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

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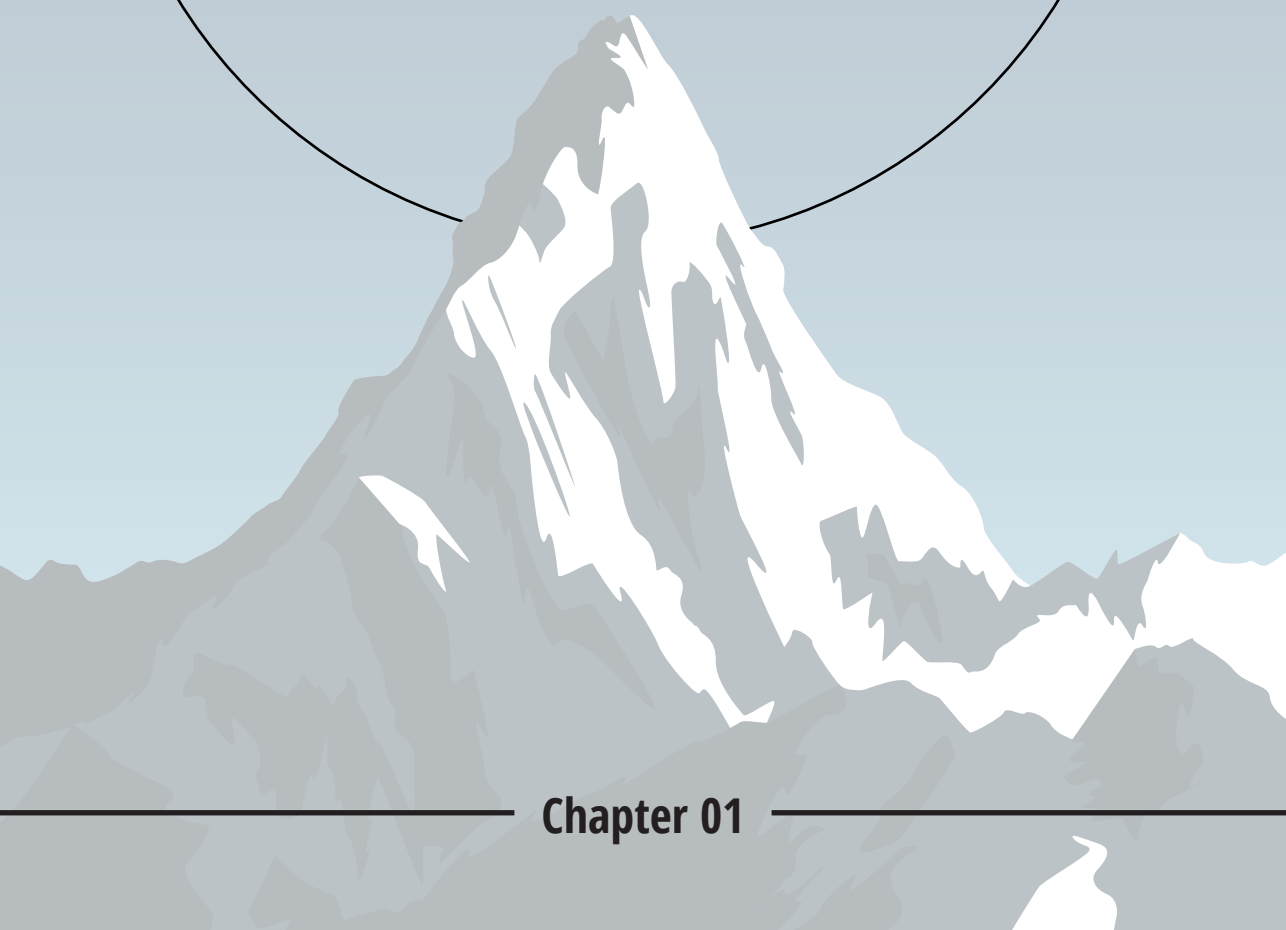
General Introduction



Chapter 01

1.1 Tuberculosis disease

Robert Koch was the first person to isolate *Mycobacterium tuberculosis* (*Mtb*), the causative pathogen of Tuberculosis (TB) disease, in 1882[1]. To date, TB remains a significant global health problem with high mortality and morbidity rates. About a quarter of the world´s population is infected with *Mtb* and more than 1 million people die from TB each year, making TB the world's deadliest infectious disease caused by a single pathogen[2]. The highest incidence of TB is in India, Asian countries and Southern Africa, collectively accounting for more than two thirds of all TB cases[2]. Although TB is considered a respiratory disease, referred to as pulmonary TB, it can also impact other parts of the body, such as the intestines, skin, lymph nodes, kidneys and the central nervous system, which is known as extrapulmonary TB. *Mtb* is transmitted via inhalation of *Mtb*-containing aerosols and mainly infects alveolar macrophages present in the lower respiratory tract (Figure 1)[3,4]. After intracellular uptake by these macrophages, *Mtb* is targeted to endosomal compartments, called phagosomes. *Mtb* has evolved several strategies to inhibit fusion of phagosomes with lysosomes. This inhibition is crucial for *Mtb* to evade destruction by the acidic environment inside lysosomes, allowing *Mtb* to replicate and survive inside the host cell[5]. *Mtb* can persist inside the phagosome and has the ability to translocate *Mtb*-derived proteins, such as CFP-10 and ESAT-6, to the cytosol via insertion of the secretion system ESX-1 into the phagosomal membrane[3,6]. The ESX-1 secretion system is also involved in the translocation of whole *Mtb* bacteria to the cytosol[6]. When *Mtb*-infected cells cross the lung epithelial barrier and enter the lung parenchyma, multicellular structures form together with recruited innate and adaptive immune cells, such as T cells, B cells, neutrophils and myeloid cells. These structures are called granulomas (Figure 1)[3]. Granulomas can contain *Mtb* infection for decades, but when the bacterial burden becomes too high and/or host immunity wanes (see below) leading to escape of immune control, *Mtb* can disseminate to the lung (pulmonary TB) or to other parts of the body (extrapulmonary TB) resulting in TB reactivation (Figure 1)[3].

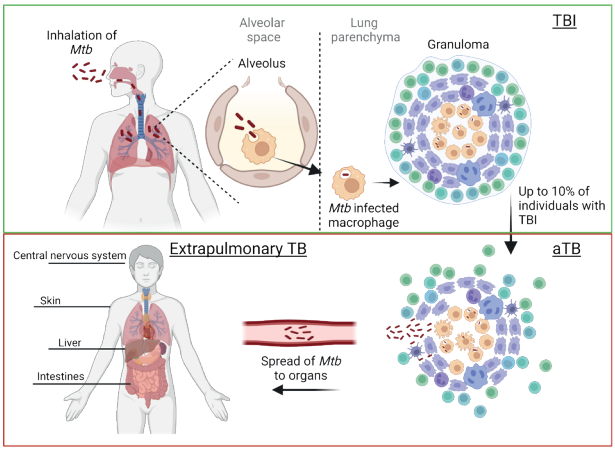


Figure 1. TB disease pathology in individuals with TBI or aTB. *Mtb* enters the lung via inhalation of *Mtb*-containing particles and infects alveolar macrophages. Multicellular granulomas form in the lung parenchyma after recruitment of other (immune) cells to *Mtb*-infected macrophages. In TBI, granulomas are contained, whereas in aTB (ca. 10% of individuals with TBI) granulomas burst allowing, in the case of extrapulmonary TB, *Mtb* to disseminate to other parts of the body. Figure created in BioRender.com, inspired by Pai et al., 2016[4].

Mtb infection in humans is measured with the tuberculin skin test (TST) or the more specific interferon-gamma (IFN-γ) release assay (IGRA), which both measure immune reactivity towards *Mtb* antigens[4]. A proportion of people can eliminate *Mtb* infection after exposure. In contrast, *Mtb*-infected individuals can develop a TB infection, called TBI, or an active form of TB, called active TB (aTB) (Figure 1). The TBI and aTB forms in fact

comprise a spectrum of disease states stratified by severity and pathogenic activity[4]. Individuals with aTB are major contributors to the spread of *Mtb*. Individuals with TBI may contain *Mtb* infection for years to decades without progressing to aTB and without displaying clinical symptoms. However, up to 10% of these individuals eventually progress towards aTB[7]. Various factors increase the risk of progressing to aTB, such as HIV co-infection, malnutrition, diabetes, aging and intake of immunosuppressants[4]. Clinical symptoms of (pulmonary) aTB include severe coughing, tiredness, night sweating and weight loss. TB can be cured by a lengthy treatment of 4 to 9 months often consisting of a combination of antibiotics, such as isoniazid in combination with rifampin[7]. However, the rise of (multi-)drug resistant *Mtb* strains complicates treatment options and is one the factors contributing to the high mortality rate of TB. Mathematical models show that development of vaccines preventing TB disease could reduce incidence rates by up to 50%, especially vaccines that target *Mtb*-infected adults[8-10]. Development of novel vaccines complementing or replacing the Bacillus Calmette-Guérin (BCG) vaccine (see below) is therefore crucial to combat the global TB pandemic and to meet the ''End TB'' goal set by the World Health Organization.

1.2 Bacillus Calmette-Guérin (BCG): a 100-year-old vaccine against TB

After Robert Koch discovered the causative pathogen of TB, attempts were made to limit the transmission of *Mtb*, such as implementation of better hygiene procedures. These efforts decreased the number of TB cases drastically[1]. A major breakthrough to prevent TB via vaccination was made by Léon Charles Albert Calmette and Camille Guérin, two French scientists at Institut Pasteur in Lille, France. Calmette and Guérin sub-cultured an isolated strain of Mycobacterium Bovis (M. Bovis) derived from TB-affected cattle for an estimated 230 times between 1908 and 1921 by continued transfer of previous M. Bovis cultures to fresh growth medium. This way, virulence of the M. Bovis strain was lost, but the ability to protect against *Mtb* in a guinea pig model of aTB was maintained[11]. This live-attenuated form of M. Bovis was named Bacillus Calmette-Guérin (BCG) after the scientists in 1921[11]. BCG was used to vaccinate against TB ever since, initially infants but later also adults, via intradermal injection into the upper arm. Soon after its discovery in France, BCG was distributed to various other countries, such as Denmark, Japan and China and was cultured under different conditions[11]. This led to the formation of more than 50 genetically distinct BCG strains, now known to have variable vaccine efficacies[12]. Currently, BCG-Denmark is the most frequently used strain for vaccination globally[12]. The BCG vaccine is included in the vaccination program for children in many countries, especially where TB incidence is high. The efficacy of BCG to protect against severe TB is high in infants, but decreases for pulmonary TB during adolescence and in adults, ranging from 0% to 80%[13]. The variation in protection efficiency is not well understood, but shows associations with BCG strain, host genetics, exposure to non-tuberculous mycobacteria and geographical factors such as latitude[13]. More than 100 years after its discovery, BCG continues to be the only licensed vaccine against TB, despite evaluation of novel strategies. Besides protection against TB, BCG also induces non-specific (i.e., heterologous) responses. For instance, BCG reduces the overall childhood mortality risk by up to 30% and protects against non-mycobacterial infections[14]. The mechanisms behind these heterologous effects are not fully understood, but they may be linked to the induction of trained innate immunity[14]. It is therefore crucial to consider these heterologous effects of BCG when a new vaccine against TB becomes available. Nevertheless, the urgent need for a novel vaccine against TB remains and is needed to curb the global pandemic.

1.3 Classical CD4+ and CD8+ T cell responses in TB

In contrast to innate immune cells which can mount broad immune responses, adaptive immune cells, i.e., B cells and T cells, can induce responses towards a specific pathogen and can develop immunological memory towards the pathogen enabling rapid expansion after a second encounter. The specificity and ability to develop memory make adaptive immune cells attractive targets for vaccination. Memory and effector T cells are generated during T cell priming. In this process, professional antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), travel to secondary lymphoid organs and present short peptide sequences from immunogenic pathogen-derived antigens called epitopes on human leukocyte antigen (HLA) molecules[15]. There are two types of HLA molecules, namely HLA-I and HLA-II, that present epitopes for recognition by respectively $CD8^+$ or $CD4^+$ T cell receptors (TCRs). The HLA locus is highly polymorphic resulting in large genetic diversities between individuals in the expressed HLA molecules and thus the epitopes that can be presented to TCRs for induction of T cell responses. This large diversity of classical HLA molecules has an evolutionary advantage as it enhances the likelihood of eradicating pathogens and, consequently, increases the survival of the human population. Besides epitope recognition, two additional signals from APCs are needed to prime and differentiate T cells. These are co-stimulatory signals and cytokines. $CD4^+$ T cells can differentiate into T helper (Th) 1, Th2 or Th17 cells, including into regulatory T cells (Tregs). $CD8^+$ T cells can differentiate into effector cells capable of inducing cytotoxic or cytolytic responses towards infected or deregulated cells. $CD4^+$ and $CD8^+$ T cell subsets have distinctive secreted cytokine and chemokine profiles and express subset specific receptors at the cell surface. Differentiated $CD8^+$ and $CD4^+$ T cells can subsequently induce functional responses towards infected cells, whereafter T cells progress into a resting or memory state, from which they can divide and respond quickly upon encountering the same antigen[16]. Hence, development of immunological memory is essential for vaccine efficacy.

$CD4^+$ T cells in particular play an indispensable role in controlling *Mtb* infections[17,18]. This is best illustrated by the increased risk of TB in HIV-infected individuals as HIV infection leads to depletion of $CD4^+$ T cells[19]. $CD8^+$ T cells are also part of the human adaptive immune response against *Mtb* as they can lyse *Mtb*-infected cells but are arguably less crucial than $CD4^+$ T cells[20]. $CD4^+$ T cells can directly interact with *Mtb*-infected cells and can exert several effector and helper functions[18]. An important $CD4^+$ T cell effector response against *Mtb* is the production and secretion of the pro-inflammatory cytokine IFN-γ. IFN-γ activates signaling pathways in macrophages leading to, e.g., the production of reactive oxygen species to limit intracellular *Mtb* replication[21]. IFN-γ is therefore commonly seen as the key cytokine associated with *Mtb* control[17]. In addition to IFN-γ, *Mtb* specific $CD4^+$ Th1 cells secrete TNF-α and IL-2, and $CD4^+$ Th17 cells IL-17. Besides Th1, $CD4^+$ Th1* (i.e., Th1/Th17) cells are another subset recently found to induce protective responses against *Mtb*[22]. $CD4^+$ T cells further secrete chemokines to recruit other immune cells to the infection site[23]. $CD4^+$ T cells are hence frequent targets for vaccination against TB. Candidate *Mtb* antigens are frequently selected based on the ability to induce IFN-γ secretion in *Mtb*-infected individuals, reflecting their antigenicity. This initially led to the identification of Ag85A-C, CFP-10 and ESAT-6, amongst other *Mtb* antigens, as possible candidates[23]. Novel techniques, such as whole genome sequencing, broadened the repertoire of possible candidate *Mtb* antigens further[24]. Nevertheless, despite validating the efficacy of these *Mtb* antigens in pre-clinical models and in humans, classical $CD4^+$ T cell targeting vaccination strategies have not shown improved efficacy over BCG thus far. Only one promising vaccine called M72/AS01 (inducing Th1 cells, Th17 cells, $CD8^+$ T cells and antibodies/B cells) showed 50% protection

against pulmonary TB in *Mtb*-infected adults and is currently evaluated in phase III clinical studies[25,26]. Alternative vaccination targets should therefore be considered.

1.4 Donor unrestricted T cells as vaccination targets

The genetic diversity of classical HLA molecules is a major hurdle to finding antigens capable of inducing T cell responses in genetically diverse populations. Unconventional T cells are a separate category of T cells and bridge the gap between innate and adaptive immunity[27]. Family members include γδ T cells, defined by the expression of γδ TCRs, MHC-related protein 1 (MR1) T cells, cluster of differentiation 1 (CD1) T cells, NK T cells and HLA-E restricted T cells, which all express αβ TCRs (Figure 2)[28]. Unconventional T cells recognize non-polymorphic antigen presentation molecules presenting small metabolites (MR1), lipids (CD1), phosphorylated antigens (BTN) and peptides (HLA-E). HLA-E, but not CD1 and especially MR1, is structurally homologous to classical MHC-Ia molecules[29]. Because of the conserved nature of these antigen presentation molecules, T cells recognizing them are unrestricted to a donor's genetic background and are called donor unrestricted T cells (DURTs)[29,30].

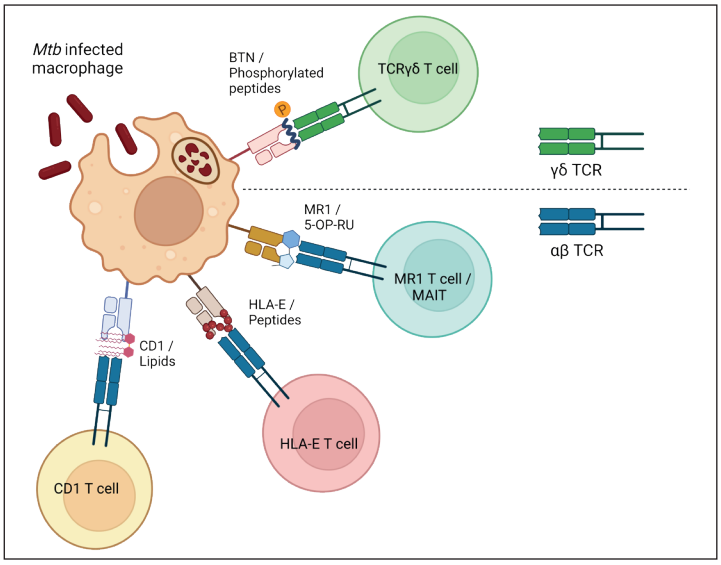


Figure 2. Overview of DURT subsets that can recognize *Mtb*-derived ligands presented by non-polymorphic antigen presentation molecules at the cell surface of *Mtb*-infected cells, such as macrophages. TCRγδ T cells recognize *Mtb*-derived phosphorylated peptides, MR1 T cells recognize *Mtb*-derived metabolites, such as 5-OP-RU, HLA-E T cells recognize *Mtb*-derived peptides, CD1 T cells recognize lipids from the *Mtb* cell wall. MR1, HLA-E and CD1 T cells express αβ TCRs and TCRγδ T cells γδ TCRs. Figure created in BioRender.com.

DURTs are potential vaccination targets for TB as they enable induction of population-wide responses irrespective of the genetic background using a single antigen (Figure 2). Mycolic acid (MA), glucose monomycolate and various other lipids derived from the *Mtb* cell wall can be presented on CD1 (Figure 2)[30]. CD1 restricted T cells recognizing MA are present in the circulation of individuals with latent or active TB, as determined using CD1 tetramers, and their frequencies are increased in the lung compared to the circulation[31]. MA was also evaluated in a pre-clinical vaccination study in mice, which revealed that MA could be deposited in alveolar macrophages upon vaccination, possibly acting as depots for long-lasting antigen delivery to CD1 restricted T cells[32]. Mucosal-associated invariant T cells (MAIT cells) are well-known MR1 restricted T cells recognizing, amongst others, the riboflavin-derived metabolite 5-OP-RU (Figure 2)[30]. This metabolite can only be produced by bacteria, including

Mtb, and not by human cells. MAIT cells preferentially home to mucosal tissue, such as the liver and lungs. In line with this, MAIT cell frequencies are higher in the lungs compared to the circulation in TB-infected individuals[33]. A pre-clinical study in mice evaluated 5-OP-RU as a vaccination modality against TB and showed that 5-OP-RU administration did not reduce lung *Mtb* loads after an initial infection, whereas *Mtb* loads were reduced after 5-OP-RU administration in chronically *Mtb*-infected mice[34]. However, this was not observed in 5-OP-RU vaccinated and *Mtb*-infected non-human primates (NHPs)[35]. In fact, MAIT cells were dysfunctional after vaccination and were unable to control *Mtb*. In addition to these findings, 5-OP-RU is unstable under physiological conditions, which further complicates MAIT cell targeting vaccination strategies. Similar to MAIT cells, TCRγδ T cells also migrate to mucosal sites and recognize phosphorylated antigens presented by the non-polymorphic antigen presentation molecule butyrophilin (BTN) or CD1 (Figure 2)[36]. Multiple *Mtb*-derived phosphoantigens, such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) and 6-O-methylglucose lipopolysaccharides (mGLP), have been identified as *Mtb*-derived BTN ligands[30]. Frequencies of TCRγδ T cells are higher in individuals with TB compared to healthy individuals and *in vitro* experiments revealed that a subpopulation of TCRγδ T cells expressing Vγ9Vδ2 TCRs could help controlling intracellular *Mtb* growth in infected macrophages[37]. Immunization of NHPs with an attenuated Listeria strain producing HMBPP prior to high dose *Mtb* challenge led to expansion of Vγ9Vδ2 T cells and these NHPs had reduced lung *Mtb* loads and extrapulmonary *Mtb* dissemination compared to control NHPs[38].

Besides these DURTs, *Mtb* specific T cells recognizing the virtually monomorphic molecule HLA-E are circulating in TB-infected individuals and can exert several functional responses. Multiple *Mtb*-derived peptides for HLA-E restricted T cells have been identified (Figure 2)[39-41]. The exploration of HLA-E/*Mtb* specific T cells as vaccination targets for TB will be described in this thesis.

1.5 The virtually monomorphic antigen presentation molecule HLA-E

HLA-E plays a crucial role in maintaining cellular homeostasis via interaction with NKG2A or -C/CD94 complexes expressed on NK cells and can present peptides to T cells, thus HLA-E is involved in both innate and adaptive immunity (Figure 3)[42,43]. Structurally, HLA-E is nearly identical to classical HLA-I molecules and is composed of a heavy chain where the peptide binding part is located associated to a β2M subunit. Although humans encode 27 allelic variants of HLA-E, only two of them are functionally expressed at the cell surface of nearly all nucleated cells[44]. These two alleles are called HLA-E*01:01 and HLA-E*01:03 and are expressed in a balanced equilibrium among the human population[45]. The highest expression of HLA-E is on endothelial cells and various immune cells, especially on monocytes. The subcellular expression of HLA-E is at the cell membrane and presumably also in endosomal compartments[46]. The two alleles differ by a single amino acid located outside the peptide binding groove at amino acid position 107: arginine for HLA-E*01:01 and glycine for HLA-E*01:03[43,47]. The location outside the peptide-binding groove (PBG) suggests limited effects on the peptide binding repertoire between alleles, hence its classification as an unconventional and virtually monomorphic antigen presentation molecule.

HLA-E can present nine amino acid long peptides derived from the signal peptide (SP) sequence of classical HLA-I molecules, which are called VL9 peptides[43] (Figure 3). The antigen presentation pathway for SP processing and HLA-E peptide-loading of VL9 peptides occurs in the Endoplasmic Reticulum (ER) via the proteolytic activity of several signal peptide peptidases and the transporter associated with antigen processing (TAP),

respectively[48,49]. This canonical antigen presentation pathway will be described in **Chapter 2**. The sequence of VL9 peptides depends on the classical HLA-I allele (i.e., HLA-A, B and C alleles) and are frequently variations of the sequence VMAPRTLLL with the highest residue variation at position 8. Affinity of VL9 peptides for HLA-E depends on the sequence and thus the HLA-I allele[50]. Residues flanking VL9 peptide sequences in the SP affect peptide processing and the subsequent availability of VL9 peptides for HLA-E peptide loading[50]. The peptide binding motifs and peptide affinities are comparable between HLA-E*01:01 and *01:03, although HLA-E*01:01 is somewhat less stringent in the preferred binding residues[51]. Loaded HLA-E/peptide complexes subsequently translocate from the ER to the cell membrane where they can be recognized by the mentioned NKG2A or -C/CD94 receptors or TCRs expressed on natural killer (NK) cells and T cells, respectively[39,52].

The PBG of HLA-E is composed of six pockets similar to classical HLA-I molecules that can accommodate specific residues of the peptide. Studies on the peptide binding motif for HLA-E revealed that position 2, 7 and 9 are the primary anchor residue positions and favor hydrophobic residues, particularly leucine and methionine, that fit into deep hydrophobic pockets of the PBG (Figure 3)[53,54]. Positions next to the anchor residues preferentially accommodate smaller residues that fit into shallow pockets, possibly facilitating binding of larger and hydrophobic residues at the anchor positions[51]. VL9 peptides adopt a classical kinked conformation in the HLA-E PBG, due to proline at position 4, exposing residues at positions 4 and 5 to the solvent[55]. These HLA-E/VL9 complexes are structurally rigid, homogenous and stable[56].

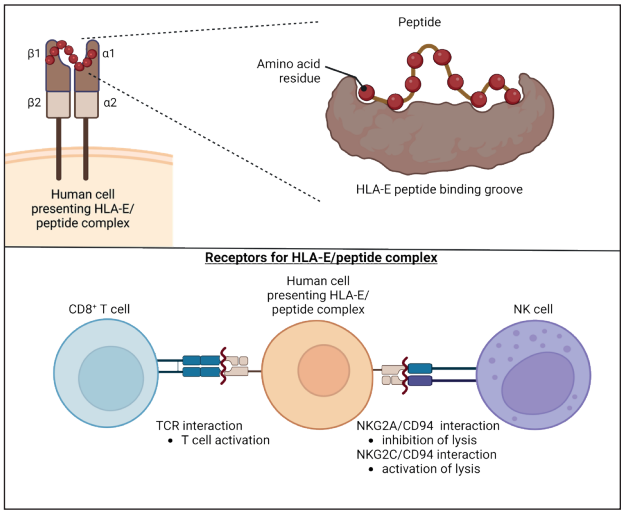


Figure 3. Schematic representation of the HLA-E/peptide complex (upper panel) and its receptors (lower panel). HLA-E contains a heavy and light chain. The peptide binding groove is located in the heavy chain part. Canonical peptides adopt a kinked motif exposing residues 4 and 5 to the solvent. Residues 1, 2, 7 and 9 are buried in the groove and are primary anchor residues. Lower panel: TCRs and NKG2A -C/CD94 are the two main receptors for HLA-E/peptide complexes. Interaction with TCRs leads to activation of $CD8^+$ T cells. Interaction with NKG2A or -C/CD94 leads to inhibition or activation, respectively, of NK cell mediated lysis of the HLA-E/peptide presenting cell. Figure created in BioRender.com.

Besides VL9 peptides, HLA-E can present a wider variety of peptides that are not only derived from endogenous sources but also from pathogens. This is known for HIV, cytomegalovirus (CMV), Salmonella Typhimurium, Epstein-Barr virus and *Mtb*, amongst other pathogens[39,57-59]. Pathogen-derived peptides can be much more diverse than VL9 peptides in terms of amino acid composition and generally have lower affinities for HLA-E[56,60]. In contrast to VL9 peptides, lower affinity pathogen-derived peptides adopt a non-classical conformation in the PBG and form structurally heterogenous HLA-E/peptide complexes, indicating formation of less stable complexes[55,56]. For example, a HIV-derived peptide adopts a conformation in the PBG with a C-terminally shifted

kinked motif, which poses constraints on a proper accommodation of the residues into the binding pockets[55]. The structural heterogeneity of these lower affinity HLA-E/peptide complexes is mainly the result of disrupted occupancy of the residue at position 7 in the E-pocket and disrupted interactions between the peptide and the α2 helix of HLA-E[56]. In contrast, a Mtb-derived peptide called Mtb44 has a comparable binding affinity for HLA-E as VL9 peptides but has a completely different sequence (i.e., RLPAKAPLL). Although the sequences are different, Mtb44 adopts a similar conformation in the PBG as VL9 peptides via compensatory interactions leading to the formation of a structurally stable HLA-E/Mtb44 complex[56].

While not studied as extensively, pathogen-derived peptides possibly use HLA-E antigen presentation pathways different from the canonical pathway involved in VL9-peptide presentation. For instance, CMV encodes TAP inhibitors to escape immune recognition by classical $CD8^+$ T cells, but can also present peptides on HLA-E for interaction with NKG2A/CD94 to prevent NK cell mediated lysis by lack of classical HLA-I expression[61,62]. These HLA-E restricted peptides must exploit alternative and TAP-independent HLA-E antigen presentation pathways. Furthermore, another study showed that HLA-E/*Mtb* complexes were present in the *Mtb*-containing phagosome in addition to components of the peptide loading complex, suggesting that HLA-E peptide loading might take place in phagosomes[46,63]. **Chapter 2** describes these possible alternative antigen presentation pathways in more detail with a focus on *Mtb*.

1.6 Interaction of NKG2A or -C/CD94 with HLA-E/peptide complexes: missing-self recognition

The NKG2A or -C/CD94 co-receptor complex expressed on NK cells were the first discovered biological receptors for HLA-E in complex with VL9 peptides (HLA-E/VL9) (Figure 3)[42,64,65]. Besides NK cells, NKG2A or -C/CD94 can also be expressed on cytotoxic $CD8^+$ T cells. These receptors belong to the family of C-type lectin receptors[52]. Dimerization between NKG2A or -C and CD94 is essential to obtain a functional receptor, and they are covalently linked via a disulfide bridge. NKG2A or -C/CD94 solely interact with HLA-E and the interaction with HLA-E/VL9 complexes is essential for surveillance of classical HLA-I cell surface expression and classical antigen presentation. These processes are frequently deregulated in malignantly transformed cells[66]. The CD94 part of the co-receptor complex makes direct interactions with the arginine at position 5 of the VL9 peptide, whereas both NKG2A or -C and CD94 interact with the HLA-E α1 and α2 helices[67]. NKG2A and -C/CD94 transduce opposing signals upon interaction with HLA-E/VL9. NKG2C/CD94 activates NK cell mediated lysis, whereas NKG2A/CD94 inhibits this process[68]. Interaction with either one of these receptors is mostly regulated by the six-fold higher affinity of NKG2A/CD94 for HLA-E/VL9 complexes compared to NKG2C/CD94[65]. CMV is thus far the only virus known to have hijacked this mechanism for its own survival via presentation of VL9-peptide mimics encoded in the UL40 protein on HLA-E for interaction with NKG2A/CD94[69]. Defects in classical HLA-I peptide presentation results in improper VL9 peptide processing and HLA-E peptide loading. The subsequent abrogated interaction between the HLA-E/VL9 complex and NKG2A/CD94 activates NK cell mediated lysis and thus clearance of defected cells. The interaction between HLA-E/VL9 and NKG2A/CD94 is therefore known as recognition of missing-self peptides and is crucial to maintain cellular homeostasis[66]. Antibodies that block the interaction between NKG2A/CD94 and HLA-E/VL9 are currently tested as a cancer immunotherapy to harness the killing capacity of NK cells[66].

1.7 Interaction of TCRs with HLA-E/peptide complexes

Besides NKG2A or -C/CD94, HLA-E can also interact with TCRs (Figure 3). TCRs recognize a broader variety of peptides in terms of sequence and origin than VL9 peptides. Various pathogens, such as HIV, *Mtb*, CMV and SARS-CoV-2, can present peptides on HLA-E for TCR recognition. CMV loads identical VL9-peptide sequences from the UL40 protein on HLA-E to prevent NK cell mediated lysis, as mentioned before, but $CD8^+$ T cells can also recognize HLA-E/UL40 complexes via interaction with the TCR[70,71]. HLA-E/UL40 specific T cells are circulating in individuals that encode non-homologous VL9 peptides (i.e., the VL9-peptide has a different sequence than the UL40-derived peptide). Biophysical studies on a T cell clone isolated from a CMV seropositive donor revealed that contact between this TCR, called KK50.4, and the HLA-E/UL40 complex is mainly mediated by the hypervariable complementary determining regions (CDR), particularly the CDR2β loop[59]. TCR KK50.4 directly interacted with the arginine at position 5 and the isoleucine at position 8 of the UL40-derived peptide via non-covalent interactions, such as salt bridges and Van der Waals interactions[59,72]. Characterization of the molecular interaction between HLA-E restricted TCRs with other pathogen-derived peptides has not been performed as extensively.

HLA-E restricted TCRs were anticipated to be (semi)-invariant, like other DURTs, as they recognize conserved antigen presentation molecules. However, 15 HLA-E restricted $CD8^+$ T cell clones recognizing a HIV-derived peptide called RL9HIV isolated from one individual revealed diversity in the variable and joining regions of both the TCRα and β chain[73]. Signaling via these TCRs could induce, albeit to variable degree, functional responses towards HIV-infected primary $CD4^+$ T cells. In addition, 13 HLA-E restricted $CD8^+$ T cell clones recognizing two SARS-CoV-2 derived peptides had a diverse TCRαβ composition as well[74]. These T cell clones were able to reduce intracellular viral replication. **Chapter 5** shows that HLA-E/*Mtb* complexes are recognized by multiple different TCRαβ sequences within and between individuals with TBI. These findings combined indicate that, in contrast to other DURT subsets, HLA-E restricted T cells are likely more polyclonal and are more similar to classical HLA-I restricted T cells. The diversity of the HLA-E restricted TCR repertoire can be advantageous for vaccination purposes as it increases the available pool of TCRs that can recognize HLA-E/peptide complexes.

1.8 Characterization of the HLA-E restricted $CD8^+$ T cell response in *Mtb* infections

Several studies have investigated the phenotype and functionality of HLA-E restricted *Mtb* specific $CD8^+$ T cells (Figure 4). These *in vitro* studies are essential for further exploration of HLA-E restricted T cells as vaccination targets for TB. *In silico* screening of the *Mtb* H37Rv genome in combination with a bioinformatics approach led to the identification of 69 potential HLA-E binding peptides, which were evaluated in HLA-E*01:03 peptide binding assays[39]. The majority of predicted peptides (55/69) could induce $CD8^+$ T cell proliferation in healthy individuals responding to *Mtb*-derived purified protein derivative (PPD) but $CD8^+$ T cell proliferation in response to one or more predicted peptides was also observed in PDD negative individuals and in umbilical cord blood samples. In addition, 4 T cell lines generated via peptide stimulation could induce cytolytic responses towards both HLA-E/peptide presenting cell lines and BCG-infected monocytes, demonstrating that T cells specific for HLA-E restricted *Mtb*-derived peptides are able to recognize mycobacterium-infected cells[39]. Next to a cytolytic response, these T cell lines could also induce a regulatory response in a TGFβ-dependent manner.

A separate study demonstrated that $CD8^+$ T cells recognizing two of these *Mtb*-derived HLA-E specific peptides were circulating in individuals with aTB and TBI, with the highest frequency in individuals with aTB and

HIV co-infection[40]. CD8+ T cell clones specific for the abovementioned HLA-E restricted *Mtb*-derived peptides were evaluated for cytotoxicity towards BCG-infected monocytes, intracellular *Mtb* growth control and regulatory functions (Figure 4)[41]. Interestingly, whereas some T cell clones displayed dual functionalities (i.e., regulatory and cytotoxicity), other T cell clones could either induce growth inhibitory and cytotoxic responses or a regulatory response. The mechanisms dictating which functional response is induced by HLA-E/*Mtb* specific CD8+ T cells remains to be discovered. Moreover, assessment of the production of Th1- and Th2-associated cytokines revealed that all HLA-E/*Mtb* specific CD8+ T cell clones could produce Th2-associated cytokines, such as IL-13 and IL-5 (Figure 4)[41]. Th1-associated cytokines, such as IFN-γ, were hardly produced under physiological conditions, such as after stimulation with BCG-infected macrophages, but could be induced using anti-CD3/CD28 beads as an artificial stimulus. Next to production of Th2-associated cytokines, the HLA-E/*Mtb* specific CD8+ T cell clones could activate B cells, demonstrating another regulatory function[41]. This dominant Th2-associated cytokine profile was not only apparent in HLA-E/*Mtb* T cell clones but also in HLA-E/*Mtb* specific CD8+ T cells circulating in individuals with aTB, which were identified using HLA-E tetramers[41]. This rather unorthodox phenotype of HLA-E/*Mtb* specific CD8+ T cells is surprising as classical HLA-I restricted CD8+ T cells recognizing *Mtb* peptides usually secrete only Th1-associated cytokines and have a cytotoxic phenotype. Furthermore, circulating HLA-E/*Mtb* specific CD8+ T cells revealed a dominant effector memory phenotype[40].

These studies combined show that *Mtb* can present peptides on HLA-E for CD8+ T cell recognition, are circulating in individuals with TB and that HLA-E/*Mtb* specific CD8+ T cells have a Th2-associated memory phenotype. These findings underscore the potential of HLA-E/*Mtb* specific CD8+ T cells as vaccination targets for TB.

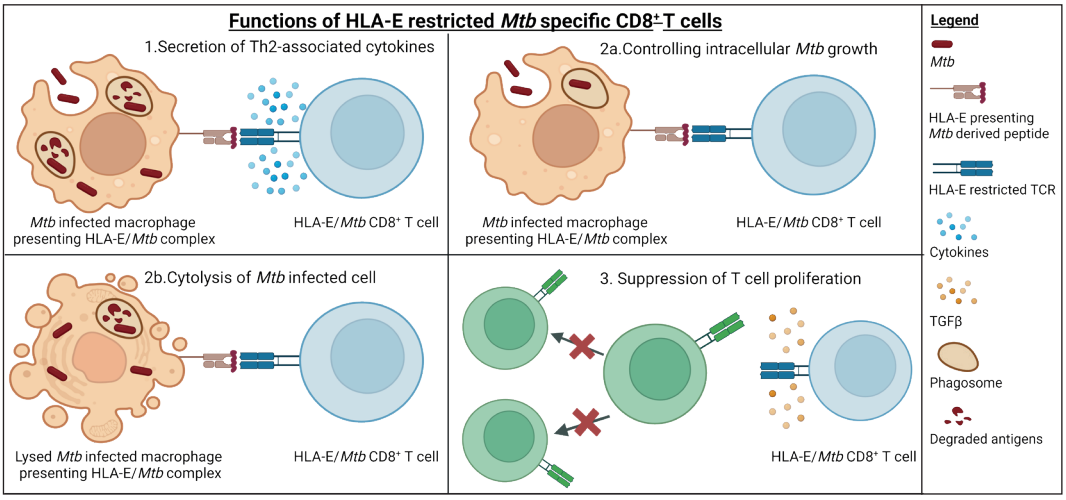


Figure 4. Overview of the functionalities of HLA-E/*Mtb* specific CD8+ T cells after recognition of the HLA-E/*Mtb* complex presented by macrophages. Macrophages engulf *Mtb* via endocytosis whereafter *Mtb* resides in a phagosome. Inside the phagosome, *Mtb* can be degraded into peptide fragments and HLA-E can present these *Mtb*-derived peptides at the cell surface for CD8+ T cell recognition. The induced functionalities of these CD8+ T cells are: 1. Secretion of Th2-associated cytokines, 2a. Control of intracellular *Mtb* growth, 2b. Cytolysis of *Mtb*-infected cells and 3. Suppression of T cell proliferation in a TGFβ-dependent manner. Functions 2a and 2b might occur via similar mechanisms. Figure created in BioRender.com.

1.9 Identification of high-affinity *Mtb*-derived HLA-E restricted peptides

A vaccine inducing HLA-E restricted *Mtb* specific $CD8^+$ T cells benefits from the inclusion of high-affinity *Mtb*-derived HLA-E restricted peptides. The development of HLA-E peptide binding motifs to scan the *Mtb* peptidome is essential to identify such *Mtb*-derived peptides. The initial motif for HLA-E was based on the interaction of NKG2A or -C/CD94 with VL9 peptides and confirmed that residues at position 2 and 9 are main anchor residue positions and are restricted to the hydrophobic residues methionine and leucine, respectively[53]. More recently, this motif was refined based on a high-throughput UV-mediated HLA-E peptide exchange assay using HLA-E monomers folded with a UV-sensitive peptide to measure the affinity of test peptides for HLA-E[51]. Large-scale assessment of HLA-E restricted peptides with substitutions at each peptide position revealed that the HLA-E PBG is more flexible than was previously thought. For instance, proline, a residue that significantly constraints the peptide conformation can be accommodated at position 7 and the larger hydrophobic amino acid tryptophan at position 9[51]. This refined and optimized peptide binding motif led to the discovery of over 100 HLA-E restricted *Mtb*-derived peptides with a much greater diversity in amino acid composition compared to VL9 peptides and with a higher affinity for HLA-E compared to the previously identified *Mtb*-derived peptides. These novel *Mtb* peptides can bind to both HLA-E*01:01 and *01:03 with a comparable affinity and to HLA-E orthologues present in NHPs and mice. Besides HLA-E binding, $CD8^+$ T cells in the circulation of individuals with TBI could recognize these HLA-E/*Mtb* complexes with a higher frequency compared to the previously identified ones[60]. These newly discovered *Mtb*-derived HLA-E specific peptides might therefore represent promising components of a vaccine formulation targeting HLA-E restricted *Mtb* specific ($CD8^+$) T cells.

1.10 The potential of HLA-E restricted T cells as vaccination targets for TB

Next to *in vitro* evaluation, *in vivo* studies are essential to understand the potential of HLA-E restricted T cells as vaccination targets for TB in a natural *Mtb* infection setting. HLA-E orthologues are present in mice ($Qa\text{-}1^b$) and NHPs, specifically Mauritian cynomolgus macaques (Mafa-E) and rhesus macaques (RMs) (Mamu-E)[51]. This conservation across species facilitates translation of pre-clinal findings to humans. NHPs in particular display similar TB pathology as humans and demonstrated to be good pre-clinical models for human TB, which is advantageous for TB vaccine development[75,76]. Moreover, HLA-E, in contrast to classical HLA-I molecules, is upregulated on HIV-infected $CD4^+$ lymphocytes *in vitro* presumably via presentation of a HIV-derived peptide that stabilizes HLA-E cell-surface expression[77]. This is beneficial for HLA-E targeting vaccination strategies as *Mtb* and HIV infections overlap in many areas[2].

The feasibility of targeting HLA-E restricted T cells via vaccination was illustrated by several pre-clinical vaccination studies in RMs against simian immunodeficiency virus (SIV), the monkey equivalent of HIV. Vaccination of RMs with a modified Rhesus CMV vector (RhCMV 68-1) encoding several immunogenic SIV-derived antigens resulted in undetectable levels of SIV in more than half (13/24) of SIV challenged and vaccinated RMs[78]. The induced $CD8^+$ T cells also had long-lasting effector memory phenotypes. Importantly, the SIV-encoded RhCMV 68-1 vaccine vector exclusively targeted unconventional MHC-E and MHC-II restricted $CD8/CD4^+$ T cells but not classical MHC-I $CD8^+$ T cells, indicating that MHC-E restricted $CD8^+$ T cells are crucial to establish protection against SIV[79]. RMs vaccinated with a RhCMV 68-1 vector encoding *Mtb* antigens had reduced signs of pulmonary TB and reduced *Mtb* loads in the lungs compared to unvaccinated RMs[80]. However, this vaccine only

elicited classical MHC-I restricted and not MHC-E restricted $CD8^+$ T cells, for reasons to be elucidated. Besides RMs, another pre-clinical study in mice revealed that Qa-1^b is upregulated on B cells, macrophages and DCs in *Mtb*-infected compared to naïve mice[81]. Qa-1^b restricted *Mtb* specific $CD8^+$ T cells derived from these *Mtb*-infected mice could produce IFN-γ and Qa-1^b knock-out mice had increased *Mtb* loads in the lungs and spleen and a higher mortality compared to wildtype mice after high-dose *Mtb* exposure[81]. These results strongly indicate that MHC-E restricted $CD8^+$ T cells contributed to this protective effect against TB in mice. Altogether, these pre-clinical studies in combination with the virtually monomorphism of HLA-E, the presence of HLA-E/*Mtb* specific $CD8^+$ T cells in individuals with TB and the sustained expression of HLA-E upon HIV infection, highlight the potential of targeting HLA-E restricted T cells as a vaccination approach against TB.

Thesis scope and outline

The aim of this thesis was to explore HLA-E restricted *Mtb* specific T cells recognizing higher affinity *Mtb*-derived peptides as novel vaccination targets for TB.

Chapter 2 reviews recent literature on HLA-E restricted antigen presentation of VL9 peptides, CMV peptides and *Mtb* peptides. This chapter proposes 3 alternative and TAP-independent peptide presentation pathways for HLA-E and describes hypothetical pathways that might be involved in the processing and presentation of *Mtb*-derived peptides on HLA-E. These insights can be of value for HLA-E targeting vaccine design.

Chapter 3 describes the frequency of circulating and local HLA-E/*Mtb* restricted T cells in *Mtb*-infected NHPs that were either vaccinated with BCG or were left unvaccinated. In addition, aligned studies were performed in BCG-infected humans. HLA-E tetramers folded with high-affinity *Mtb*-derived peptides were used in combination with several memory markers to stain both peripheral blood mononuclear cell (PBMC) and bronchoalveolar lavage (BAL) samples.

Chapter 4 focuses on the in-depth exploration of the immunophenotype of HLA-E/*Mtb* specific T cells circulating in individuals with TBI and with aTB in the presence or absence of HIV. PBMCs were stained with a 30-marker cell surface spectral flow cytometry panel in combination with HLA-E/*Mtb* tetramers following procedures previously developed and published by our group[82].

Chapter 5 tests the hypothesis that HLA-E/*Mtb* specific TCRs are (semi-)invariant like other DURT subsets or are more polyclonal. HLA-E/*Mtb* tetramers were used to sort HLA-E/*Mtb* specific $CD8^+$ T cells from individuals with TBI followed by single-cell TCR sequencing using TCRα and β specific primers targeting the variable and constant regions. The GLIPH2 algorithm was used to identify the most peptide-specific TCRαβ pairs and these TCRs were evaluated for functionality by determining the activation of the TCR signaling molecule Zap70.

Chapter 6 compares the interaction requirements of two HLA-E specific TCRs and NKG2A/CD94 with HLA-E/peptide complexes. Position substituted peptides were synthesized and folded into HLA-E tetramers using the temperature-exchange method, which were used to evaluate recognition by both TCRs and NKG2A/CD94. Peptide variants were also evaluated for the capacity to induce receptor signaling using reporter cell lines expressing either TCRs or NKG2A/CD94. Computational models were built based on the recognition and signaling data to identify the most selective residue positions for interaction with TCRs or with NKG2A/CD94.

Chapter 7 presents the identification of novel receptors for HLA-E/peptide complexes using high-throughput CRISPR/Cas9 overexpression screens. HLA-E tetramers folded with VL9 peptides as well as *Mtb* and CMV-deri-

ved peptides were used to stain CRISPR/Cas9 induced overexpressing cells followed by sorting of the cells that were bound to HLA-E tetramers. Functional assays were performed to delineate the possible biological function of the interaction between HLA-E/peptide complexes and these identified receptors.

Chapter 8 summarizes and discusses the findings of this thesis and provides novel perspectives for future research.

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