proteins, instead of adding proteins exogenously. As a result, these experiments could provide a better understanding of trafficking and processing properties of proteins which give rise to cytoplasmic class II-restricted epitopes. Such understanding may facilitate improved vaccine design against microbial and tumour antigens, as well as help understand the factors which influence resulting T_H cell responses to cytoplasmic microbial and tumour antigens acquired from non-MHC class II expressing cells.

Next one could determine quantitative differences in class II epitope presentation by wild type, TAP- and ERAAP-deficient bone marrow DCs (or RAW 264.7, J774 or DC-like line KG1cell line) by incubating DBY, RBP1 and p60 proteins, or transducing cells with constructs containing such proteins. After different times of incubation, cells would be lysed, class II molecules isolated by using columns containing MHC class II-specific antibodies, as well as irrelevant, control antibodies (285). Next, class II-restricted peptides would be eluted using reversed-phase RP-HPLC. Next, each collected fraction would be analysed using LC/MS/MS in order to obtain high quality spectra, which are indicative of peptide sequences. These proposed studies could directly indicate differences in peptide presentation by wild type and mutant cells. Such results may provide direct evidence that specific components of the class I pathway, TAP and ERAAP, negatively affect presentation of cytoplasmic class II-restricted antigens. This goal is aimed at providing evidence that could support the hypothesis that listerial antigens are also affected by TAP and ERAAP.

Since quantification using mass spectrometry may be somewhat limited, an alternative approach could be employed to examine quantitative differences in class II peptide presentation from three types of cells listed above. RP-HPLC separated peptide fractions may be used to pulse K^bD^b-deficient professional APC. Peptide pulsed APCs can stimulate T_H cells from mice previously immunized with minor HAg-disparate donor splenocytes or, in the case of p60, from mice challenged with *L. monocytogenes.* T_H cell stimulation can be measured using ELIspot assay in order to test differences in T cell responses.

TAP- and ERAAP-deficient fractions might induce increased stimulation of immune T_H cells compared to class II peptides eluted from wild type cells. Such result would be direct evidence that ERAAP destroys peptides imported via TAP into the ER lumen.

B cell-mediated immunity plays a crucial part in fighting certain pathogens, such as *Borrelia burgdorferi* and *Ehrlichia muris* (308-310). Such pathogens elicit negligible CTL and unproductive T_H cell responses (311). Therefore, a second area of future research is aimed at promoting B cell-mediated antibody responses by uncovering low abundant or previously not characterized class II-restricted epitopes that are normally destroyed by concerted action of TAP and ERAAP. Such class II epitope(s) may be utilized to boost T_H cell responses and as a means to ultimately design novel and efficient subunit vaccines. Such studies could also benefit from using not only ERAAP, but IRAP-deficient mice. In order to define novel class II-restricted epitopes, $CD8^{-/-}$, $CD8^{-/-}$;$TAP^{-/-}$ and $CD8^{-/-}$;$ERAAP^{-/-}$ mice could be used, or $\beta 2m^{-/-}$ single and double knockouts, in order to exclude any minor involvement of CTL responses. Low abundant class II epitopes could be isolated from from TAP and ERAAP knockouts using the techniques described in previous section, followed by RP-HPLC fractionation and mass spectrometry analysis.

Novel class II epitopes may be used in vaccine design by creating complex subunit vaccines consisting of a mixture of (*i*) microbial proteins which give rise to low abundant and/or previously uncharacterized class II epitopes that are normally destroyed by TAP and ERAAP, (*ii*) glycoproteins and/or lipoproteins

that stimulate B cell responses and antibody production, (*iii*) glycolipids that activate NKT cells and (*iv*) a TAP inhibitor or an ERAAP-inhibitor (perhaps leucine-thiol (292)), in order to prevent degradation of novel class II epitopes and increase their presentation by professional APCs.

To test such an approach, bone marrow-derived murine DCs could be incubated with above mixture of agents. The next step would be to inoculate experimental animals with the relevant pathogen to test microbial clearance and protection by measuring T_H and B cell responses. If studies with experimental animals yield positive results, such candidate vaccine may be tested on human subjects.

A third future direction is to investigate the possible role of cryptic class II epitopes in the development of keratitis following infection by herpes simplex virus. Herpetic stromal keratitis (HSK) is a chronic inflammatory disease and a leading cause of blindness in infants resulting from an infection in the mother (312, 313). MHC class II-restricted T_H cell responses play a critical role in chronic HSK (312, 314). Nonetheless, which class II-restricted antigen(s) trigger the activation of pathogenic T_H cells during HSV infection and how such antigens are presented remain poorly defined. I hypothesise that TAP blockade by HSV-encoded ICP47 alters the processing and presentation of cytoplasmic, class II-restricted self and viral peptide antigen(s) during herpes infection and activates pathogenic T_H cell responses that mediate HSK.

In order to test this hypothesis, viral peptides associated with HLA-DR1 (B1*0101) molecules expressed by KG-1 DC-like cells infected with wild type or

ICP47-null mutant HSV-1 infected could be isolated and characterized. Since pathogenic T_H cell responses are observed in HSK, self-peptides associated with HLA-DR1 (B1*0101) molecules expressed by uninfected as well as cells infected with wild type or *ICP47*-null HSV-1 would be isolated and characterized.

Next, one could test whether TAP blockade by ICP47 will result in altered T_H cell responses to novel HLA-DR1 (B1*0101)-restricted epitopes, thereby causing HSK. In order to test this hypothesis, transgenic mice that express both human TAP-1 and TAP-2 genes (hTAP^{tg}) could be generated. Next, I would determine whether the HSV-1-specific T_H cells cross-react with HLA-DR1-restricted self-peptides presented by uninfected cells or altered self-peptides presented upon herpes infection. I would also determine whether infection of DR1^{tg};hTAP^{tg} mice with *ICP47*-null mutant HSV-1 causes HSK.

Upon successful completion of the proposed studies, we would have determined whether TAP blockade alters class II-restricted antigen processing and presentation under pathologic conditions. We would have also determined whether TAP blockade impacts the T_H cell responses to cytoplasmic antigens encoded by a pathogenic virus.

Finally, one could use a similar approach to identify novel class II antigens for another bacterial pathogen. Chronic infection with the bacterial pathogen *Helicobacter pylori* is frequent in humans and causes gastritis which can lead to an increased risk of gastric cancer (315-317). *H. pylori,* which carries the Cag pathogenicity island, causes patients to have increased risk of developing gastric

cancer (317-320). Recently, it was demonstrated that the cytotoxin-associated gene A (CagA) protein carries several class II-restricted T_H cell epitopes (317). Interestingly, T_H cell activation upon exposure to the pathogen causes severe gastric pathology and has poor prognostic outcome (315-317, 321, 322).

The results of my research could be employed to identify novel *H. pylori* specific class II epitopes derived from secreted or membrane anchored proteins which may induce T_H cell activation and possibly cause gastric cancer. Since research presented here suggests that the class I pathway negatively affects presentation of class II-restricted epitopes, human DCs and Mϕ treated with ICP47 (TAP inhibitor) or leucine-thiol (ERAAP-inhibitor) could be used in vitro to identify low abundant and/or novel class II epitopes derived from *H. pylori* proteins. Upon their identification, the next goal would be to in vitro expand reactive T_H cell clones which provide information regarding TCR usage. The ultimate goal would be to eliminate T_H cell clones specific for *H. pylori* in humanized mouse models (and potentially in *H. pylori* infected patient samples or patients themselves) in order to improve prognostic outcome. In order to delete such T_H cells, MHC II tetramers conjugated with Actinium 225 can be used to create an agent for T_H cell clonal deletion. Actinium 235 can serve as an alpha-emitting atomic nanogenerator, capable of single-hit killing from the cell surface (323). It is expected that deletion of T_H cells would provide better prognostic outcome for affected patients. Characterization of novel class II epitopes following TAP and/or ERAAP inhibition in patient samples could provide better

information regarding low abundant and or novel class II epitopes which may provide better protection against *H. pylori* infection and gastric cancer.

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