Exploring HLA-E restricted Mycobacterium tuberculosis specific T cells as vaccination targets for Tuberculosis

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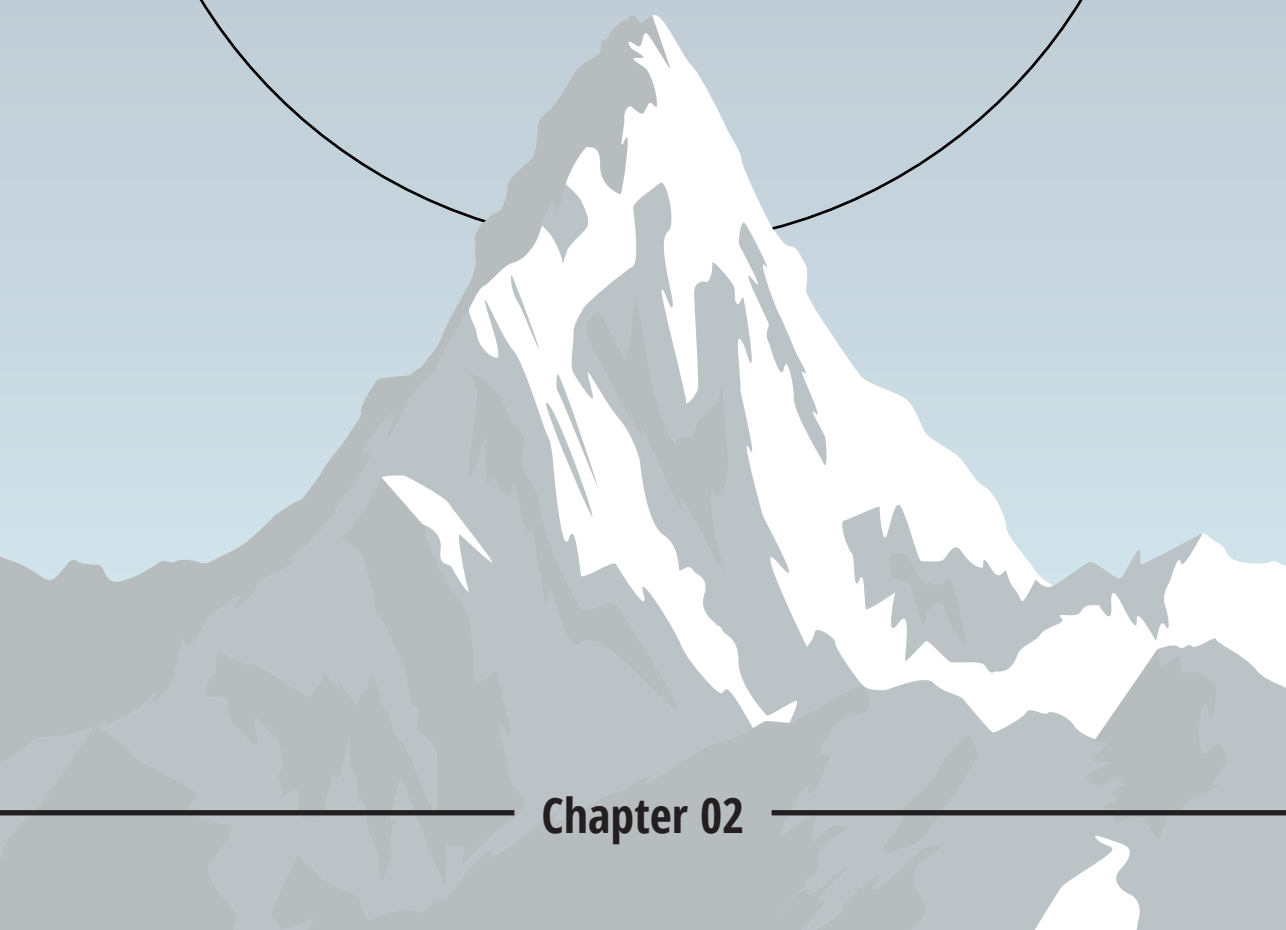

Chapter 02

Antigen presentation by MHC-E: a putative target for vaccination?

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Highlights

- Human HLA-E molecules are 'unconventional' MHC molecules that have limited allelic diversity but which can present a wide variety of pathogen- and tumor-derived peptides.
- The pathways responsible for peptide loading onto HLA-E and the subcellular spaces where this takes place are largely unknown.
- The absence of TAP is associated with the presentation of tumor epitopes by HLA-E, but HLA-E peptide presentation also occurs in the presence of TAP, suggesting additional, independent pathways.
- The presentation of HLA-Ia leader sequences in HLA-E avoids CD94/NKG2A inhibitory receptor recognition and natural killer cell-mediated lysis, which is likely a mechanism hijacked by tumors and pathogens that downregulate HLA class Ia presentation.
- The presentation of de novo epitopes in tumors and infections elicit HLA-E-restricted $CD8^+$ T cells that play an important role in immunity.
- $CD8^+$ T cells can recognize HLA-E/peptide complexes with polyclonal TCRs despite the essentially monomorphic molecule HLA-E.

Abstract

The essentially monomorphic human antigen presentation molecule HLA-E is an interesting candidate target to enable vaccination irrespective of genetic diversity. Predictive HLA-E peptide-binding motifs have been refined to facilitate HLA-E peptide discovery. HLA-E can accommodate structurally divergent peptides of both self and microbial origin. Intracellular processing and presentation pathways for peptides by HLA-E for T cell receptor (TCR) recognition remains to be elucidated. Recent studies show that, unlike canonical peptides, inhibition of the transporter associated with antigen presentation (TAP) is essential to allow HLA-E antigen presentation in cytomegalovirus (CMV) infection and possibly also of other non-canonical peptides. We propose three alternative and TAP-independent MHC-E antigen presentation pathways, including for *Mycobacterium tuberculosis* infections. These insights may help in designing potential HLA-E targeting vaccines against tumors and pathogens.

The unconventional antigen presentation molecule MHC-E

Endogenous and exogenous **peptides** (see Glossary) are presented at the cell surface by **major histocompatibility complex (MHC)** class I and II molecules (human leukocyte antigens, HLAs) to $CD8^+$ or $CD4^+$ T cells, respectively. MHC-class I includes classical (MHC-Ia) and non-classical molecules (MHC-Ib). HLA-Ia molecules are highly polymorphic and present diverse peptide repertoires, whereas HLA-Ib molecules, such as HLA-E, have limited allelic polymorphism[1]. Two dominant functional alleles, HLA-E*01:01 and HLA-E*01:03, cover >99% of all human populations[2,3]. The alleles differ in one amino acid outside the peptide-binding groove (PBG) at position 107 - Arg in HLA-E*01:01 and Gly in HLA-E*01:03[4]. HLA-E regulates innate immune responses via presenting HLA-Ia leader sequences (**canonical** peptides, VL9) to CD94/NKG2(A,C) co-receptors on natural killer (NK) cells and by presenting pathogen and tumor-derived peptides (non-canonical peptides) to TCRs and possiblyCD94/NKG2(A,C) (Figure 1A-E)[5,6].

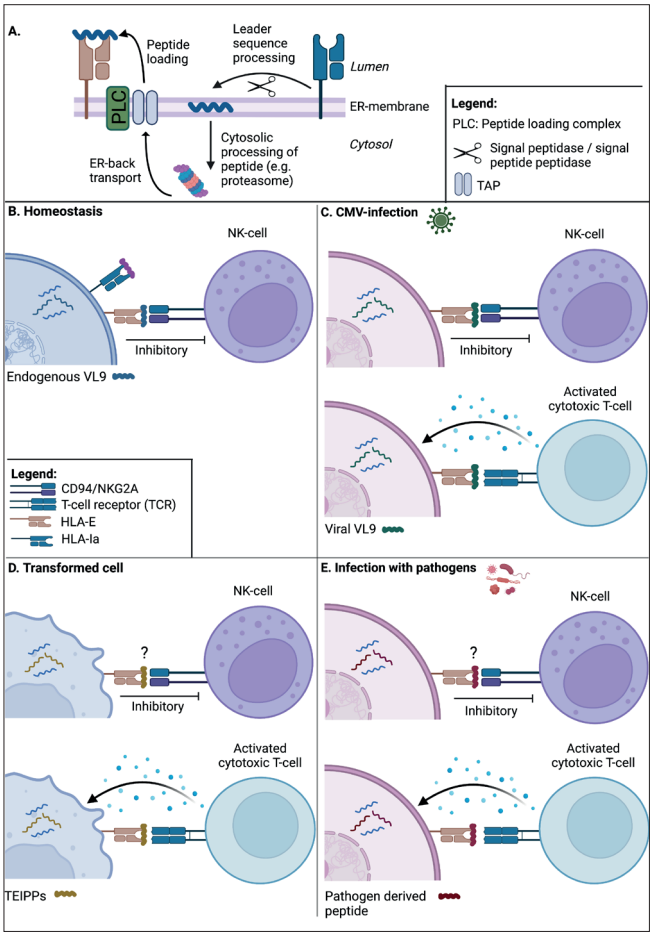

Figure 1. HLA-E involvement in innate and adaptive immunity. HLA-E is best known as a ligand for the CD94/NKG2-A, -B or -C heterodimeric receptor complex expressed by NK cells and a subset of $CD8^+$ T cells in humans. (A) Presentation of canonical HLA-E ligands derived from HLA-Ia leader sequences via the SPase/SPPase pathway[21]. (B) HLA-E binds nonameric self-peptides derived from HLA-Ia leader sequences (VL9 peptides) to either inhibit the cytolytic activity of NK cells via engagement with the CD94/NKG2A or -B receptor or activate their cytolytic activity via engagement with the CD94/NKG2C receptor[5,13]. (C) In addition to VL9-peptides, CMV exploits this same axis to ensure the survival of CMV-infected cells. CMV encodes a TAP inhibitor called US6 that can downregulate canonical peptide loading onto HLA-Ia molecules, rendering CMV-infected cells susceptible to NK-mediated lysis[13]. To avoid lysis and permit engagement with the CD94/NKG2A coreceptor, CMV expresses an HLA-E-restricted, VL9 peptide mimic derived from the UL40 protein ('viral VL9')[14]. This peptide and potentially other CMV-derived peptides can also interact with TCRs. (D,E) In addition to its role in innate immunity, HLA-E can also present peptides from pathogens and tumors to TCRs, and possibly to CD94/NKG2 coreceptors. These activated HLA-E-restricted T cells can elicit potent cytotoxic and cytolytic responses against tumor cells and pathogen-infected cells[6,12,48,62,69]. Figure created with Biorender.com. Abbreviations: CMV, cytomegalovirus; NK cell, natural killer cell; SPase, signal peptidase; SPPase, signal peptide peptidases; TAP, transporter associated with antigen presentation; TCR, T cell receptor; TEIPPs, T cell epitopes associated with impaired peptide processing.

The conservation of HLA-E across vertebrates can facilitate the identification of cross-species peptides to design and evaluate HLA-E-based vaccination strategies[7,8]. Recently, improved predictive peptide-binding motifs confirmed that positions 2 and 9 are crucial anchors for the HLA-E PBG, with preference for hydrophobic residues (Box 1)[7,9,10]. In addition to peptide binding, it is important to define the determinants that govern TCR recognition of HLA-E/peptide complexes and to understand HLA-E antigen processing and presentation in more detail. We hypothesize that the transporter **associated with antigen presentation (TAP)** is involved in loading peptides onto HLA-E because cytomegalovirus (CMV) peptides (CMV actively inhibits TAP) are only presented at the cell surface and recognized by TCRs and CD94/NKG2A by routes that are independent from TAP (Figure 1C)[11-14]. Moreover, aside from insights into peptide loading, elucidating the details of TCR recognition of HLA-E/peptides will be important in understanding immune activation via HLA-E[11,15,16]. We briefly review the pathways potentially contributing to peptide loading onto MHC-E, including subcellular localizations and intracellular transport. We then discuss the limited current knowledge on T cell recognition of peptides presented by MHC-E. We also propose ways in which this knowledge might contribute to future HLA-E-based vaccines against tumors and pathogens.

How and where are self- and non-self peptides loaded onto MHC-E?

The **proteasome** processes intracellular proteins to generate peptide fragments that are transported into the endoplasmic reticulum (ER) lumen via TAP for MHC-Ia-loading[17]. Peptides can be additionally trimmed by ER-associated aminopeptidases or directly loaded onto MHC-Ia in the luminal part of the ER membrane via the **peptide-loading complex (PLC)**. Stabilized MHC-Ia/peptide complexes travel to the cell surface via the Golgi apparatus[17,18]. MHC-E molecules are also present in the ER-membrane, but peptide processing during homeostasis occurs differently, as discussed in the following text[19,20].

1. The signal peptidase (SPase) pathway

Canonical VL9-peptides are generated via ER-resident SPases that cleave off the N-terminal region of HLA-Ia leader sequences. Signal peptide peptidases (SPPase) further cleave hydrophobic regions to release nonameric peptides into the cytosol[19]. The peptides can then be processed in a proteasome-dependent (and potentially also a proteasome-independent) manner for backtransport into the ER via TAP for loading onto MHC-E (Figure 1A)[21]. Peptides other than canonical VL9 might not require TAP for peptide loading onto MHC-E, as suggested in tumor cell lines, such as K562, and also confirmed for the CMV **UL40-derived peptide** (see following text)[11,16]. Moreover, HLA-Ia-expressing human tumor cells such as lung carcinoma and melanoma present an alternative repertoire of (non-mutated) tumor-peptides: **T cell epitopes associated with impaired peptide processing (TEIPPs)**[22]. The majority of TEIPPs lack ER-targeting signal sequences, suggesting that other pathways can be involved in peptide loading[23]. Two alternative pathways, furin and autophagy, can load MHC-Ia independent from TAP, and these two pathways might also be exploited by MHC-E.

2. The furin pathway

The trans-Golgi protease furin has endoproteolytic activity and cleaves pro-proteins at a specific C-terminal cleavage site[24]. $TAP^{+/+}$ or $^{-/-}$ mice were immunized with two types of hepatitis B virus (HBV)-derived vectors, in which the SIINFEKL epitope was inserted behind a furin cleavage site. One vector contained an HBV carrier protein

to access the secretory pathway (HBe) whereas the other lacked this protein (HBc). The HBe vector activated SIINFEKL-specific $CD8^+$ T cells in both $TAP^{+/+}$ and $^{-/-}$ mice, whereas the HBc vector only did so in $TAP^{+/+}$ mice, demonstrating the importance of intracellular localization to access processing pathways[25]. In vitro, MHC-Ia/SIINFEKL complex formation was significantly reduced by peptidyl chloromethylketone decanoyl-RVKR-CMK, a furin inhibitor, following infection with the HBe vector in $TAP^{-/-}$ human lymphoblastoid cells (T2) transfected with mouse MHC-Ia (Kb). By contrast, furin inhibition marginally reduced MHC-Ia/SIINFEKL complex formation in HBe-infected $TAP^{+/+}$ murine fibroblasts, which instead relied on the proteasome. Infection with HBc induced MHC-Ia/SIINFEKL complexes in $TAP^{+/+}$ cells only, independently from furin, and these were instead fully reliant on the proteasome[25]. Moreover, the furin pathway was less efficient in inducing SIINFEKL-specific IFN-γ in $CD8^+$ T cells compared to the TAP and proteasome pathways. We argue that furin involvement in TAP-independent processing may vary with the cell type and antigen involved (e.g., if it contains a furin cleavage site). However, the intracellular compartments loading furin-processed peptides onto MHC-I as well as the degree of involvement of the PLC or other translocons remain elusive.

3. The autophagy pathway

Autophagy encapsulates intracellular proteins and organelles into double-membrane vesicles which fuse with lysosomes to form autophagolysosomes for degradation. Autophagolysosomal processing was found to be the dominant pathway for MHC-Ia peptide processing in $TAP^{-/-}$ T2 cells infected with a CMV-UL138-encoding adenovector[26]. In human CMV-infected monocyte-derived dendritic cells (moDCs), peptide processing occurred via both autophagolysosomes and proteasomes as $CD8^+$ T cell activation was reduced by the proteasome inhibitor lactacystin, the autophagy inhibitor 3-MA, and the endosome acidification inhibitor chloroquine[26]. This suggests that peptide processing is likely skewed to the proteasome-independent autophagolysosome pathway when TAP is inhibited[26-28]. We posit that TAP-independent MHC-Ia peptide loading in this pathway might occur in the (auto) phagosome because peptide loading has been found to be independent of the ER where TAP is localized. The ER membrane might be a source of MHC-Ia and PLC components to form autophagosomes, as demonstrated in yeast cells treated with dithiothreitol (DTT) to induce ER stress[29]. Indeed, electron microscopy on DTT-treated yeast cells detected Sec61 - an ER-localized translocon – as well as ribosomes in the ER autophagosomes[29]. MHC-E has also been detected in endosomal compartments; however, whether peptide-processing and loading can also occur in these compartments remains elusive[30]. Combined, these findings suggest that peptides in **endosomal compartments** may be loaded onto MHC-E, but further experiments will be necessary to validate this.

Taken together, we propose that the SPase/SPPase, furin, and autophagy-mediated pathways are three potential TAP-independent pathways that can contribute to peptide loading onto MHC-E in mammalian cells.

MHC-E peptide presentation: tumors and pathogens

Tumor cells interfere with human HLA-Ia antigen presentation by downregulating TAP1/2 subunits to avoid T cell recognition and clearance[31]. In K562 cells devoid of endogenous HLA-Ia and transfected with HLA-E*01:03, surface expression of HLA-E*01:03 could be maintained in the presence of the TAP inhibitor UL49.5[31]. This suggested that HLA-E peptides might exploit non-classical pathways if the classical pathway and ER-based TAP are not available. Of note, analysis of the presented peptides revealed novel, diverse, and unique sequences

similar to the TEIPPs in TAP$^{-/-}$ human cancer cells[22,32]. Murine TAP$^{-/-}$ T cell lymphoma cells (RMA/S) presented alternative and immunogenic peptides on Qa-1^{b} (the mouse HLA-E ortholog), and Qa-1b-restricted CD8^{+} T cell clones produced IFN-γ upon coculture with fibrosarcoma tumor cells derived from TAP$^{-/-}$ mice[33,34]. Adoptive transfer of a human TEIPP-specific CD8^{+} T cell clone into naïve mice injected with TEIPP-presenting RMA/S cells resulted in tumor infiltration and delayed RMA/S tumor growth[35]. TEIPP presentation by HLA-E by tumor cells might, similarly to the presentation of the UL40-derived peptide during CMV infection, result in evasion from NK cell clearance via ligation of HLA-E/TEIPP with CD94/NKG2A on NK cells[14]. Moreover, presentation of these novel, TAP-independent, epitopes might evoke specific effector cytotoxic T lymphocyte (CTL) responses that may contribute to tumor clearance. However, the relative effect of inhibitory CD94/NKG2A and cytolytic CD8^{+} TCR recognition of HLA-E/TEIPP on tumor killing need to be determined. In particular, we argue that TEIPPs with strong T cell-activating properties might be promising targets in future vaccines against cancer (Figure 1D)[33,36].

Rhesus macaques (RMs) immunized with a RhCMV vector encoding the simian immunodeficiency virus gag protein (RhCMV/SIVgag) elicited SIVgag/MHC-E-restricted CD8^{+} T cells that cleared infection in 9/16 RMs (no PCR detectable virus in plasma) after repeated limiting-dose SIV challenge, demonstrating the feasibility of MHC-E vaccine targeting[16,37]. Furthermore, CD8^{+} T cells recognizing HLA-E/peptide complexes were found in individuals with Salmonella serovar Typhimurium, *Mycobacterium tuberculosis* (*Mtb*), human immunodeficiency virus (HIV-1), CMV, and Epstein-Barr virus (EBV) infections, confirming the diversity of HLA-E binding peptides[38-44]. Most pathogens reside in intracellular compartments and manipulate host cell signaling pathways, possibly including antigen presentation pathways. *In silico* discriminant analysis predicted 69 *Mtb*/HLA-E peptides which induced CD8^{+} T cell proliferation from donors also recognizing whole mycobacterial extract (PPD) *in vitro*[6]. HLA-E restriction was demonstrated by comparing CD8^{+} T cell proliferation in response to peptide-pulsed K562-cells with and without HLA-E[6]. *Mtb*/HLA-E CD8^{+} T cells showed an unorthodox **type 2 T helper (Th2) phenotype** that had both regulatory and cytolytic effector functions and inhibited intracellular *Mtb* in macrophages *in vitro*[44,45]. Moreover, in contrast to HLA-Ia, HLA-E cell-surface expression was not susceptible to downregulation in primary CD4^{+} T cells infected with HIV-1. Targeting HLA-E may thus be interesting to induce effector CD8^{+} T cell responses in Tuberculosis (TB)/HIV-1 coinfected individuals, a common coinfection[46]. We argue that the strong effector responses of *Mtb*/HLA-E CD8^{+} T cells, and possible application in *Mtb*-HIV-1 coinfections, make HLA-E an interesting vaccine target in the combat against TB[46,47].

Pathogen HLA-E-specific CD8^{+} T cells have also been identified during other intracellular infections. Loading of Salmonella Gro-EL-derived peptides onto HLA-E^{+} B cell lines induced granzyme-B and IFN-γ production in CD8^{+} T cells[48]. In vitro expanded effector cells from volunteers vaccinated with a live attenuated typhoid vaccine were able to lyse S. serovar Typhimurium infected HLA-E^{+} B cells[48]. Similar *in vitro* cytotoxic phenotypes of human HLA-E-restricted CD8^{+} T cells were found in CMV, HIV-1, and EBV infections; however, such CD8^{+} T cells have not yet been characterized in detail, unlike *Mtb*/HLA-E CD8^{+} T cells, including the unusual Th2 phenotype[39,42,43].

T cells can recognize peptides presented by HLA-E, but the specific determinants for TCR recognition remain unknown. A few HIV-1 and CMV-specific TCRs have been sequenced and their functional characteristics determined (Box 2). However, this report comprises a limited dataset which will require expansion by including other pathogen- and tumor-specific TCRs[14,49,50]. Nevertheless, together, these studies indicate that cytotoxic CD8^{+} T cells can

recognize pathogens via HLA-E and contribute to controlling intracellular infections *in vitro*; this information warrants further investigation into the putative protective responses *in vivo*, which may inform potential HLA-E vaccine targeting.

Antigen processing and MHC-E presentation of membrane-compartmentalized bacteria

Phagosomal pathogens such as *Mtb* can activate MHC-Ia-restricted and MHC-E-restricted T cells in humans and mice (Figure 1E)[6,44,45,51-55]. Treatment of *Mtb*-infected moDCs with epoxomicin (a proteasome inhibitor) and brefeldin A (an ER-to-Golgi transport inhibitor) showed that IFN-γ production by an HLA-E-/*Mtb*-specific $CD8^+$ T cell clone mainly depended on proteasomal processing and only partly on ER-to-Golgi transport; by contrast, activation of a classical HLA-B/*Mtb*-restricted $CD8^+$ T cell clone relied on both mechanisms[56]. However, the proteasome inhibitor may have interfered with other cellular processes. In MelJuso cells, the proteasome inhibitor MG132 induced a redistribution of ubiquitin molecules from he nucleus to cytosol leading to aggregation, possibly following loss of ubiquitin on histones in the nucleus. This suggested that proteasome inhibitors can have 'off-target' effects at the epigenetic level[57]. The inhibitor studies on *Mtb*-infected moDCs[56] further suggested that *Mtb* antigens could translocate from phagosome to the cytosol for peptide processing, possibly via the proteasome, and be peptide-loaded onto HLA-E in the phagosome of *Mtb*-infected macrophages via retrotransport[52]. However, mechanisms via which *Mtb* antigens cross the phagosomal membrane into the cytosol for peptide processing are unknown but might involve **retrotranslocation** machinery. This was suggested because reduced IFN-γ production by a $CD8^+$ HLA-E T cell clone directed against *Mtb*-infected moDCs was observed in the presence of the retrotranslocon inhibitor ExoA. Alternatively, the *Mtb* virulence-associated ESX Type-VII translocon might participate in the transport of *Mtb* antigens into the cytosol, as evidenced by low amounts of cytosolic *Mtb* in moDCs infected with ESX translocon-deficient *Mtb* mutants relative to controls[52,58,59]. These results suggest that *Mtb* might exploit ER-independent HLA-E peptide-loading pathways that require cytosolic access.

MHC-E peptide-loading in phagosomal compartments

Phagosomes might fuse with the ER early after, or during, phagocytosis - a process termed **ER-mediated phagocytosis**. MHC-I peptide-loading machinery components such as TAP, calreticulin, calnexin, and tapasin, including MHC-I, have been reported to be present in isolated phagosomal membranes from ovalbumin (OVA) bead-loaded macrophages (J774-cells)[60]. *Mtb*-containing phagosomes in moDCs stained positive for Rab5, transferrin receptor, MHC-I, and TAP[52]. These *Mtb* phagosomal membranes contained higher amounts of HLA-E than HLA-Ia, with relatively low amounts of HLA-E at the cell membrane, as detected by Western blotting, suggesting that peptide loading occured in the phagosomal compartment[52]. Accumulation of HLA-E in intracellular compartments, especially during the differentiation of primary monocytes and monocytic cell-lines, was confirmed by Western blotting and cell-surface staining[30]. Moreover, TAP in *Mtb* phagosomes was hypothesized to facilitate peptide transport across phagosomal membranes into the lumen after cytosolic processing because *Mtb* peptides had a reduced capacity to enter the phagosomal lumen in purified phagosomes and in *Mtb*-infected moDCs treated with the TAP inhibitor UL49.5; one of these peptides also reduced IFN-γ production by a *Mtb*-specific HLA-E $CD8^+$ T cell clone[61]. However, this finding was based on an artificial biotin-streptavidin linkage of *Mtb* to UL49.5 and a single T cell clone. Therefore, the contribution of TAP to peptide transport into phagosomes requires further sub-

stantiation[61]. Combining current literature on *Mtb* infection and MHC-E-restricted T cells, we propose a model for MHC-E peptide processing and loading following phagocytosis of *Mtb* in macrophages (Figure 2, Key Figure).

Collectively, the results described here illustrate significant knowledge gaps concerning non-homeostatic peptide loading onto MHC-E. However, we propose that different intracellular pathways are involved in MHC-E peptide processing depending on the intracellular localization and on pathogen-mediated interference with host cell processing mechanisms.

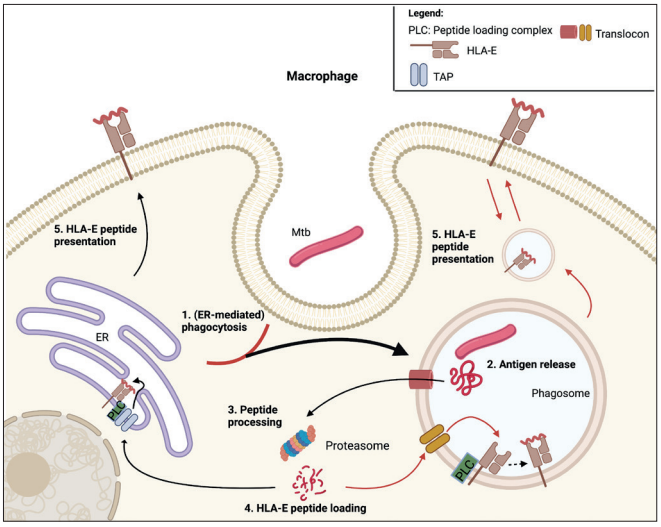

Figure 2. Schematic representation of hypothetical HLA-E loading of *Mycobacterium tuberculosis (Mtb)* peptides in macrophages. (Step 1) *Mtb* is taken up via endoplasmic reticulum (ER)-mediated phagocytosis, leading to proximity to the HLA-I loading machinery (i.e., calreticulin, tapasin, TAP1/2) and possibly to the ER retrotranslocation machinery in the phagosomal membrane[52]. (Step 2) Dismantling of the *Mtb* membrane leads to the 'escape' of *Mtb* antigens from the phagosome and the subsequent entry of these antigens into the cytosol via a translocon. This translocon can be the ER retrotranslocation machinery or the ESX type VII translocon inserted by *Mtb* itself[52,58]. (Steps 3 and 4) *Mtb* antigens are processed by the proteasome, and the resulting peptide fragments are either transported back to the phagosome via translocation machineries or to the ER where the peptides are loaded onto HLA-E molecules via the peptide-loading complex (PLC)[56]. (Step 5) Peptide-loaded HLA-E molecules translocate to the cell membrane via an unknown mechanism. Empty HLA-E molecules could originate from a recycling process at the cell membrane, the ER, or during the phagocytosis event itself. Unknown or undefined routes are indicated by red arrows, whereas established routes are indicated by black arrows. Figure created with BioRender.com. Abbreviation: TAP, transporter associated with antigen presentation.

Antigen processing and MHC-E presentation in viral infections

CMV, HIV-1, and EBV can evoke cytotoxic MHC-E-restricted T cell responses[14,38-40,42]. In CMV infections, MHC-E is loaded with viral VL9 during CMV-induced TAP inhibition to prevent NK-mediated lysis of CMV-infected cells resulting from MHC-I downregulation[11,13,14]. Viral VL9 is also essential for upregulating MHC-E cell-surface expression and ER release given that higher MHC-E cell-surface staining was detected on rhesus macaque fibroblasts cotransfected with the TAP inhibitor UL49.5 and viral VL9 compared to UL49.5 alone[11]. Moreover, in RM fibroblasts cotransfected with UL49.5 and viral VL9, MHC-E localized to early endosomal compartments lacking the lysosomal maturation marker LAMP-1[15]. Presumably, MHC-E peptide loading might have occurred in these compartments to avoid competition with higher-affinity canonical VL9 peptides in the ER. Recent studies further suggested that, at least in CMV infection, TAP inhibition likely plays an essential role in inducing MHC-E T cell responses[15] (Box 3).

Moreover, in chronically HIV-1 infected patients, two HLA-Ia-restricted HIV gag epitopes elicited a

HLA-E-restricted **polyfunctional T cell response** by $CD8^+$ T cells *ex vivo* and *in vitro*[62]. Allele-specific as well as shared TCR sequences were identified, suggesting that TCR crossreactivity might underlie this dual recognition - a possibility that warrants further investigation. Of note, the overall frequency of $CD8^+$ T cells from chronically infected donors producing IFN-γ and expressing CD107a upon HLA-E/peptide stimulation was lower compared to HLA-Ia, and the overall contribution and significance of HLA-E-restricted $CD8^+$ T cells in HIV-1 infections therefore remains to be determined.

Together, these results show that HLA-E-restricted $CD8^+$ T cells directed against pathogens are induced following natural viral and bacterial infections and can potentially be targeted via vaccination if specific conditions are met for HLA-E restriction. We consider that the effector profile of HLA-E-restricted $CD8^+$ T cells is encouraging. We strongly support further investigation of MHC-E-restricted TCRs and their functional properties, diversity, and peptide-ligand interactions.

Concluding remarks

Understanding MHC-E peptide-binding requirements together with the search for immunogenic epitopes and pathway(s) involved in antigen processing and peptide loading might expediate the development and improvement of MHC-E-targeting vaccines. MHC-E is highly conserved in humans and vertebrates, and this facilitates the evaluation of candidate vaccines as well as mechanistic studies in relevant disease models. Nevertheless, many questions remain to be answered (see **Outstanding questions**). Recently, MHC-E peptide binding and presentation were suggested to also occur independently from TAP[11]. Accordingly, we propose that the SPase/SPPase-mediated, furin-mediated and autophagy-mediated pathways represent alternative, potentially TAP-independent, MHC-E peptide-loading mechanisms. This hypothesis merits further assessment because alternative antigen-presentation pathways may require specific targeting to elicit MHC-E-restricted effector T cells. In humans, HLA-E targeting may be of limited applicability if peptide-loading pathways are not generally accessible; however, detection of HLA-E-restricted T cells upon natural infection is reassuring. Further work will be necessary to dissect whether TAP-independence is a general feature of MHC-E antigen presentation, and whether loading pathways depend on intracellular antigen localization, the pathogen or tumor type, as well as on the transformed/infected cell type. Future work should also dissect the molecular details of TCR recognition of MHC-E/peptide complexes to identify the most immunogenic and translatable peptides for potential vaccination protocols. We propose that an important first step should be detailed profiling of HLA-E-restricted TCR sequences followed by functional characterization of the identified TCRs and T cells; this should help to understand the requirements for T cell recognition as well as the functional and phenotypic properties of HLA-E-restricted T cells along the spectrum of **hypervariable T cells** to **invariant T cells**[63]. Moreover, it will be important to dissect the requirements of MHC-E/TCR versus MHC-E/CD94/NKG2(A/C) to determine the balance between T cell activation and NK cell inhibition, and to determine whether these interactions are molecularly distinct. We argue that answering these key questions will advance MHC-E-centered vaccine technology approaches because MHC-E peptide loading and T cell restriction likely require unique pathways to induce potent effector immune cell responses against tumors and pathogens.

Box 1. Generation of HLA-E peptide-binding motifs

The human HLA-E peptide-binding repertoire was initially thought to be relatively limited, and peptides derived from HLA class Ia leader sequences were known to bind strongly to HLA-E[64-66]. Crystal structures of HLA-E in complex with such leader sequences from several HLA-Ia alleles revealed a conserved configuration of these translated peptides in the peptide-binding groove (PBG), with primary anchor residues 2 and 9 buried in the groove and a kinked, solvent-exposed conformation at positions 4 and 5[67]. In contrast to these leader sequences, recently resolved crystal structures of HLA-E in complex with *Mycobacterium tuberculosis* (*Mtb*)-derived Mtb44 (RLPAKAPLL) and HIV-1-derived RL9HIV (RMYSPTSIL) showed that peptide-binding to HLA-E was less canonical and more flexible than was previously thought[9]. A recently developed high-throughput HLA-E/peptide-binding assay based on UV-mediated cleavable peptide exchange allowed a detailed and larger-scale assessment of HLA-E-binding peptides, including combinatorial peptide libraries, and resulted in an improved predictive HLA-E/peptide-binding motif[7,10]. These studies confirmed the previously described main anchor positions 2 and 9 for HLA-E-binding peptides, and a preference for large hydrophobic residues (Met and Leu) was established through sequence alignment of the identified peptide sequences. It also demonstrated the binding of peptides containing additional main anchor residues such as hydrophobic Val and Phe, or polar Gln, at positions 2 and 9[7]. Analysis of amino acid substitutions indicated that the tolerability for alternative main anchor residues could be supported by the presence of a central Pro, a rigid amino acid which might promote a compensatory peptide configuration suitable for HLA-E binding[7]. In addition, residues at central position 5 of HLA-E-binding peptides could be large hydrophobic Trp or small Ala, potentially accommodating a deeper or shallower conformation of positions 6 and 7, respectively, in line with published crystal structures[9]. Additional structural analyses will be necessary to further elucidate HLA-E pocket occupancy and exposure of central residues outside the pocket, including interactions with the side chains of alternative binding peptides composed of non-canonical anchor residues, complemented by affinity and avidity measurements. Additional insights on the molecular characteristics of HLA-E-binding peptides will be crucial for optimizing HLA-E binding motifs and improving predictive algorithm-based selection of novel HLA-E-presented peptides derived from tumors or pathogens such as SARS-CoV-2, among others.

Box 2. Understanding T cell recognition of HLA-E/peptide complexes

TCR sequencing of $CD8^+$ T cells primed with an HLA-E-restricted peptide derived from HIV-1, called RL9HIV showed no dominant or preferential TCRα or -β V-gene segment usage, suggesting that this constitutes a polyclonal TCR repertoire despite recognizing an HLA-I molecule with limited allelic variation[49]. Although each of these TCRs can recognize HLA-E/RL9HIV, the TCRs differ in their activation thresholds and signaling efficiency[49]. Further support for a polyclonal TCR repertoire in MHC-E T cells was derived from CMV-specific $CD8^+$ T cells and three TEIPP-specific Qa-1^b-restricted T cell clones in mice; these harbored conserved TCRα V-segments, but diverse TCRβ V- and J-segments[14,33,35]. These initial results suggested that the diversity of HLA-E-restricted TCRs might be comparable to TCRs that recognize polymorphic MHC-Ia molecules[14,33,35,62]. However, to further substantiate this, extensive TCR profiling of T cells recognizing a broader HLA-E/peptide repertoire will be needed. In addition to TCR repertoire analysis, TCR affinity is important; studies on the CMV UL40-derived peptide (viral VL9) revealed that specific human TCRs differed in their affinity for HLA-E/viral VL9 complexes[14]. Perhaps to avoid autoreactivity with canonical HLA-E peptides, lower- and higher-affinity TCRs specific for HLA-E/viral VL9 complexes were found to regulate T cell activation via differential NK cell receptor

expression on HLA-E-restricted T cells. T cells expressing lower-affinity TCRs for HLA-E/viral VL9 complexes, concomitantly expressed strong activating NK cell receptors such as the CD94/NKG2C coreceptor, and cells with lower-affinity TCRs expressed inhibitory receptors such as CD94/NKG2A (important for minimizing autoreactivity) by coregulation of $CD8^+$ T cells by NK cell receptors, activated in response to pathogen-derived signals[50]. It will be interesting to investigate whether TCRs that recognize other more divergent HLA-E peptides use a similar mechanism to control T cell activation.

Box 3. Detection of MHC-E-restricted T cells during human CMV infections

The induction of $CD8^+$ T cell responses to peptides presented by MHC-E, including the possible role of TAP remains elusive and understudied. One study assessing the presentation of SIVgag protein on MHC-I molecules by B cell lymphoblastoid cells showed that inhibition of TAP via UL49.5 negatively affected $CD8^+$ T cell recognition and the expression of MHC-Ia molecules, but not of MHC-E, *in vitro*[68]. However, inhibition of TAP was not sufficient to direct $CD8^+$ T cell responses towards MHC-E in rhesus macaques (RMs). These findings suggest that mechanisms other than TAP inhibition may be involved in directing $CD8^+$ T cell responses to MHC-E *in vivo*, but this remains to be tested. Another study provided insights into these additional viral determinants[15]. RMs that were vaccinated with the Rhesus CMV strain 68-1 vector (RhCMV68-1) encoding a SIVgag protein induced broad and effective MHC-E- and MHC-II-restricted SIVgag-specific $CD8^+$ T cell responses, as evidenced by deconvolution of recognized epitopes. The difference between RhCMV68-1 and wild-type RhCMV is the absence of several genetic sequences that affect the function of a pentameric receptor complex (PRC) that is used to infect non-fibroblast cells[15]. These genes, Rh157.5, Rh157.4, Rh158, Rh161 (UL128, UL130, UL146 and UL147 respectively in human CMV infections), encode CXC chemokine-like proteins. When these four genes were deleted from the wild-type RhCMV strain, SIVgag-specific $CD8^+$ T cell responses were restricted to MHC- II and MHC-E *in vitro*[15]. Consequently, although the viral VL9 peptide is required for MHC-E ER egress and cell-surface expression, inhibition of the RhCMV pentameric complex is likely needed for the induction of MHC-E-restricted $CD8^+$ T cell responses during CMV infection, pending further investigation to fully understand how MHC-E antigen presentation might occur during CMV infections. The exact (intracellular) mechanism(s) through which these CXC chemokine-like proteins and the PRC can direct T cells towards MHC-Ia or MHC-E remain to be determined, as well as whether pathogens other than CMV might use PRC-like strategies to direct VL9 mimics or other sequences into HLA-E to elicit NK recognition.

Glossary

- **Canonical:** standard or common features of a pathway or molecule. Non-canonical molecules represent alternatives but can exploit the same pathway as canonical molecules.
- **Endosomal compartments:** double or single membrane-enclosed organelles that can be formed following endocytosis of extracellular particles.
- **ER-mediated phagocytosis:** the endoplasmic reticulum (ER) acts as a membrane source for the formation of phagosomes following phagocytosis of extracellular particles.
- **Hypervariable T cells:** MHC-restricted T cells that can recognize highly polymorphic MHC-molecules.
- **Invariant T cells:** T cells, not necessarily MHC-restricted, that recognize ligands presented on genetically res-

tricted antigen presentation molecules.

- **Major histocompatibility complex (MHC):** MHC molecules can present peptides to T cells. The genetic locus encoding MHC molecules is polymorphic, resulting in diverse allelic MHC variants in an individual and between individuals.
- **Peptides:** short sequences of amino acids derived from proteins that are generated during antigen processing. These peptides can be translocated to the ER for MHC loading and displayed at the cell surface.
- **Peptide-binding groove (PBG):** the part of MHC molecules that accommodates peptides. MHC-restricted peptides have several anchor residue positions that occupy pockets in the PBG for optimal positioning.
- **Peptide-loading complex (PLC):** a multi-subunit complex consisting of the proteins calreticulin, ERp57, tapasin and the TAP1/2 subunits. The PLC is localized to the ER-membrane and is crucial for loading peptides onto MHC molecules.
- **Polyfunctional T cell response:** T cells that can mediate effector (e.g., cytotoxic) responses and can produce cytokines to activate humoral immune responses.
- **Proteasome:** a cylindric cytosolic enzymatic protein complex consisting of several subunits. The core subunits comprise the active site and have proteolytic activity to degrade (misfolded) proteins. Proteins destined for proteasomal degradation are tagged with ubiquitin.
- **Retrotranslocation:** the translocation of misfolded proteins or peptides from the ER lumen to the cytosol.
- **T cell epitopes associated with impaired peptide processing (TEIPPs):** epitopes expressed by tumor cells with impaired TAP function that can be recognized by T cells. TEIPPs are non-mutated and represent a separate category of neoepitopes in tumor cells.
- **Transported associated with antigen presentation (TAP):** consist of two subunits, TAP1 and 2, that are localized to the ER membrane to transport of peptides from the cytosol to the ER lumen. These peptides can then be loaded onto ER-resident MHC-using the PLC.
- **Type 2 T helper (Th2) phenotype:** a subclass of $CD4^+$ T helper cells that are mainly involved in regulating and activating humoral immune responses such as B cell activation. Th2 cells secrete specific cytokines (e.g., IL-4, IL-5 and IL-13).
- **Ubiquitin:** small molecules that are added to substrates in eukaryotic cells to alter their activity or direct substrates for proteasomal degradation.
- **UL40-derived peptide:** a cytomegalovirus (CMV) peptide that is sequentially similar to endogenous VL9 peptides derived from MHC-Ia leader sequences; UL40 peptide engagement with inhibitory natural killer (NK) cell receptors ensures the survival of CMV-infected cells.

Outstanding questions

- How are HLA-E-restricted peptides loaded onto endosomally localized HLA-E molecules? HLA-E molecules are present in *Mtb*-infected phagosomes, and activation of HLA-E-restricted T cells partly depends on ER-to-Golgi transport in the presenting cells. Moreover, CMV induces upregulation of HLA-E molecules at the cell surface but also colocalized to endosomal compartments. Further research should dissect whether peptide loading can occur in these endosomal compartments.
- Are endosomally localized HLA-E molecules empty and, if so, how are these complexes stabilized? It is un-

known whether HLA-E molecules present in endosomal compartments are stabilized by peptide ligands or remain empty. Classical HLA-I molecules collapse in the absence of 'rescuing' peptide, but it remains unknown whether empty HLA-E complexes are stable or whether they require accessory molecules or rescue peptides.

- Does the HLA-E antigen presentation route depend on the intracellular localization of the antigen and the infected cell type? Pathogens usually have a preferred cell type which they (persistently) infect or are taken up by antigen-presenting cells for destruction and the induction of T cell responses. Is there a dominant conserved pathway for HLA-E antigen processing and presentation that is common to all cell types and pathogens, or is this (partly) dependent on the cell type and pathogen?
- Can all HLA-E-presented peptides also interact with the CD94/NKG2 coreceptors, and what is the functional consequence of this interaction? Recent studies have identified novel HLA-E peptides that can be recognized by TCRs[6,42,48,62]. However, it remains to be determined whether these peptides can be recognized by NK cells and what the consequences of this 'dual' response might be.
- What is the unique contribution of HLA-E-restricted T cells in combatting infectious agents? HLA-E-restricted T cells have been identified in several infections, and in some cases these T cells have been essential for pathogen clearance. It remains to be determined why these HLA-E-restricted T cells are indispensable in the combat against particular infectious agents.

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Declaration of interests

The authors declare no competing interests.

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