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DEVELOPMENT OF MHC CLASS I AFRICAN ALLELES AND *EX VIVO* DETECTION OF *M.TUBERCULOSIS*-REACTIVE CD8+ T-CELLS

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ABSTRACT

The T-cell mediated adaptive immune response is important in controlling infection with *Mycobacterium tuberculosis* (*M.tb*). *S*everal types of T-cells participate in the anti-*M.tb* defense, including CD4+ and CD8+ cells. CD8+ T-cells recognize small parts, so-called epitopes, of foreign antigens as well as self-derived antigens in association with MHC class I molecules. Identification of T-cell epitopes might therefore aid in the development of diagnostic markers and vaccine candidates. They may also guide to monitor CD8+ T-cell responses in disease settings where CD8+ T-cells play a role in biologically and clinically relevant immune responses.

In this thesis, we evaluated the previously identified *M.tb*-derived T-cell epitopes (*Paper V*), as well as identified novel *M.tb*-derived CD8+ T-cell epitopes from several proteins (TB10.4, Ag85B, ESAT-6, glycosyl transferase I, glycosyl transferase 2 and cyclopropane fatty acid synthase) (*Papers I–III*). The epitopes were restricted by a wide range of MHC class I allotypes, including some of the most common alleles in Caucasian, Asian and African population groups. Most of the MHC class I alleles common in the African groups were not commercially available. Therefore, they were cloned and subsequently expressed as recombinant proteins in order to be used in peptide binding detection and to construct peptide-MHC class I multimeric complexes for the first time (*Papers II–III*).

We studied peptide-MHC interactions to 13 different allotypes by using overlapping peptide libraries. A variable broadness of peptide binding patterns was identified. Some alleles showed a diverse pattern, allowing binding of many epitopes, while others displayed a more restricted peptide binding pattern. Another interesting feature was the very frequent occurrence of promiscuous binding epitopes. Subsequent evaluation of the binding characteristics of a majority of the 672 identified binding epitopes showed a wide range of affinities and dissociation rates with both inter- and intra-allelic differences (*Papers I–III*).

An extensive panel of 62 MHC class I multimers was constructed in order to validate some of the previously identified binding epitopes as being CD8+ T-cell epitopes. We also used these reagents to characterize *M.tb*-specific CD8+ T-cell responses in patients with pulmonary tuberculosis (TB) with diverse ethnic background (Caucasian, Asian and African). Generally, a low CD8+ T-cell response reflecting a diverse *M.tb*-specific reactivity could be detected, with only a few immunodominant epitopes. The majority of the *M.tb*-specific CD8+ T-cells had a precursor-like phenotype (CD45RA+CCR7+), despite expressing high frequencies of the degranulation marker CD107a, indicating that antigen-experienced effector cells reside in this population (*Papers II–IV*). One explanation for the high number of specific 'naïve-like' T-cells might be that they belong to a compartment of memory cells with 'stem-cell like' features, including expression of c-kit (CD117) and CD95 (*Paper IV*).

This thesis shows that both MHC class I allotypes and epitope-derived proteins might influence immune recognition on several levels including peptide-MHC binding, T-cell receptor (TCR) engagement as well as T-cell effector functionality and phenotype of the antigen-specific T-cells (*Paper III*); The T-cell phenotype and *M.tb*-specific T-cell frequency appear to be determined by both the restricting allele *and* the antigen.

In conclusion, we identified and validated many novel CD8+ T-cell targets from *M.tb*-derived proteins restricted via a broad range of MHC class I molecules, with the hope that these tools will aid future diagnostics and prevention strategies in different disease settings.

SAMMANFATTNING PÅ SVENSKA

Det T-cellsförmedlade adaptiva immunförsvaret är viktigt för att hålla en infektion med *Mycobacterium tuberculosis* (*M.tb*) under kontroll. Flera olika typer av T-celler, t.ex. CD4+ och CD8+, ingår i vårt immunförsvar mot tuberkulos (TBC). CD8+ T-celler känner igen små delar, så kallade epitoper, av främmande antigen eller proteiner härledda från egna kroppen, bundna till MHC klass I-molekyler. Identifiering av T-cellsepitoper kan därför hjälpa till vid utvecklandet av nya diagnostiska markörer och vaccinkandidater. De kan också hjälpa till att övervaka CD8+ T-cellssvar vid sjukdomstillstånd där CD8+ T-celler har betydelse för immunförsvaret, både biologiskt och kliniskt.

I denna avhandling har vi utvärderat tidigare identifierade epitoper härledda från *M.tb*-proteiner (*delarbete V*) samt identifierat nya CD8+ T-cellsepitoper härledda från *M.tb*-proteiner (TB10.4, Ag85B, ESAT-6, glykosyltransferas I, glykosyltranferas 2 och cyklopropan-fettsyra-syntas) (*delarbete I–III*). Epitoperna presenterades av många olika MHC klass I-alleler, inklusive några av de mest vanliga allelerna i kaukasiska, asiatiska och afrikanska befolkningsgrupper. På grund av att de flesta MHC klass I-alleler som är vanliga i afrikanska befolkningsgrupper inte finns att få tag på kommersiellt, klonade och konstruerade vi dem själva för första gången och använde dem för att detektera peptidbinding och för att konstuera peptid-MHC-multimerkomplex (*delarbete II–III*).

Vi studerade peptid-MHC-bindning till 13 olika MHC klass I-alleler genom att använda överlappande peptidbibliotek. Variationer i bredden på peptidbindningen kunde identifieras. Vissa alleler hade ett brett bindningsmönster som tillät bindning av många vitt skilda epitoper, medan andra alleler uppvisade ett mer begränsat peptidbindingsmönster. En annan intressant upptäckt var vanligt förekommande korsbinding av epitoper mellan olika MHC klass I-alleler. Efterföljande analys av bindningsegenskaperna hos en majoritet av de 672 identifierade bindningsepitoperna visade ett brett intervall av affiniteter och dissociationshastigheter som varierade både inom och mellan olika alleler (*delarbete I–III*).

En omfattande panel bestående av 62 MHC klass I-multimerer konstruerades för att bekräfta att några av de sedan tidigare identifierade bindningsepitoperna var CD8+ T-cellsepitoper. Vi använde också dessa reagenser för att karakterisera det *M.tb*-specifika CD8+ T-cellssvaret i patienter med lung-TBC från olika etniska befolkningsgrupper (kaukasiska, asiatiska och afrikanska). Generellt sett kunde ett lågt men brett *M.tb*-specifikt CD8+ T-cellssvar detekteras, med enbart ett fåtal immunodominanta epitoper. Majoriteten av de *M.tb*-specifika CD8+ T-cellerna hade en naiv fenotyp (CD45RA+CCR7+), trots att de uttryckte höga frekvenser av degranuleringsmarkören CD107a, vilket indikerar att antigen-erfarna effektorceller finns i denna population (*delarbete II–IV*). En förklaring till de höga frekvenserna av antigenspecifika celler med naiv fenotyp kan vara att de tillhör en grupp minnesceller med stamcellslika egenskaper t.ex. uttryck av ytmarkörerna c-kit (CD117) och CD95 (*delarbete IV*).

Denna avhandling visar vidare att både de presenterande MHC klass I-allelerna och de proteiner som epitoperna kan härledas ifrån kan påverka immunförsvarets igenkänning på flera nivåer såsom peptid-MHC-bindning, igenkänning via T-cellsreceptorer samt effektorfunktionalitet och fenotyp av de antigenspecifika T-cellerna (*delarbete III*); T-cellernas fenotyp och antalet *M.tb*-specifika T-celler verkar påverkas av både den presenterande MHC-allelen *och* av den tuberkuloshärledda antigenen.

Sammanfattningsvis har vi identifierat och validerat många nya CD8+ T-cellseepitoper härledda från *M.tb*-proteiner och presenterade via många olika MHC klass I-molekyler, med förhoppning om att dessa ska hjälpa till i utvecklingen av nya diagnostiska metoder och förebyggande åtgärder för flertalet sjukdomar i framtiden.

LIST OF PUBLICATIONS

I. REBECCA AXELSSON-ROBERTSON, Frank Weichold, Donata Sizemore, Markus Wulf, Yasir A. W. Skeiky, Jerry Sadoff and Markus J. Maeurer

Extensive major histocompatibility complex class I binding promiscuity for *Mycobacterium tuberculosis* TB10.4 peptides and immune dominance of human leucocyte antigen (HLA)-B*0702 and HLA-B*0801 alleles in TB10.4 CD8+ T-cell responses. Immunology. 2010 Apr;129(4):496-505

II. REBECCA AXELSSON-ROBERTSON, Raija K. Ahmed, Frank F. Weichold, Marthie M. Ehlers, Marleen M. Kock, Donata Sizemore, Jerry Sadoff, and Markus Maeurer

Human Leukocyte Antigens A*3001 and A*3002 Show Distinct Peptide-Binding Patterns of the *Mycobacterium tuberculosis* Protein TB10.4: Consequences for Immune Recognition. Clin Vaccine Immunol. 2011 Jan;18(1):125-34

III. REBECCA AXELSSON-ROBERTSON, Andre Loxton, Gerhard Walzl, Marthie M. Ehlers, Marleen M. Kock, Alimuddin Zumla and Markus Maeurer

MHC class I alleles determine the effector CD8+ T-cell phenotype in *Mycobacterium tuberculosis* specific immune response in patients with active Tuberculosis. Manuscript

IV. REBECCA AXELSSON-ROBERTSON, Ji Hyeon Ju, Ho-Youn Kim and Markus Maeurer

M.tuberculosis specific and MHC class I-restricted CD8+ T-cells exhibit a stem-cell precursor-like phenotype in patients with active TB. Manuscript

V. REBECCA AXELSSON-ROBERTSON, Isabelle Magalhaes, Shreemanta K Parida, Alimuddin Zumla and Markus Maeurer

The Immunological Footprint of *Mycobacterium tuberculosis* T-Cell Epitope Recognition. J Infect Dis. 2012 Mar 23

CONTENTS

1 Introduction 1
1.1 Immunology 2
1.1.1 Major histocompatibility complex (MHC) 2
1.1.2 T-cells 8
1.1.3 MHC-TCR interaction 9
1.1.4 Phenotype and effector functions of T-cells 10
1.2 Multimers 13
1.2.1 Structure 13
1.2.2 Function 13
1.3 Tuberculosis 14
1.3.1 The bacterium 14
1.3.2 The disease 14
1.3.3 Adaptive immune responses to *M.tb* 15
1.3.4 Genetic variability and immune responses against *M.tb* 16
1.3.5 *M.tb* epitopes 16
1.3.6 Genetic diversity in *M.tb* 16
1.3.7 Immunogenic *M.tb* proteins 17
2 Aim of the thesis 20
3 Methodological development 21
3.1 Patients with pulmonary TB 21
3.2 Development of novel materials and methods 21
3.2.1 African MHC class I alleles 21
3.2.2 Recombinant MHC class I molecules 21
3.2.3 Peptide binding assay 22
3.2.4 Multimers 23
3.2.5 Flow cytometry 24
4 Results and discussion 26
4.1 MHC class I binding epitope identification 26
4.2 Characterization of the peptide-MHC binding properties 30
4.3 Enumeration of antigen-specific T-cells 30
4.4 Characterization of the antigen-specific T-cells 33
4.5 Variant *M.tb* epitopes 35
5 General conclusions 37
6 Future perspectives 38
7 Acknowledgements 40
8 References 42

LIST OF ABBREVIATIONS

APC	Antigen-presenting cells
β_2m	Beta-2-microglobulin
BCG	Bacillus Calmette-Guérin
CDR	Complementary determining regions
CFA	Cyclopropane fatty acid
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cell
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICS	Intracellular cytokine staining
IFN	Interferon
IL	Interleukin
MHC	Major histocompatibility complex
M.tb	*Mycobacterium tuberculosis*
PBMCs	Peripheral blood mononuclear cells
PLC	Peptide loading complex
SNP	Single nucleotide polymorphism
TAP	Transporter associated with antigen processing
TB	Tuberculosis
TCR	T-cell receptor
Th	T-helper

1 INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis (M.tb)*. The bacterium has infected humans for more than 4 000 years, yet there were still 8.8 million new cases of TB, and 1.45 million deaths reported in 2010. Although the highest number of cases can be found in south-east Asia, the highest incident by far can be found in sub-Saharan Africa (Figure 1) [1]. Factors contributing to the epidemic include inadequate vaccine efficacy, inadequate diagnostics [2] as well as drug resistance and the human immunodeficiency virus (HIV) epidemic [3]. Therefore, more information about what constitutes and regulates protective immunity in TB needs to be gained.

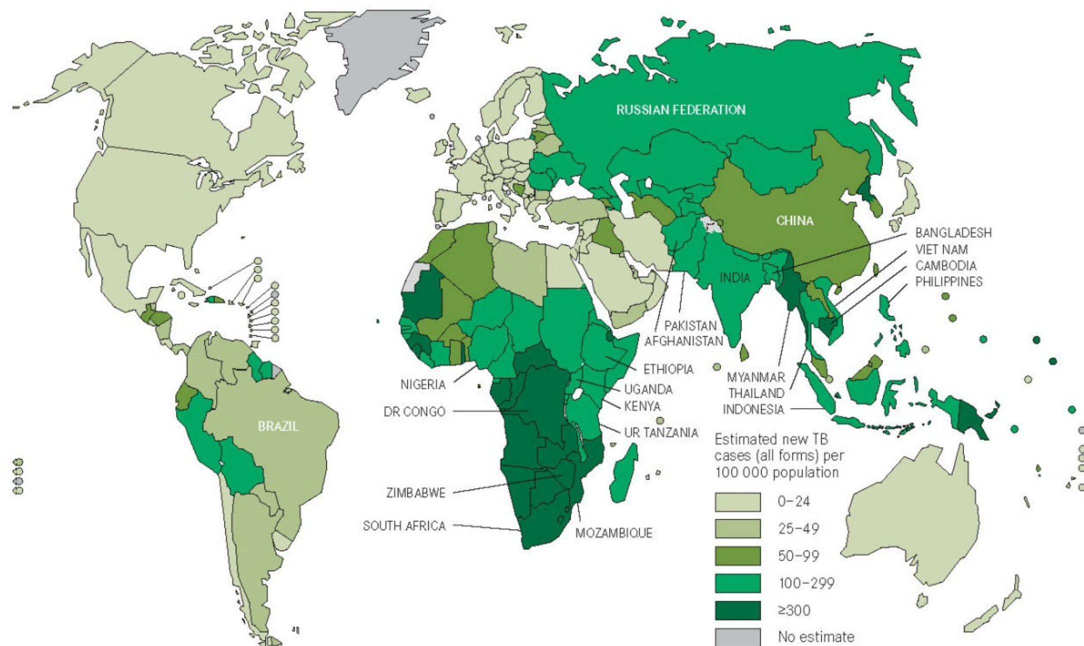

Figure 1. Estimated TB incidence rates 2010.

Source: World Health Organization (WHO) [1].

The cell-mediated adaptive immune response is important in controlling *M.tb* infections. An effective anti-*M.tb* response has been shown to be mainly cell-mediated, with different compartments of T-cells: CD4+ and CD8+ [4, 5] yet NK-cells [6], NK-T-cells [7] and γδ-T-cells [8], also play an important role at different stages of the infection. T-cells recognize, and are activated by, small parts (so-called epitopes) of protein-derived antigens presented via major histocompatibility complex (MHC) glycoproteins. Identification of epitopes is highly valuable in diagnostics as well as for the development of novel vaccine regiments and for gauging of vaccine take. Although

the CD4+ T-cell response is more well-studied, previous studies have shown that cytotoxic CD8+ T-cells are important in the defense against TB [5] and that they are induced early in the infection [9]. Identification of novel antigenic peptides recognized by CD8+ T-cells presented via the MHC class I complex, will therefore be of great value to better understand the cytotoxic T-lymphocyte (CTL)-mediated anti-*M.tb* immune response.

1.1 IMMUNOLOGY

1.1.1 Major histocompatibility complex (MHC)

The MHC multigene cluster contains more than 200 genes. In humans, this cluster is called human leukocyte antigen (HLA), since the proteins were first discovered as transplantation antigens that varied among individual patients. The gene family is divided into three sub-groups (class I, II and III). The first two classes (class I and II) encode glycoprotein receptors involved in antigen-presentation of protein-derived antigens towards T-cells (MHC class I and II molecules) as well as antigen processing molecules and proteins involved in peptide loading such as transporter associated with antigen processing (TAP) and tapasin. The class III gene family encodes other proteins involved in the immune system, such as complement components (e.g. C4, C2 and factor B) and cytokines (e.g. tumor necrosis factor-α and lymphotoxin) [10].

The classical MHC class I molecules, HLA-A, -B and -C (non-classical: e.g. HLA-E, -F and -G) are encoded by three loci located on chromosome 6 in humans and they present predominantly endogenously derived antigens to CD8+ T-cells. MHC class II molecules are subdivided into HLA-DP, DQ and DR (non-classical HLA-DM and -DO) and present mainly exogenous antigens toward CD4+ T-cells (T-helper [Th] cells). Exogenously derived peptides might also be presented via MHC class I molecules via cross-presentation [11, 12]. MHC class I molecules are expressed at different levels on all nucleated cells, while MHC class II molecules are only expressed on specific antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages and B-cells. The glycoproteins are not only important for antigen-presentation, but also for self-recognition.

1.1.1.1 Diversity of the MHC class I

The MHC class I glycoprotein is the most polymorphic molecule in the human body, with more than 5 000 different versions (alleles) identified. The individual MHC class I

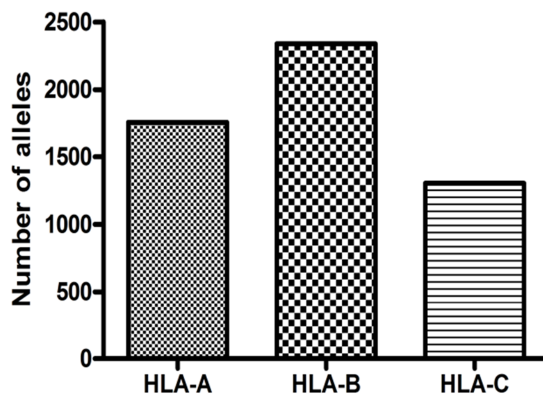

Figure 2. Number of MHC class I alleles for the different HLA loci.

Adapted from numbers derived from IMGT/HLA database [13].

alleles can differ by up to 20 amino acids, making each variant unique. The HLA-B locus is the most diverse with more than 2 000 different alleles, followed by HLA-A (~1 700) and HLA-C (~1 300) (Figure 2) [13]. Each individual can carry and co-dominantly express up to six different alleles, since in addition to being poly-morphic the molecules are also polygenic, with each chromosome containing one HLA-A, one HLA-B and one HLA-C allele. Both haplotypes (allele-combinations found on a chromosome) are subsequently co-dominantly produced and expressed on the cell-surface.

Table 1.
Average frequencies (%) of a number of selected MHC class I alleles for different population groups. Adapted from numbers derived from the *Allele frequency net* database [14].

Allele	*Caucasians*	*Asians*	*Africans*
A*01:01	12	4	4
A*02:01	25	7	9
A*03:01	13	3	5
A*11:01	6	18	1
A*24:02	10	19	3
A*30:01	1	3	6
A*30:02	1	1	7
A*68:01	3	2	3
B*07:02	10	2	5
B*08:01	8	1	4
B*15:01	5	3	1
B*58:01	2	7	5
C*07:01	14	4	17

The frequencies of many alleles are evenly distributed between different population groups (e.g. A*68:01 and B*58:01). However, different distribution of MHC class I alleles can be found based on geographic location or ethnicity for most of the MHC class I alleles (e.g. A*02:01, A*11:01 and A*30:02) [14] (Table 1).

1.1.1.2 Structure of the MHC class I glycoprotein

The MHC class I glycoprotein consists of an invariant 12 kDa light chain (96 amino acids) (β_2-microglobulin [β_2m]) encoded outside the HLA-locus (on chromosome 15), non-covalently linked to a 43 kDa (340 amino acids) membrane anchored polymorphic heavy chain. The heavy chain consists of three sub-domains (α_1, α_2 and α_3), of which the first two form the walls and bottom of the peptide binding cleft and the third ends in the transmembrane region anchoring the molecule to the cell-membrane, as well as containing a short cyto-plasmic tail. A protein-derived peptide fragment, usually between 8 and 10 amino acids long, constitutes the third part of the trimeric complex (Figure 3). Without the peptide, the trimeric complex remains unstable and the molecules will rapidly dissociate [15].

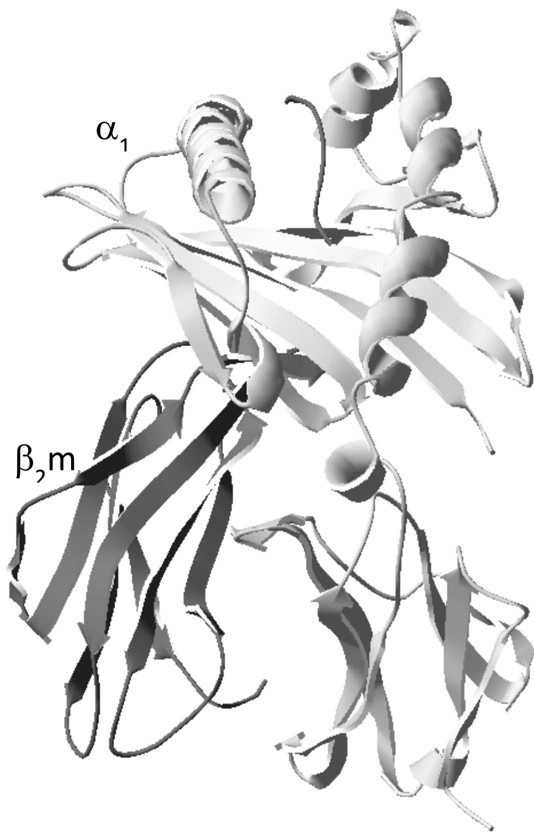

Figure 3. Structure of the peptide-β_2m-MHC class I complex.

Adapted from PDB file 1AKJ [49].

1.1.1.3 The peptide binding cleft and the peptide motif

Two α-helixes, forming the walls on top of an antiparallel 8-stranded β-sheet forming the floor, create the peptide binding cleft of an MHC class I molecule. The cleft is closed at the ends by certain inter-allelic conserved tyrosine residues which interact with the amino and carboxy terminal ends of the presented peptide, limiting, in most of the cases, the size of binding peptides to 8–10 amino acids [16]. The peptides bind in an elongated conformation within the cleft and ‘kinking’ of the peptides explains peptide binding of additional length associated with some MHC class I alleles, e.g. HLA-35 [17]. Most of the polymorphism that defines the different MHC class I alleles, lies within the peptide binding clefts and, more specifically, within amino acid residues interacting directly with the peptide [18]. These residues form 6 binding pockets labeled A–F, that interact with specific amino acids positions within a potential peptide

(Figure 4) [19, 20], thereby allowing each individual MHC class I allele to bind a large repertoire of peptides that varies inter-allelic. Each MHC class I allele will therefore only accommodate a certain repertoire of peptides with properties (electrostatic, hydrophobicity or bulkiness) that match the specific biochemical environments within these 6 binding pockets. Some positions within the peptide are more important for interaction with the MHC molecule than others; they are called 'anchor residues'. Anchor residues are therefore often conserved. They are usually situated at position two (P2) and at the carboxy-terminal (P9) end of a peptide interacting with pocket B and F. Yet, inter-allelic variations exist with some alleles preferring peptides with anchor-residues at position 3 (P3) and C-terminus (P9) (e.g. HLA-A*01), or only at the C-terminal end (P9) (e.g. HLA-A*11:01 and A*31:01) [21]. However, these anchor residues are not sufficient for high-affinity binding, since not all peptides with suitable anchor residues bind to the respective MHC molecule. In addition, most alleles show auxiliary (or secondary) anchor residues critical or important for peptide binding. In the case of HLA-A*02:01 (one of the most well-studied human MHC class I molecules), the anchor residues have been described to be either leucine or methionine at P2 in the binding peptide and either valine or leucine at P9 [22]. In addition, the amino acid situated at P3 in the presenting peptide has been shown to be a secondary anchor residue [23]. Altogether, the primary and auxiliary anchor residues form the allele-specific peptide motif. The motif is not absolute, as numerous peptides without the appropriate primary and auxiliary anchor residues have been identified as well as longer [24] or shorter peptides [25]. However, by using the sequence motif one can accurately predict peptide binding, at least for the more well-studied MHC class I alleles [21, 26, 27].

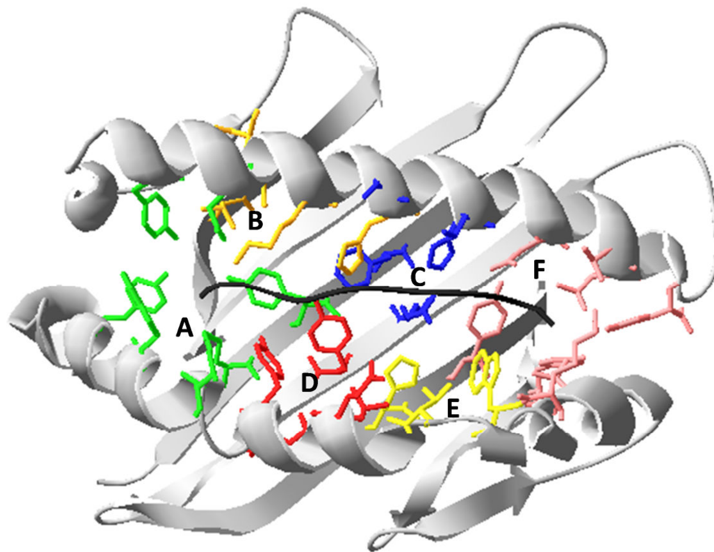

Figure 4. The peptide binding cleft of a MHC class I molecule, including the peptide binding pockets A-F.

Adapted from PDB file 1AKJ [49] .

1.1.1.4 *Antigen processing and antigen-presentation*

The correct assembly and folding of the heavy chain of the MHC class I molecules take place in the endoplasmic reticulum (ER). This step is stabilized by the chaperon calnexin and is followed by incorporation of β_2m and the formation of the peptide-loading complex (PLC). The PLC is composed of two MHC-encoded components, TAP and tapasin, as well as two 'house-keeping' ER proteins, calreticulin and ERp57 [28]. They help in loading a broad range of high-affinity peptides into the peptide binding clefts of the MHC class I molecules [29].

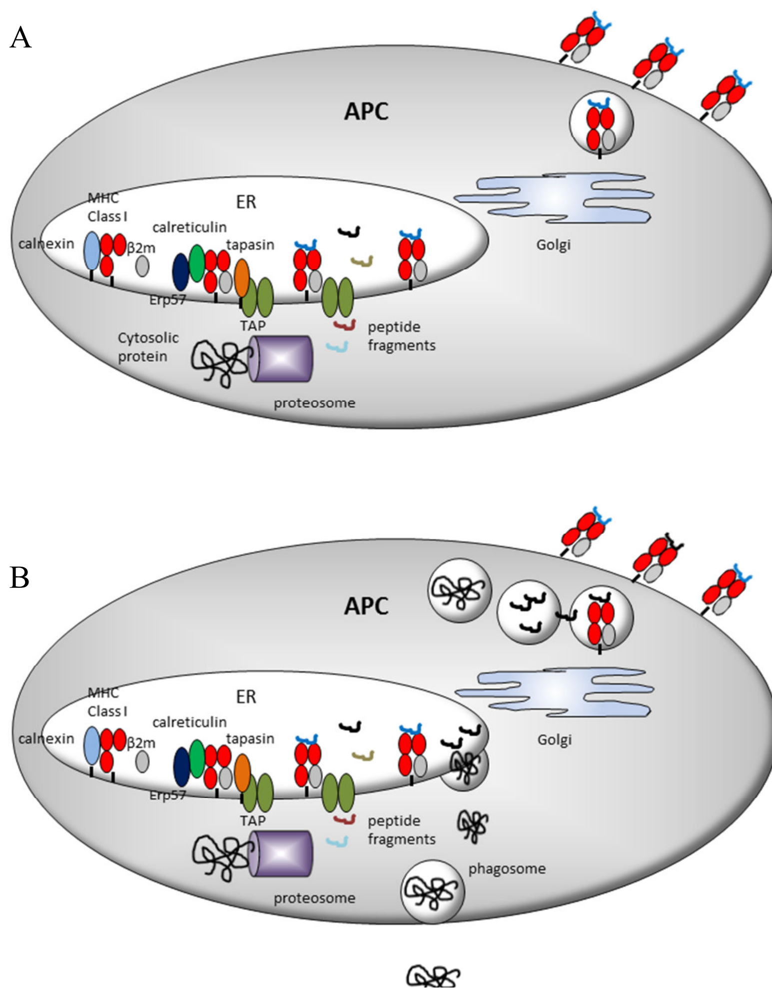

Figure 5. (A) The classical antigen presenting pathway and (B) pathways for cross-presentation of exogenously generated peptides.

In the direct antigen processing pathway, endogenous peptides, i.e. 'self', viral or bacterial proteins formed in the cytosol of the cell, are presented on MHC class I glycoproteins. Misfolded or foreign proteins are first tagged for destruction by ubiquitylation. Ubiquitinated proteins are then degraded in the proteasome and the peptides formed are transported to the ER via the ATP-dependant dimeric TAP-transporter system [30] before they are occasionally additionally trimmed by aminopeptidases [31]. In the ER, peptides of the approximate length of 8–10 amino acids bind to the MHC class I molecules and the trimeric complex is transported via the secretory pathway through the Golgi apparatus to the cell-surface. The peptides are subsequently presented to CD8+ T-cells on the cell-surface [32] (Figure 5A).

Signal sequences of proteins, designated for the cell-surface, are cleaved off in the ER and peptides originating from them can be loaded directly in the ER, independently of the TAP transport [33]. Retrograde translocation of ER-associated proteins back to the cytosol is another mechanism for endogenous peptide generation [34].

Exogenously generated peptides, e.g. most bacterial proteins, induce predominantly B-cell (humoral) and CD4+ T-cell responses; by recognition of the nominal antibody molecule and MHC class II restricted presentation respectively. Exogenously derived antigens can, via different pathways, also associate with MHC class I molecules, a phenomenon referred to as 'cross-presentation' [35]. In most cases, the peptides originate from intra-cellular pathogens escaping to the cytosol, phagocytosed pathogens or from phagocytosed apoptotic bodies. After uptake by e.g. DCs or macrophages, the proteins are retranslocated to the cytosol, cleaved by the proteasome and delivered to the ER, similar to the classical pathway (TAP-dependant pathway) [36]. Cytosolic access occurs either via leakage, pore formation, active transport through the vacuolar membrane, or via direct fusion of the vacuole to the ER. An alternative way of cross-presentation occurs when MHC class I molecules take up and present phagosome and vacuolar-degraded antigens directly within the vacuolar system [37, 38] (TAP-independent pathway) (Figure 5B).

1.1.1.5 Epitopes

An antigenic determinant, 'epitope', is the part of an antigen that the adaptive immune system (mainly T-cells and B-cells) recognizes. CD8+ T-cell epitopes consist of peptides bound to MHC class I molecules recognized by the T-cell receptor (TCR). The immunogenicity of an epitope depends on several criteria; 1) the epitope must be generated and transported to the endoplasmic reticulum, by overcoming limitations in proteasome dependent proteolysis [39] and restrictions related to the TAP-system [40] or by cross-presentation [41], 2) the epitope must bind to the MHC haplotype with appropriate affinity and dissociation rate, 3) the peptide-MHC complex must then be recognized by the individual's specific TCR repertoire and subsequent trigger T-lymphocytes.

Different strategies could be utilized for identification of CD8+ T-cell epitopes. Either one could start with identification of a positive T-cell response (e.g. by measuring

proliferation or cytokine production) and then track down the specific epitope. The opposite strategy could be utilized as well, starting with the identification of MHC-binding peptides from an immunogenic protein (using e.g. *in silico* selected or overlapping peptide libraries and recombinant MHC molecules or T-cell stabilization assays [42, 43]) followed by assessing the ability of these epitopes to elicit a T-cell response, e.g. by using MHC-multimers [44], or functional assays (e.g. enzyme-linked immunosorbent assay [ELISA], enzyme-linked immunosorbent spot [ELISPOT], intra-cellular cytokine staining [ICS] or cytotoxicity assays). By utilizing these methods, a high number of CD4+ and CD8+ T-cell epitopes have been identified in many different disease settings. However, we still lack knowledge of T-cell epitope identification in many areas. For instance, knowledge of the exact restricting MHC allele is frequently missing or, if it is known, one can detect a clear bias towards epitopes presented on a limited number of well-studied 'Caucasian' MHC class I alleles such as HLA-A*02:01, A*03:01, A*11:01 and B*15:01 [45].

1.1.2 T-cells

T-cells are differentiated in the thymus from bone-marrow-derived common lymphoid progenitor cells. During this differentiation process, thymocytes undergo a series of defined maturation steps, including that each progenitor T-cell makes its own specific TCR by gene rearrangement. To create a self-tolerant T-cell repertoire which is able to recognize foreign antigens, T-cell clones carrying TCRs that recognize MHC molecules too weakly or self-antigens (too strongly) are subsequently deleted via positive and negative selection, respectively. Thereafter, the thymocytes are committed to either CD4 or CD8 linage before entering the circulation as mature T-cells. Upon antigenic encounter, naïve non-antigen-experienced T-cells need to be primed against their cognate antigen by APCs, before becoming competent effector cells as well as long-lived memory T-cells. Antigenic encounter is mediated via the TCR which recognizes only antigens in association with an individual's own MHC molecules, the 'MHC restriction' [46].

1.1.2.1 The T-cell receptor

In most T-cells, the TCR is a heterodimer composed of disulfide-linked α and β polypeptide chains (a minority of T-cells, so-called γδ-T-cells, uses γ and δ polypeptide chains instead), each having separate constant and variable domains. The constant domains of the TCR are anchored in the cell-membrane of the T-cell. Each

variable region of the TCR is assembled from a number of randomly selected gene segments called V, D and J, at the junctions of these segments additional diversity is introduced through imprecise joining thereby creating a large repertoire of putative TCRs. In addition, the variable domain contains three hypervariable regions or complementary determining regions (CDRs) that are responsible for antigen recognition. The CDR1s interact predominantly with the N and C terminal ends of the peptide; the CDR2s interact with the α-helixes of the MHC molecules and the CDR3s, which are the most hypervariable loops, interact with the central part of the presented peptide [47].

1.1.2.2 CD8 co-receptor

Another cell-surface glycoprotein that is present on the cell-surface of certain T-cells (CD8+ T-cells), as well as on other immune cells (e.g. natural killer cells and dendritic cells), is the CD8 molecule. In most cases, CD8 represents a heavily glycosylated disulfide-linked heterodimer consisting of an α- and a β-chain, but a homodimer consisting of two α-chains is expressed on a certain subset of T-cells (αα T-cells). On T-cells, CD8 serves as a co-receptor for the TCR by recruiting essential intracellular signaling molecules, e.g. Lck [48] to the TCR complex, as well as interacting with the constant regions of the α_2 and α_3 domains of the peptide-MHC complex [49, 50]. The interaction keeps the TCR of the CD8+ T-cell and the target cell bound closely together, thereby allowing for prolonged engagement. During antigen-specific activation, this is thought to increase the sensitivity of a T-cell to its antigen a hundredfold.

1.1.3 MHC-TCR interaction

Both CD8+ T-cell priming and target recognition of T-cells in the effector phase of the immune response are dependent on MHC-TCR interactions. T-cell priming needs a variety of receptor-associated signaling events to take place between the T-cell and the APC, including the formation of the peptide-MHC-TCR complex (signal 1), co-stimulatory signaling (B7-1/B7-2 and CD28) (signal 2) as well as cytokine signaling (e.g. interleukin-12 [IL-12]) [51] (signal 3), while effector cells can respond to their target cells without co-stimulatory signals [52]. The frequency of individual peptide-MHC complexes on the cell-surface of infected cells is thought to be very low; only a few (10–50) TCR-MHC interactions are thought to be needed for T-cell activation, and even less for subsequent CD8+ T-cell mediated cytotoxicity [53].

1.1.3.1 *The immunological synapse*

The initial contact between APC and naïve T-cells is mediated by cell-adhesion molecules like intracellular adhesion molecules (ICAMs) [54]. The binding between an individual MHC molecule and a TCR is characterized by low affinity and fast dissociation rate, which is required for enabling serial contacts of each TCR molecule to bind to multiple MHC-peptide ligands [55]. The interaction between the TCR and the peptide-MHC complex is a two-step process. In the first step, the TCR docks on the MHC molecule in a peptide-independent fashion. This is followed by contact between the TCR and the peptide, which stabilizes or abrogates the MHC-TCR complex [56]. The TCR orients itself diagonally across the peptide-MHC complex, with the TCRα-chain lying above the amino-terminal end of the bound peptide and the α_2 domain of the MHC class I molecule and the TCRβ-chain interacting with the carboxy terminal end and the α_1 domain. Certain residues within the TCRs loops then interact with individual protuberating residues on the MHC complex, small alterations within the peptide might consequently alter the T-cell response. Because of this, it is believed that certain variable TCR genes have co-evolved with certain MHC alleles [57].

1.1.3.2 *Immunodominance*

The immune recognition by antigen-specific CD8+ T-cells in many diseases is often directed against a few of the many potential epitopes originating from a complex pathogen. This 'skewing' of the immune system gives rise to immunodominant epitopes, which might be accompanied by less recognized sub-dominant epitopes [58]. The driving force behind immunodominance and sub-dominance in different disease settings is not completely elucidated, but factors that are considered crucial include 1) availability (time and receptor numbers) of the peptide-MHC complex on the cell-surface [59], 2) the stability and the conformation of the presented epitope [17] and 3) the frequencies of T-cell precursors with the possibility of recognizing the epitope [60]. Recognition of certain epitopes might as well dampen the immune recognition of other epitopes, a process called 'immunodomination'.

1.1.4 Phenotype and effector functions of T-cells

Before activation, naïve CD8+ T-cells are immunologically pluripotent with the possibility to differentiate either into effector or memory subsets upon activation [61].

Therefore, the degree and time of antigenic stimulation, cytokine availability, co-stimulatory environment and the presence of CD4+ T-cell help are important factors for complete activation to occur as well as for determining the fate of a precursor CD8+ T-cell [51, 62]. Initially during infections, a massive heterogeneous response of antigen-specific T-cells is elicited. Only a small percentage of these initially clonally expanded cells survive and proceed to long-lived heterogeneous memory subsets after clearance of the pathogen.

1.1.4.1 T-cell phenotypic compartments

Based on a number of cell-surface markers such as CD45RA and CCR7, mature CD8+ T-cells can be divided into four different phenotypic compartments [63, 64]. CD45RA is an isoform of the protein tyrosine phosphatase receptor type C and functions as a regulator of receptor signaling in T-cells and B-cells, while CCR7 (CD197) is a chemokine receptor responsible for migration of naïve and central memory lymphocytes to secondary lymphoid organs. Antigen non-experienced precursor T-cells express both markers on their cell-surface and are able to give rise to memory or effector T-cells upon antigen encounter. There are two different compartments of memory T-cells based on expression of the chemokine receptor CCR7. Central memory T-cells express this receptor, but have switched the expression of the CD45RA isoform to another isoform (CD45RO). They are lymph-node homing antigen-experienced cells with high proliferative capacity, and are believed to lack immediate effector functions (except IL-2 production) and upon rechallenge need co-stimulation in order to expand. The other memory compartment consists of effector memory T-cells, which have down-regulated both cell-surface markers and exhibit a high degree of effector functionality, low proliferation rate and preferentially reside as sentinels in tissues. Terminally differentiated effector cells (T_{EMRA}) (CD45RA+CCR7-), which home to disease sites, constitute the last and most differentiated compartment. They are mainly effector cells with limited proliferating capacity [65].

1.1.4.2 CD8+ T-cell effector functions

T-cells produce two main classes of effector functions: cytotoxins and cytokines. CD8+ T-cells are called cytotoxic T-cells based on their main effector functions, i.e. cytolytic killing of infected cells by the help of cytotoxic granules containing cytotoxins (e.g. perforin, granzyme and granulysin). Perforin makes contact with the target cell and helps to release granzyme to the cytoplasm where the protease is activated to induce

apoptosis in the target cell [66]. However, many CD8+ T-cells also carry the Fas-ligand (CD178) by which they might induce apoptosis in Fas receptor (CD95/APO-1) expressing cells. A third effector function of the cells is cytokine production, for example interferon-γ (IFN-γ), IL-2 and TNF-α. IL-2, IFN-γ and TNF-α have a central role in the cellular immunity; IFN-γ is for example important for activation and differentiation of immune cells as well as up-regulation of MHC molecules and TNF-α is an important activating cytokine for macrophages and endothelial cells.

Upon chronic infection, dysfunctional T-cells might arise. They are either characterized by the loss of proliferative capacity, due to telomere erosion or DNA damage (senescence) or characterized by expression of inhibitory receptors and an altered transcriptional profile associated with compromised effector functions (T-cell exhaustion). T-cell exhaustion is associated with high antigenic load and the exhausted cells most often display an effector memory phenotype characterized by the up-regulation of inhibitory receptors such as programmed death 1 (PD1) receptor, cytotoxic T-lymphocyte antigen 4 (CTLA4) and lymphocyte activation gen 3 (LAG3) [67]. The effector functionality of these cells might become compromised starting with a decreased IL-2 production, followed by TNF-α production, decreased cytotoxic activity and finally a loss of IFN-γ production. This might occur generally, or more specifically in certain antigen-specific T-cell clones [68, 69].

1.1.4.3 Other cell-surface markers

Evaluation of cytotoxic effector functions of CD8+ T-cells can be done without measuring the intracellular cytolytic molecules themselves, by enumerating the frequencies of cells expressing CD107a (LAMP-1) on the cell-surface. CD107a is associated with the membrane of cytolytic granules carrying perforin and granzyme, and this marker can be measured on the cell-surface of T-cells upon exocytosis of these vesicles [70]. The measurements can be done either in *ex vivo* assays (which reflect the activation of immune cells without *in vitro* manipulation) [71] or, alternatively, after antigenic stimulation *in vitro*.

The cytokine IL-7 is important for T-cell homeostasis, growth, maturation and memory formation [72]. It acts by signaling through the IL-7R, which is a heterodimer composed of the IL-7Rα (CD127) and the cytokine common γ-chain (CD132). This

receptor is expressed, and can be measured, on the cell-surface of a majority of differentiated CD8+ T-cells, but it is transiently down-regulated during proliferation in response to antigenic stimuli [73].

Other important cell-surface markers expressed by different immune cells including T-cells are e.g. CD95, which is a receptor involved in modulation of T-cell activation and in pro- and anti-apoptotic signaling cascades [74]. CD117 is a cell-surface receptor expressed on different types of hematopoietic progenitor cells and plays a role in cell survival, proliferation, and differentiation [75].

1.2 MULTIMERS

1.2.1 Structure

Due to too low avidity between individual MHC complex and TCRs, it is not possible to visualize individual MHC-TCR interactions directly in *ex vivo* sampled blood specimens by flow cytometry. Instead, multimerization of the MHC molecules allows for simultaneous binding of multiple MHC molecules to TCRs on the cell-surface of T-cells, thereby increasing the avidity. Multimers (e.g. tetramers, pentamers and dextramers) are flow cytometry reagents initially developed in 1996 [44] that are used for detecting antigen-specific T-cells *ex vivo.* They consist of at least 4 soluble MHC class I molecules presenting the same peptide-epitope, linked together via a fluorescent labeled linker (e.g. streptavidin, avidin or dextran) (Figure 6). Today, multimers exist commercially for most of the common 'Caucasian' and 'Asian' MHC class I alleles. However, we do not have sufficient access to soluble MHC class I molecules covering the most frequent 'African' MHC class I alleles.

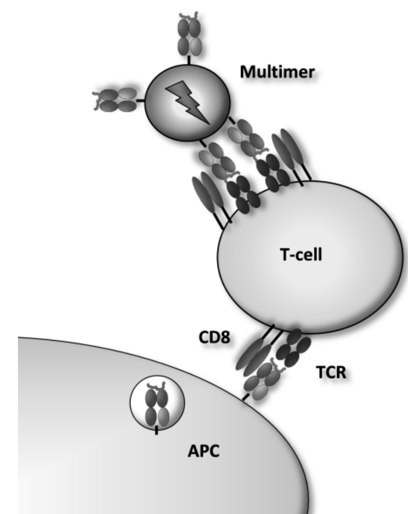

Figure 6. Multimer recognition of an antigen-specific T-cell.

1.2.2 Function

Multimers provide an efficient way to identify, enumerate, visualize and sort antigen-specific CD8+, and to a lesser extent CD4+, T-cells directly in blood, in frozen peripheral blood mononuclear cells (PBMCs) or in tissue samples [44, 76, 77]. They can be combined with other cell-surface markers to derive important information about the phenotype, effector functions, activation status, homing capacity, proliferation and

apoptosis of T-cells [78]. The reagents can be used for measuring antigen-specific response in infection and after vaccination. They can also be utilized for sorting and purification of antigen-specific T-cells for subsequent analysis, therapeutic transfer [79] or TCR analysis. One advantage of the use of multimers is the fact that it is possible to assess T-cell frequencies without any *in vitro* manipulation [44]. The major drawback with this method is that it does not provide any direct evidence of function, e.g. killing capacity or cytokine production. However, MHC class I multimeric analysis can be combined with analysis of cytokine production, although the currently applied protocols may not allow accurate estimation of MHC class I-TCR interactions, since detection of intracellular cytokines requires fixation and permeabilization of the cell-membrane and this may result in lower frequency of multimer positive events.

1.3 TUBERCULOSIS

1.3.1 The bacterium

M.tb was isolated and identified as the causative agent of TB by Robert Koch in 1882. *M.tb* is a large rod-shaped bacterium (2–4 µm in length and 0.2–0.5 µm in width) belonging to the genus *Mycobacterium*. *M.tb* is a facultative intracellular parasite that usually infects human macrophages. The generation time is long, usually 15–20 h, defining it as a slow-growing pathogen. The bacteria are classified as acid-fast due to their complex cell-walls impermeability to a number of dyes. The cell-wall consists of peptidoglycan and complex lipids (e.g. mycolic acids, acyl glycolipids and sulfolipids) and is an important virulence factor of the bacteria [80]. *M.tb* has circular chromosomes about 4 200 000 nucleotides long containing about 4 000 genes [81]. Many of these genes are still unknown, although intensive research has been performed since the genomic sequence was revealed in 1998 [82]. Among the proteins with identified function, an unusual high amount participates in the fatty acid metabolism. Another distinctive feature of the mycobacterial genome is the large group of unrelated proteins containing specific repeats of proline (P) and glutamic acid (E) (PE and PPE proteins) [83] .

1.3.2 The disease

M.tb is able to cause disease in almost all organs in the human body (e.g. lung, central nervous system, the bones and the joints). However, since the bacteria are mainly transmitted via the airborne route, pulmonary TB is the most prevalent form of disease. Extrapulmonary TB is most common in immunosuppressed individuals, e.g. those

infected by HIV and young children. Not all individuals exposed to aerosols contaminated with *M.tb* acquire infection, usually repeated contact is required. An infection starts with the aspiration of bacteria to the pulmonary alveoli where they infect resident macrophages. Macrophages phagocyte the bacteria, but instead of being degraded in the hostile environment of the phagolysosomes that normally form and degrade phagocytosed pathogens, *M.tb* stops the fusion of the phagosomic and lysosomic compartments, thereby creating an immune privilege niche were they can survive and replicate [84]. Early during infection, professional APCs like alveolar macrophages, monocyte-derived macrophages and DCs take up the bacteria, subsequently DCs travel to the draining lymph nodes and present *M.tb* antigens to naïve T-cells [85]. The T-cells become activated and afterwards home to the infected lung tissues where they orchestrate the formation of granulomatous structures, together with neutrophils and monocytic cells, which may contain the *M.tb* bacilli. In a majority of cases, the host immune system is able to control the initial infection and keep the bacteria in a dormant, non-replicating state. In this state, the infected individuals are neither sick nor infectious, i.e. they are carrying latent TB. During a lifetime there is in general a 10 % risk of reactivation of latent TB into active disease. The only vaccine used today against *M.tb* is the *M.bovis*-derived *Bacillus Calmette Guérin* (BCG) originating from 1921. Billions of people have been vaccinated with this vaccine but its efficacy is questioned, as it seems to give limited effect against pulmonary TB in adult individuals, dependent on the geographic regions and previous exposure of environmental mycobacteria [86].

1.3.3 Adaptive immune responses to *M.tb*

As previously mentioned, *M.tb* is an intracellular pathogen; therefore the adaptive immune response is primary directed by Th1 cytokines (e.g. IL-2, IFN-γ and TNF-α), i.e. cell-mediated. Many different T-cell subsets have been shown to be important, including CD4+ T-cells [4], CD8+ T-cells [5] and unconventional and regulatory T-cells. However, anti-*M.tb* directed humoral immune responses have recently gained increased interest in protection against mycobacteria, both regarding modulation of the cell-mediated immune response as well as in a direct protecting role against *M.tb* infection [87, 88]. Another kind of adaptive immune response against *M.tb* infection is the IL-17 mediated Th17 response [89], associated with recruitment and activation of neutrophils [90].

1.3.4 Genetic variability and immune responses against *M.tb*

The outcome of infectious diseases depends on a delicate balance between host (e.g. genetic variants of factors in the immune system and different MHC alleles) and pathogen-derived factors (e.g. virulence factors and escape mutations), as well as other external factors like co-existing diseases and environmental factors (including stress, malnutrition, poverty and helminth infections).

Host-derived factors, differences in ethnicity and certain MHC class I alleles have been shown to be important for the specific T-cell responses in many disease settings, including hepatitis B [91], hepatitis C [92] and HIV [93]. Considering *M.tb* susceptibility, associations between specific lineages of *M.tb* and certain populations indicate local host-pathogen co-adaptation [94]. More recently, increasing evidence of host-derived gene polymorphisms (e.g. IFN-γ [95], DC-SIGN, vitamin D receptor [96], toll like receptor variants [97] and nitric oxide synthase [98]) affecting TB susceptibility have been identified. In addition, different studies have linked individual MHC class II molecules to increased or decreased susceptibility to *M.tb* infection in different population cohorts (e.g. HLA-DR2 in Indian and Russian populations [99, 100] and HLA-DR4 in the Italian population [101]). Despite the fact that no significant association between MHC class I alleles and *M.tb* susceptibility has been found, inter-allelic variations in the MHC class I locus may still have impact on immune recognition due to their possibility to present different peptide repertoires and thereby influencing immune recognition by antigen-specific T-cells.

1.3.5 *M.tb* epitopes

The sequencing of the entire H37Rv *M.tb* reference genome [82] opened many new possibilities to identify molecularly defined protein targets for CD4+ and CD8+ T-cell epitopes. Yet, a majority of the identified T-cell epitopes derived from *M.tb* proteins originates from a limited set of well-studied targets, like the early expressed virulence factors ESAT-6, CFP10, TB10.4 and the Ag85complex [102-104], as well as the very abundant PE and PPE family proteins [105].

1.3.6 Genetic diversity in *M.tb*

The genetic diversity in the *M.tb* genome between different bacterial strains seems to be quite low, but some differences (insertions, deletions and point mutations) have been identified [106]. However, these variations may have both phenotypical and

immunological impact, e.g. by changing the ability of the infected individuals to induce a cellular immune response [107, 108]. Most variations in the TB genome reside in non-essential genes and it has previously been shown that human T-cell epitopes show little sequence variability in proteins derived from *M.tb*. This seems to hold true for many proteins, except for the small immunogenic protein TB10.4 being one exception [109].

1.3.7 Immunogenic *M.tb* proteins

1.3.7.1 TB10.4 (Rv0288)

TB10.4 (Rv0288) (also known as ESAT-6-like protein esxH, CFP7) is a well-studied early expressed and secreted TB virulence factor of 10 kDa (96 amino acids). It consists of two major α-helixes and forms a dimer with TB9.8 (Rv0287) (Figure 7). It is expressed in both the vaccine strain BCG as well as in virulent *M.tb* strains [110]. TB10.4 is part of the 6 kDa early secretory antigenic target (ESAT-6) gene family which encodes a number of secreted immunodominant molecules such as TB10.3 and TB12.9 [103]. A few CD8+ T-cell epitopes have previously been described for this protein [111, 112]. TB10.4 is also a component in several novel TB-vaccine candidates [113].

Figure 7. Structure of *M.tb* protein TB10.4 (left part) and TB9.8 (right part). Adapted from PDB file 2KG7 [176].

1.3.7.2 Ag85B (Rv1886c)

The 30 kDa (325 amino acids) Ag85B (Rv1886c) (also known as α antigen or MPT59) is the most abundant protein expressed by *M.tb*. The secondary structure of the protein consists of 7 α-helixes encircling a central parallel β-sheet (Figure 8). It is both a secreted and cell-wall associated protein that seems to be primarily expressed during the early stages of infection and is expressed both by *M.tb* and by the *M.bovis*-derived vaccine strain BCG as well as in mycobacteria other than tuberculosis (MOTT). The protein is one of three closely related mycolyl transferase molecules (the others are Ag85A and Ag85C) contributing to cell-wall biosynthesis synthesis by catalyzing the transfer of the fatty acid mycolate from one trehalose monomycolate to another. This

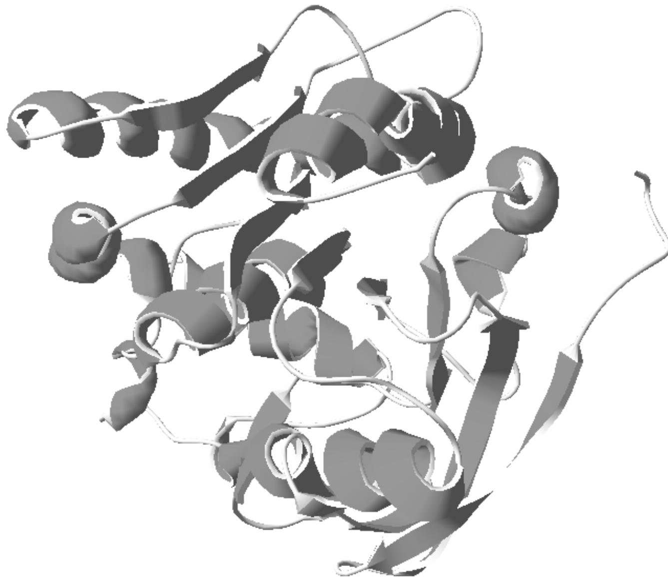

Figure 8. Structure of *M.tb* protein Ag85B.
Adapted from PDB file 1F0N [177].

results in trehalose 6,60-dimycolate and free trehalose [114]. The protein has been shown to be among the most potent antigens identified [115] and is a major target of human T-cell responses to *M.tb* [104], and it is also part of many novel TB-vaccine candidates [113, 116].

1.3.7.3 ESAT-6 (Rv3875)

ESAT-6 (*esxA,* Rv3875) is a 95 amino acid long *M.tb*-derived protein which forms a dimer with another *M.tb* protein: CFP10 [117]. It belongs to the RD1 region which is only present in a limited number of mycobacterial species including *M.tb* and e.g. *M. marinum* and *M. kansasii*, but is absent in BCG and many other mycobacterial species [118]. This makes it an interesting diagnostic marker for differentiating between vaccinated and *M.tb* infected individuals [119]. It belongs to an extended family (~23 members) of small (~100 amino acids) proteins from which many CD4 and CD8 T-cell epitopes have already been identified and most patients infected with *M.tb* recognize the antigen. The protein also contributes to *M.tb* pathogenicity, as deletion of the gene leads to attenuation of the bacteria [120]. ESAT-6 is expressed early in infection and contributes to apoptosis of macrophages and lung epithelial cells, thereby permitting cell-to cell spread of *M.tb.* In addition, immunomodulatory effects on macrophages, like perturbation of intracellular signaling pathways affecting antigen-presentation and cytokine production have been reported [121-123].

1.3.7.4 Glycosyl transferases and cyclopropane fatty acid synthase

Non-secreted *M.tb*-proteins like glycosyl transferase (1) (Rv2958c), glycosyl transferase (2) (Rv2957) and the cyclopropane fatty acid synthase (CFA synthase) (Rv0447c) are expressed predominantly in slow growing bacteria such as mycobacteria. The glycosyl transferases are enzymes involved in glycosylation steps downstream of branched lipids like phenolic glycolipid (which are associated with *M.tb*

hypervirulence [124]), and esters like glycosylated *p*-hydroxybenzoic acid methyl esters biosynthesis [125] (products that seems to be associated with resistance to intracellular killing by macrophages [126]). The CFA-synthase belongs to a group of enzymes involved in the creation of important components for making the cell-wall impermeable (i.e. cyclopropanation of the mycolic acids) [127].

The proteins are known to be immunogenic, since they have been identified to be targets for humoral responses [128] and HLA-class II presentation [129], as well as T-cells responses measured in whole blood assays, assessing CD4+ and CD8+ T-cell responses [130]. In addition, these proteins are mainly expressed by slow growing bacteria and might therefore show a different pattern of immunogenicity compared to the early expressed *M.tb* antigens.

2 AIM OF THE THESIS

- Identification of MHC class I binding epitopes in the TB protein TB10.4 using a panel of Caucasian MHC class I molecules.

- Construct monomers for some of the most frequent 'African' MHC class I alleles: HLA-A*30:01, A*30:02, A*68:01, B*58:01 and C*07:01.

- Construct a peptide binding assay and identify MHC-peptide binding epitopes for the *M.tb*-associated antigens TB10.4, Ag85B and ESAT-6.

- Further characterize the binding properties of the identified peptide binding epitopes from TB10.4, Ag85B and ESAT-6.

- Construct MHC class I multimers presenting *M.tb* protein-derived epitopes (TB10.4, Ag85B, ESAT-6, glycosyl transferase 1, glycosyl transferase 2 and cyclopropane fatty acid synthase) to enumerate MHC class I restricted CD8+ T-cells.

- Employ the multimers for characterization of *M.tb*-specific T-cells directly in blood obtained from patients with *M.tb* infection with different ethnic background (Caucasian, Asian and African).

- Evaluate the number of existing T-cell epitopes from *M.tb*-derived proteins.

3 METHODOLOGICAL DEVELOPMENT

3.1 PATIENTS WITH PULMONARY TB

In this thesis, PBMCs from patients with active pulmonary TB were used to enumerate and characterize antigen-specific T-cells in different settings. These patients had different genetic background and originated from different geographic regions. In *Paper I* we used T-cells from Caucasian patients originating from Mainz, Germany, in *Paper IV* we used cells from TB patients from South Korea and in *Papers II and III* we used cells from patient with a sub-Saharan African background, originating from two different sites in South Africa (Pretoria and Cape Town).

3.2 DEVELOPMENT OF NOVEL MATERIALS AND METHODS

3.2.1 African MHC class I alleles

Today, the most frequently expressed MHC class I alleles in a Caucasian and Asian population exist commercially, but since different African population groups have a very diverse expression of MHC class I alleles, the lack of commercially available tools for many of the most common alleles in this area represents a major problem to directly enumerate T-cells in blood from patient with TB. In order to overcome this challenge, we constructed and produced some of the most common African MHC class I alleles [14, 131] recombinantly for *Papers II and III* (e.g. HLA-A*30:01, A*30:02, A*68:01, B*58:01 and C*07:01). This represents a major part of this thesis.

3.2.2 Recombinant MHC class I molecules

In addition to the above listed 'African' MHC class I molecules, several previously described MHC class I alleles (e.g. HLA-A*02:01, A*24:02 and B*07:02) were produced recombinantly during this thesis and used in *Papers II, III and IV*. Bacterial expression vectors (pET24d+ and pHN1) containing the nucleotide sequences for the soluble part of the heavy chain of the MHC class I alleles and the light-chain β_2m were either newly cloned or contributed by Beckman Coulter, San Diego, CA. All recombinant MHC class I molecules ('African' and 'Caucasian/Asian') were produced as previously described [132]. In summary, heavy and light chains were produced in *Escherichia coli* Bl21 DE3 pLys as inclusion bodies and solubilized in an 8 M urea buffer, pH 6.5, and folded to correct trimeric structure in a pH 8.0 Tris-EDTA-arginine buffer together with allele-specific candidate peptides (Table 2).

Folded MHC class I-peptide monomers were biotinylated using the enzyme BirA. The biotinylated monomers were affinity purified using an avidin column.

Table 2.
The MHC class I molecules included in this thesis and their control peptides.

Allele	*Control Peptide*	*Original protein*
A*01:01	EVDPIGHLY	MAGE-A3
A*02:01	FLPSDFFPSV	HBV CORE
A*03:01	KVFPCALINK	Consensus peptide modified at position 5
A*11:01	AVFDRKSDAK	EBV EBNA 3B
A*24:02	RYLKDQQLL	HIV env
A*30:01	KTKDIVNGL	F-actin capping protein beta
A*30:02	KIQNFRVYY	HIV-integrase
A*68*01	KTGGPIYKR	Influenza virus nucleoprotein
B*07:02	TPRVTGGGAM	CMV pp65
B*08:01	EIYKRWII	HIV p24
B*15:01	AMKGLPIRY	DENGUE VIRUS NS3
B*58:01	IAMESIVIW	HIV RT
C*07:01	KYFDEHYEY	CDC28 protein

3.2.3 Peptide binding assay

In *Paper I*, a commercially available peptide binding assay (iTOPIA, Beckman Coulter, San Diego, CA) was used for the most common 'Caucasian' MHC class I alleles. A similar peptide binding assay was constructed for several MHC class I alleles (A*02:01, A*24:02, A*30:01, A*30:02, A*68:01, B*07:02, B*58:01 and C*07:01) and used in *Papers II and III*. Briefly, ninety-six-well plates were first coated with biotinylated bovine serum albumin, followed by avidin and biotinylated monomer (0.5 μg/ml). During the first step of the assay, the monomer-coated plates were stripped of the placeholder peptide leaving the heavy chain free to reassociate with an added candidate peptide after addition of β_2m. Peptide binding was detected as fluorescence, after 18 h of incubation at 21 °C with a conformational antibody (either fluorescein isothiocyanate [FITC]-conjugated anti-HLA-A, -B and -C [A*02, A*24, A*68, B*07, B*58 and C*07] [Beckman Coulter, San Diego, USA] or anti-HLA-A30 [One Lambda Inc, Canoga Park, USA]) (Figure 9). The binding of each candidate peptide was compared with the binding of an appropriate control peptide (Table 2).

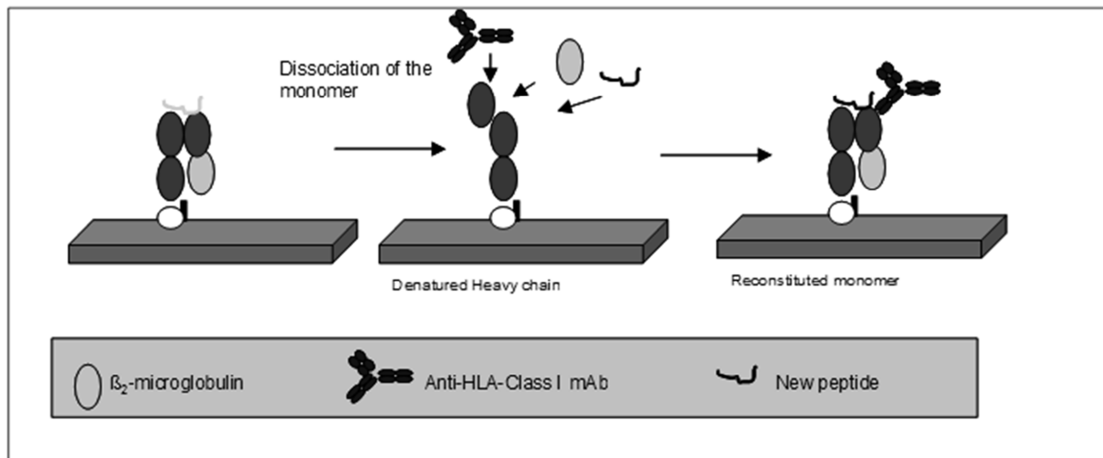

Figure 9. Schematic structure of the peptide binding assay. In the first step, both the placeholder peptide and β_2m are stripped away using an acidic buffer. By subsequent addition of a candidate peptide as well as new β_2m, the trimeric complex is able to refold if the candidate peptide binds to the selected MHC class I allele. Formation of the trimeric complex can be measured using a conformation-dependent antibody.

This assay was not only used for assessing peptide binding, but also for assessing peptide-MHC affinity and dissociation rate (*Papers I–III*). The dissociation rate was measured as a $t_{1/2}$ value, which is defined as the time point when 50 % of the initial peptide concentration has dissociated from the MHC-peptide molecule complexes. The measurements took place after incubating the refolded MHC molecules at 37 °C at eight different time points (0 h, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h and 8 h). Affinity was measured incubating titrated peptide concentrations (10^{-4} to 10^{-9} M) overnight and then calculating the peptide quantity needed to achieve 50 % binding saturation.

3.2.4 Multimers

In this thesis (*Papers I–IV*), 62 *M.tb*-derived multimers (tetramers and dextramers) were constructed, of which 50 were never previously described (12 were described in [104, 133-137]) (Table 3). They were either purchased (Beckman Coulter, San Diego, USA and Immudex, Copenhagen, Denmark) or constructed in our laboratory as previously described [44]. A particular focus was made to construct *M.tb* multimers based on MHC class I alleles not commercially available and common in an African population (e.g. HLA-A*30:01, B*58:01 and C*07:01).

Table 3.
List of constructed multimers for this thesis, including epitope-derived protein, epitope sequence and the restricting MHC class I allele.

Antigen	*Sequence*	*Allele*	*Study*	*Antigen*	*Sequence*	*Allele*	*Study*
Rv0288$_{2-10}$	SQIMYNYPA	A*02:01	I	Rv0447c$_{331-337}$	KYIFPGGLL	A*24:02	III, IV
Rv0288$_{3-11}$	QIMYNYPAM	A*11:01	I	Rv0447c$_{396-404}$	RMWELYLAY	A*30:02	III
Rv0288$_{3-11}$	QIMYNYPAM	A*30:01	II, III	Rv1886c$_{77-85}$	YLLDGLRAQ	A*02:01	III
Rv0288$_{3-11}$	QIMYNYPAM	A*30:02	II, III	Rv1886c$_{100-108}$	WYYQSGLSI	A*24:02	III
Rv0288$_{4-12}$	IMYNYPAML	A*02:01	I, III, IV	Rv1886c$_{110-118}$	MPVGGGSSF	B*07:02	III
Rv0288$_{4-12}$	IMYNYPAML	A*24:02	I, III, IV	Rv1886c$_{133-141}$	QTYKWETFL	B*58:01	III
Rv0288$_{4-12}$	IMYNYPAML	A*30:02	II, III	Rv1886c$_{140-146}$	FLTSELPQW	A*24:02	III
Rv0288$_{4-12}$	IMYNYPAML	B*07:02	I	Rv1886c$_{145-153}$	LPQWLSANR	A*68:01	III
Rv0288$_{5-13}$	MYNYPAMLG	A*24:02	III	Rv1886c$_{183-191}$	FIYAGSLSA	A*02:01	III, IV
Rv0288$_{5-13m3}$	MYNYP**T**ML**D**	A*24:02	III	Rv1886c$_{184-192}$	IYAGSLSAL	A*24:02	III, IV
Rv0288$_{10-18}$	AMLGHAGDM	A*02:01	III	Rv1886c$_{184-192}$	IYAGSLSAL	B*07:02	III
Rv0288$_{10-18m2}$	AML**D**HAGDM	A*02:01	III	Rv1886c$_{200-208}$	GPSLIGLAM	B*07:02	III
Rv0288$_{11-19}$	MLGHAGDMA	A*02:01	III	Rv1886c$_{237-245}$	IPKLVANNT	B*07:02	III
Rv0288$_{11-19m1}$	ML**D**HAGDMA	A*02:01	III	Rv1886c$_{239-247}$	KLVANNTRL	A*02:01	III, IV
Rv0288$_{20-28}$	GYAGTLQSL	A*24:02	I	Rv1886c$_{241-249}$	VANNTRLWV	A*30:01	III
Rv0288$_{43-51}$	WQGDTGITY	B*15:01	I	Rv1886c$_{241-249}$	VANNTRLWV	A*30:02	III
Rv0288$_{50-58}$	TYQAWQAQW	A*24:02	I	Rv1886c$_{242-250}$	ANNTRLWVY	C*07:01	III
Rv0288$_{54-62}$	WQAQWNQAM	B*15:01	I	Rv1886c$_{307-315}$	WGAQLNAMK	A*68:01	III
Rv0288$_{60-68}$	QAMEDLVRA	A*02:01	I	Rv2957$_{26-34}$	SIIIPTLNY	A*02:01	III, IV
Rv0288$_{61-69}$	AMEDLVRAY	A*03:01	I	Rv2957$_{137-145}$	LVYGDVIMR	A*68:01	III
Rv0288$_{61-69}$	AMEDLVRAY	A*30:02	II, III	Rv2957$_{183-191}$	PYNLRYRVL	A*24:02	III, IV
Rv0288$_{65-73}$	LVRAYHAMS	A*30:01	II, III	Rv2957$_{249-257}$	IVLVRRWPK	A*30:01	III
Rv0288$_{70-78}$	HAMSSTHEA	A*68:01	III	Rv2958c$_{22-30}$	AAPEPVARR	A*68:01	III
Rv0288$_{78-86}$	ANTMAMMAR	A*11:01	I	Rv2958c$_{110-118}$	KYIAADRKI	A*24:02	III, IV
Rv0288$_{78-86}$	ANTMAMMAR	A*68:01	III	Rv2958c$_{138-146}$	SARLAGIPY	A*30:02	III
Rv0288$_{81-89}$	MAMMARDTA	B*07:02	I	Rv2958c$_{288-296}$	ALADLPVTV	A*02:01	III, IV
Rv0288$_{82-90}$	AMMARDTAE	B*07:02	I	Rv3875$_{28-36}$	LLDEGKQSL	A*02:01	III, IV
Rv0288$_{83-91}$	MMARDTAEA	A*02:01	I	Rv3875$_{50-58}$	AYQGVQQKW	A*24:02	III, IV
Rv0288$_{83-91}$	MMARDTAEA	B*08:01	I	Rv3875$_{64-72}$	ELNNALQNL	A*24:02	III, IV
Rv0447c$_{31-39}$	AASAAIANR	A*68:01	III	Rv3875$_{82-90}$	AMASTEGNV	A*02:01	III, IV
Rv0447c$_{110-118}$	VLAGSVDEL	A*02:01	III, IV	Rv3875$_{82-90}$	AMASTEGNV	A*30:02	III

3.2.5 Flow cytometry

The multimers were used in flow-cytometric experiments together with other cell-surface markers (Table 4). Panel 1 was used in *Paper I*, while panel 2 was used in *Papers II, III and IV*. In *Paper IV*, panels 3 and 4 were used.

Table 4.
List of cell-surface markers used for defining T-cell phenotypes and effector functions.

Panel 1 – Small multimer panel	*Panel 2 – Large multimer panel*	*Panel 3 – 'Stem-cell' markers*	*Panel 4 – ICS*
CD3	CD3	CD3	CD3
CD4	CD4	CD4	CD4
CD8	CD8	CD8	CD8
Multimer	CD45RA	CD45RA	IL-17
	CCR7	CCR7	IL-2
	CD127	*CD95*	IFN-γ
	CD107a	*CD117*	TNF-α
	Multimer(s)	Multimer(s)	

4 RESULTS AND DISCUSSION

The main aim of this thesis was the identification of T-cell epitopes restricted via MHC class I molecules common in different ethnical population groups, and subsequent analysis of the nature of the antigen-specific T-cells that recognize them. Therefore, we have been studying *M.tb*-derived epitopes on several levels including: 1) peptide-MHC interaction, 2) T-cell epitope recognition and 3) phenotype and effector functions of the antigen-specific T-cells.

4.1 MHC CLASS I BINDING EPITOPE IDENTIFICATION

Many human T-cell epitopes originating from *M.tb*-derived proteins have been identified until today. We have, by using the immune epitope database [45], evaluated more than 800 human epitopes. By investigating the epitope-derived protein, we could see that all the *M.tb*-derived epitopes that have been identified thus far originated from only 170 out of an estimated total of ~4 000 proteins, i.e. only 4 % of the *M.tb* genome [81] (*Paper V)*. This leaves most of the genome still unexplored regarding T-cell epitopes. For the majority of the 170 epitope-containing proteins, only a single epitope has been found. For other protein-derived virulence factors, a significant number (>20) of epitopes have already been identified (e.g. Ag85A, Ag85B, TB10.4, ESAT-6 and Cfp10) (*Paper V*). Although quite a number of epitopes have been identified, a bias and a lack of proper knowledge still exist regarding the nature of many of these epitopes. In line with previous findings regarding the nature of human *M.tb* epitopes [138], we have pinpointed challenging areas concerning *M.tb* epitope identification: 1) A majority of the epitopes are MHC class II restricted CD4+ T-cell epitopes based on the length (>11 amino acids), 2) information is missing about the disease/vaccination status (active/latent *M.tb* and/or BCG vaccination) of the individual from which an immune response is originating, 3) information about the epitope-derived *M.tb* strain is not known, 4) information is missing about the function and expression pattern of the epitope-derived protein and, finally, 5) information about the restricting allele is in many cases either missing or it represents one of the alleles common in the Caucasian population groups, such as HLA-A*02:01. The last bias is particularly problematic, since high TB burden exists in many parts of the world, where the population groups express predominantly other MHC class I allotypes than HLA-A*02:01, for example populations in sub-Saharan Africa and Southeast Asia [1].

4.1.1.1 TB10.4-, Ag85B- and ESAT-6-derived MHC class I binding epitopes

Due to the limited knowledge of *M.tb*-derived epitopes binding to a broad variety of human MHC class I alleles, we aimed at identifying novel immunogenic epitopes recognized by individuals with different genetic backgrounds. Therefore, *Papers I–III* reported the identification of a large amount of novel peptide binding epitopes from

Figure 10. Identified binding epitopes in the *M.tb*-derived protein TB10.4. Binding of different epitopes to a panel of 13 different MHC class I molecules; HLA-A*01:01 (light purple), A*02:01 (red), A*03:01(apricot), A*11:01 (pink), A*24:02 (dark purple), A*30:01 (orange), A*30:02 (dark green), A*68:01 (middle blue), B*07:02 (light blue), B*08:01 (light green), B*15:01 (dark blue), B*58:01 (grey) and C*07:01 (black) (*Papers I–III*).

several different *M.tb*-derived proteins (TB10.4, Ag85B and ESAT-6) to a diverse panel of MHC class I molecules (HLA-A*01:01, A*02:01, A*03:01, A*11:01, A*24:02, A:30:01, A*30:02, A*68:01, B*07:02, B*08:01, B*15:01, B*58:01 and C*07:01) covering the most common alleles in Caucasian, Asian and African population groups [14]. In *Paper I*, we identified 53 binding epitopes in TB10.4 for 8 MHC class I alleles common in the Caucasian population (Figure 10). In *Paper II*, we continued to study binding epitopes from the same *M.tb*-derived protein in association with two of the most common sub-Saharan African MHC class I alleles (HLA-A*30:01

and A*30:02). In this study, we could identify 25 novel epitopes, including the first *M.tb*-associated epitopes binding to HLA-A*30:02 (Figure 10). In *Paper III*, we identified 29 additional TB10.4 epitopes associating with three other 'African' alleles (HLA-A*68:01, B*58:01 and C*07:01) (Figure 10) as well as 292 peptide epitopes originating from Ag85B and 40 epitopes from ESAT-6 associating with HLA-A*02:01, A*24:02, A*30:01, A*30:02, A*68:01, B*07:02, B*58:01 or C*07:01. The 30 epitopes associating with HLA-C*07:01 were the first ever identified disease-related epitopes for this allele.

4.1.1.2 Difference in MHC class I restricted peptide binding repertoires

As has previously been reported [139, 140], a discrepancy in the peptide binding capacity exists between different MHC class I alleles (Table 5). We found that some of the alleles included in these studies exhibited a broad (A*30:02, A*02:01 and A*24:02), some an intermediate (A*68:01 and B*58:01) or some a restricted (B*07:02 and C*07:01) peptide binding pattern. Particularly interesting is the fact that although the difference is only four amino acids [13], there is a remarkable discrepancy in the binding between the two alleles belonging to the A*30 family (i.e. A*30:01/A*30:02) (*Paper II*), in part due to structural constrains, electrostatic charges and hydrophobicities within the binding pockets responsible for the binding repertoire [20].

Table 5.
Number of identified epitopes restricted by selected MHC class I molecules for the *M.tb*-derived proteins TB10.4, Ag85B and ESAT-6.

	Number of Epitopes			
Allele	TB10.4	Ag85B	ESAT-6	Total
A*01:01	2	–	–	2
A*02:01	17	57	11	85
A*03:01	2	–	–	2
A*11:01	3	–	–	3
A*24:02	10	41	2	53
A*30:01	3	8	0	11
A*30:02	22	78	21	121
A*68:01	11	29	3	43
B*07:02	7	25	0	32
B*08:01	0	–	–	0
B*15:01	12	–	–	12
B*58:01	14	29	2	45
C*07:01	4	25	1	30

For the majority of the most common MHC class I alleles, the peptide binding motif has been determined. Based on similar preferences for specific amino acids at certain positions, the MHC class I alleles have been divided into groups called supertypes [141]. In this thesis (*Papers I–III*), we identified epitopes matching perfectly with their assigned motif but also epitopes that matched only partially or did not match at all with the assigned MHC-peptide binding motif. For example, the very well-studied alleles HLA-A*02:01 and A*24:02 matched quite well concerning actual peptide binding data and peptide-MHC class I motifs (*Papers I and III*) [22, 142]. Also for the alleles HLA-A*68:01, B*07:02 and B*58:01, the majority of identified epitopes correlate at least partly with their assigned motif [142-144]. On the other hand, for alleles with less well-studied or disputable motifs (e.g. HLA-A*30:01), the majority of epitopes does not match the assigned motifs at all [145, 146]. Therefore, the data indicates that A*3001 may, as has previously been suggested, be able to use different arrangements of primary and secondary anchor residues [27]. For the allele HLA-A*30:02, we could identify a very broad peptide binding repertoire with diverse amino acids (hydrophobic, charged and polar) at the anchor positions, indicating that HLA-A*3002 utilizes multiple secondary anchor residues in the C- and N-terminal regions and may therefore show a promiscuous binding motif. However, the allele also seems to prefer tyrosine (Y) residues at P9 (*Papers II–III*, all epitopes with Y at P9 bound strongly) which is in line with the determined peptide motif [145]. For C*07:01, no motif exists to our knowledge, but based on the identified binding epitopes (*Paper III*) we are able to identify a preference for tyrosine or phenylalanine at P9.

4.1.1.3 Promiscuous MHC class I binding epitopes

Another very interesting discovery documented in *Papers I–III* was the frequently occurring ‘cross-binding’ of epitopes to different MHC class I alleles. The epitope TB10.4$_{4-12}$ is, for example, recognized by no less than 9/13 tested MHC class I alleles including both HLA-A, -B and -C alleles (*Papers I–III*), indicating a potential very broad immune recognition (Figure 10). Promiscuous binding has previously been identified for other pathogens like HIV, HPV and tumor associated antigens [140, 147, 148] and is likely to have clinical implications. In the field of vaccine design, the fact that a single peptide epitope is presented by a diverse repertoire of MHC alleles might facilitate the development of peptide vaccines, as fewer epitopes are needed to cover large population cohorts. On the other hand, a narrower focus on a few selected epitopes might facilitate immune escape variants, as described for viral pathogens (e.g.

HIV [149] and HCV [150]) although epitope-variations have only rarely been seen in *M.tb* thus far [109].

4.2 CHARACTERIZATION OF THE PEPTIDE-MHC BINDING PROPERTIES

The affinity and dissociation rate (off-rate) of a peptide-MHC complex determine the time-frame in which an epitope is available for T-cell priming on the cell-surface and for target recognition (in the effector phase) of the immune response. In *Papers I–III*, we determined these two parameters for most of the identified binding epitopes and could identify a range of intra-allelic differences (different peptides bound different to the same MHC class I allele) as well as inter-allelic differences (same peptides bound differently to different alleles).

The stability (ED50 value) generally ranged between 800 µM and 5 nM, which is in line with previous studies [104, 139], and we could see no significant variations between the different MHC class I alleles (*Papers I–III*). The dissociation rate, on the other hand, ranged between *0.1 h and 27 h* and varied considerably between different MHC class I alleles. HLA-A*02:01 showed the broadest range of off-rate with peptides dissociating very rapidly (~0.3 h) as well as very slowly (27 h). Generally, peptides that bound to the alleles with few identified binding epitopes (e.g. HLA-A*30:01, B*07:02 and C*07:01) tended to dissociate more rapidly, a fact that might originate from intrinsic structural features of the allotype (*Papers I–III*). A more restricted binding repertoire, in addition to a faster dissociation rate, might suggest a disadvantage in immune recognition in individuals with certain MHC class I allelic background. However, a fast peptide off-rate seems to be able to be counter-balanced by a high affinity, as described in *Paper III*.

4.3 ENUMERATION OF ANTIGEN-SPECIFIC T-CELLS

Prior to this thesis, only a limited number of MHC class I *M.tb*-specific multimers had been utilized in different settings (active-latent TB, children, HIV co-infected individuals and vaccine settings). We evaluated them in *Paper V* and could detect that the vast majority of the multimers were constructed using the common ‘Caucasian’ MHC class I allele: HLA-A*02:01 [14]. In addition, we could detect a bias towards a limited set of epitope-derived proteins, like the Ag85 proteins (Rv3804, Rv1886c and Rv2905c) [104, 133-135, 137, 151-153], ESAT-6 (Rv3875) [135, 137], 16kDaAg

(Rv2031c) [137], Hsp65 (Rv0440) [137], 19kDaAg (Rv3763) [135, 154], probable membrane protein (Rv1490) [137] and lgt (Rv1614) [137], despite the fact that *M.tb* contains around 4 000 different proteins [81].

4.3.1.1 Detection of antigen-specific T-cells using novel MHC class I multimers

In order to gauge new immunogenic epitopes derived from *M.tb* antigens, we enumerated and characterized the antigen-specific T-cell repertoire in patients with acute pulmonary TB. A broad array of (62) novel *M.tb*-specific multimers (*Papers I–IV*) was constructed, in order to test T-cells in peripheral blood from these patients.

Generally, we could identify a low, yet clearly detectable, broad recognition of several co-dominant epitopes derived from different *M.tb* proteins in the peripheral circulation of most patients with acute pulmonary TB, independent of their genetic background (Caucasians 0–2.1 % [*Paper I*], Africans 0–3.9 % [*Papers II–III*] and Asians 0–6.5 % [*Papers IV*]). The frequencies of antigen-specific CD8+ T-cells varied between the recognized epitopes (0–6.5 %), with the majority being recognized at least in some patients with acute pulmonary *M.tb* infection. This broad recognition is in line with another recent publication identifying multimer-specific novel A*02:01-restricted epitopes from another set of novel *M.tb*-derived proteins [155]. However, we could also identify some epitopes that appeared to be associated with higher T-cell frequencies, including the ESAT-6-derived epitope ELNNALQNL restricted by HLA-A*24:02 and the Ag85B-derived epitope KLVANNTRL restricted by A*02:01 (*Paper III–IV*). In *Paper I* (but not in *Paper III* due to too few HLA-B epitopes included) we were able to observe a predominantly HLA-B restricted T-cell response in line with previous studies on TB [104, 156], as well as in viral diseases like HIV [157], EBV [158] and CMV [159]. An HLA-B restricted dominant response might be linked to either differences at the MHC expression level on APCs and/or differences in the TCR repertoire that is available to recognize the respective MHC class I-peptide complex. Another reason for HLA-B dominance may be a selective interference of pathogens with HLA-A processing or up-regulation of HLA-B alleles [156].

4.3.1.2 *MHC class I allotype and epitope-derived protein might influence T-cell recognition*

When a large cohort of different MHC class I restricted multimers was used to characterize the *M.tb*-specific CD8+ T-cell response (*Paper III*), allele-specific recognition patterns could be identified. This observation also held true when combining the frequencies of antigen-specific T-cells detected in *Papers I–IV*. As can be seen in Figure 11A, HLA-A*03:01 and A*24:02 as well as HLA-B*07:02 and

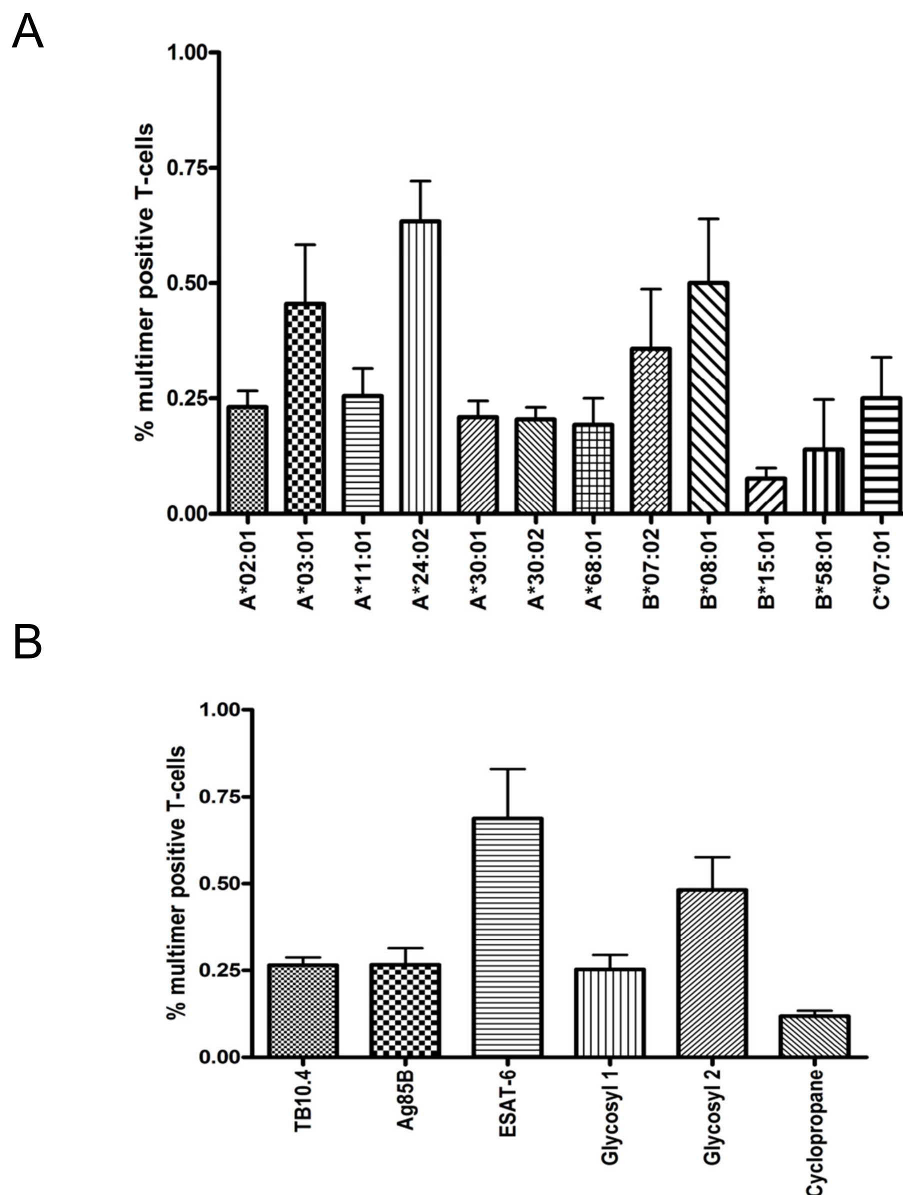

Figure 11. Combined detection of multimer positive CD8+ T-cells (*Papers I–IV*) stratified by restricting MHC class I allotype (A) and epitope-derived protein (B).

B*08:01 restricted epitopes were generally detected by a higher proportion of CD8+ T-cells than for example HLA-A*02:01, A*30:02, A*68:01 and B*15:01 restricted epitopes. Certain immunodominant epitopes might of course skew these results to a

certain extent, but the fact that we have included up to 16 different epitopes restricted by the same allotype strengthens the observed differences.

In addition to allele-specific differences in the frequencies of antigen-specific T-cell recognition, differences based on epitope-derived antigens could be identified in *Papers I–IV*. For example within the group of well-characterized early expressed *M.tb* antigens (TB10.4, Ag85B and ESAT-6), we could detect higher frequencies of antigen-specific T-cells recognizing ESAT-6-derived epitopes (0.69 %) compared to TB10.4 (0.26 %) and Ag85B (0.27 %). However, we believe that this result is biased by the strong recognition of the immunodominant epitope A24-ESAT-$6_{ELNNALQNL}$. While comparing antigen-specific recognition to other (not yet reported) *M.tb*-derived antigens, such as two glycosyl transferases and cyclopropane fatty acid synthase, expressed predominantly in slow-growing mycobacteria, additional differences in frequencies of antigen-specific T-cells could be seen. The glycosyl-transferase 2 (Rv2957) was for example more strongly recognized (0.48 %) than some of the previously well-studied immunodominant antigens (TB10.4 and Ag85B) [103] (Figure 11B).

4.4 CHARACTERIZATION OF THE ANTIGEN-SPECIFIC T-CELLS

4.4.1.1 Phenotypic characterization

In *Papers II–IV*, we also examined the phenotype and effector functions of the antigen-specific CD8+ T-cells. The phenotype of CD8+ T-cells can be defined by the usage of the cell-surface markers CD45RA and CCR7. *M.tb* antigen-specific T-cells have previously been described to predominantly belong to different T-cell compartments including terminally differentiated compartments CD45RA+CCR7- [137], memory compartments (CD45RA-) [104, 152] as well as precursor like compartments (CD45RA+CCR7+). In this thesis, we could detect that a majority of the antigen-specific CD8+ T-cells belongs to a compartment with a precursor-like phenotype, despite the different genetic background of the *M.tb*-infected individuals. This is in line with a number of previous studies which have identified a large frequency of *M.tb*-specific or BCG reactive T-cells in both adults and children expressing a 'naïve'-like phenotype [134, 160, 161]. Reasons for the high number of antigen-experienced cells with a precursor-like phenotype might include an increase in lymphopoiesis in patients with TB as well as a recycling of terminally differentiated antigen-experienced cells to the precursor CD8+ T-cell compartment by re-expression of

CCR7, as has been suggested in the field of HIV [162]. Another explanation might be that these cells belong to a stem-cell memory compartment, as we suggest in *Paper IV*, based on expression of CD45RA and CCR7, along with the stem-cell markers CD95 and c-kit (CD117). This has previously been seen in the context of other viral diseases and in the context of tumor antigens, where recent studies have identified 'naïve'-like T-cell populations based on the expression of the cell-surface markers CD45RA, CCR7, and CD127 as well as memory and stem-cell like markers such as CD95 and/or c-kit. It is hypothesized that these T-cells represent a long-lived subset of antigen-experienced multipotent cells [163, 164]

The frequency of antigen-specific T-cells that reside in a certain phenotypic compartment varies, as could be seen in *Paper III*, in association with the restricting MHC class I allele. Restriction by certain alleles like HLA-A*30 and C*07 lead to higher proportions of antigen-specific, terminally differentiated CD8+ T-cells, which may be important in controlling the infectious disease [5]. In addition, T-cells restricted via these allotypes showed increased expression of the degranulation marker CD107a, indicating increased cytotoxic functionality. The nature of the epitope-derived *M.tb* protein also influences the phenotype of the antigen-specific CD8+ T-cells. For example, in *Paper IV* we could detect a significant difference in the proportion of cells belonging to the terminally differentiated and precursor compartments between T-cells recognizing epitopes derived from TB10.4, Ag85B and ESAT-6 as compared to epitopes derived from the glycosyl transferases and cyclopropane fatty acid synthase. This might be due to differences in expression pattern (secreted/intracellular retained and/or expressed early in infection/during active/latent infection) of the antigenic proteins influencing immune recognition and thereby the priming event of specific CD8+ T-cells.

4.4.1.2 Expression of survival markers and effector functions

As expected, CD107a is up-regulated on antigen-specific T-cells independent of the restricting MHC class I allele and independent of the presented epitope (*Papers II–IV)*, indicating an increased cytolytic function of these antigen-experienced cells. However, as previously mentioned, T-cells restricted by certain MHC class I allotypes show higher expression of this cell-surface marker as well as expression of the IL7-Rα T-cell survival marker (*Paper III)*.

In *Paper IV*, we investigated the frequencies of antigen-specific cytokine-producing cells (IL-17, IL-2, IFN-γ and TNF-α) and were able to detect a low, yet clearly detectable, cytokine production (0–0.2 % of the CD8+ T-cells) in response to stimulation with the *M.tb* antigens TB10.4 and ESAT-6. The frequencies of cytokine-producing cells were similar or lower as compared to cytokine-producing *M.tb*-specific CD8+ T-cells identified in other studies [165-167]. In addition, we were able to compare the frequencies of cytokine-producing CD8+ T-cells to the frequencies of multimer-specific CD8+ T-cells in response to the same *M.tb*-derived antigen (TB10.4 and ESAT-6). Although the cytokine production was measured in response to overlapping peptides covering protein we were often able to detect higher frequencies of CD8+ T-cells specific to a single epitope (derived from the same protein) using multimers. The fact that we were able to detect higher frequencies by e*x vivo* identification of multimer reactive T-cells (probably cytotoxic based on high CD107a expression) has previously been reported in the context of HIV, where the CD8+ T-cells tend to exhibit either of these functions (cytokine production or cytotoxicity), but rarely both [168]. These results imply that functional T-cell assays based solely on detection of Th1 cytokines might underestimate the number of *M.tb* reactive T-cells.

The biological consequence concerning T-cell effector functions needs to be further studied. Of note, the PBMCs analyzed in our studies derived from patients with *active* TB and may therefore not represent the potential breadth of a strong protective CD8+ T-cell response, due to decreased CD8+ T-cell effector functionality in active TB [137].

4.5 VARIANT *M.TB* EPITOPES

The genome of *M.tb* is regarded as rather constant, with only minor polymorphism [169]. However, recently more frequent strain-to-strain variations have been reported. Most polymorphisms have been identified in non-essential genes, yet also in essential genes and in human T-cell epitopes from immunogenic *M.tb* proteins. One of the *M.tb*-derived proteins with the highest frequency of single nucleotide polymorphism (SNP), compared to its size, is the well-studied immunogenic protein TB10.4 [109]. In *Paper III*, we compared MHC class I binding and subsequent CD8+ T-cell recognition of TB10.4 epitopes from the reference genome H37Rv with epitopes from SNP-containing clinical isolates [170]. We showed: 1) Alteration of an epitope predominantly reduces its binding to the assigned MHC class I allele based on either an increased dissociation rate or a decreased affinity between the peptide and MHC

complex. 2) Variations in epitope sequences affect T-cell recognition. 3) Epitope variability leads to alteration of the phenotype and expression of cell-surface markers like CD107a and CD127 of the antigen-specific CD8+ T-cells. 4) Different T-cell repertoires seem to recognize the wild-type and variant epitopes (*Paper III*).

5 GENERAL CONCLUSIONS

- The usage of recombinant MHC class I molecules is a fast and objective way of identifying novel T-cell epitopes, which can be confirmed by the usage of multimers.

- Small differences in the peptide binding cleft between different MHC class I allotypes (e.g. HLA-A*30:01 and A*30:02) can give rise to completely different peptide binding repertoires.

- Promiscuous binding of *M.tb*-derived epitopes to many different MHC class I allotypes (including both HLA-A, -B and -C alleles) occurs and the epitopes give rise to T-cell responses restricted via different alleles.

- With the novel epitopes derived from *M.tb* proteins, it is possible to construct *M.tb* multimers and subsequently enumerate antigen-specific T-cell responses *ex vivo*. In combination with usage of other cell markers it is also possible to determine the phenotype and possible effector functions of *M.tb*-specific T-cells.

- The frequencies of antigen-specific T-cells recognizing different *M.tb* epitopes are usually quite low but broad. Non-immunodominant recognition of several different epitopes can be seen in blood from most patients with active pulmonary TB.

- The antigen-specific T-cell responses against *M.tb* tend to be HLA-B restricted but some immunodominant HLA-A epitopes could also be detected.

- The 1) restricting MHC class I allotype, 2) epitope-derived protein and 3) specific peptide sequence (including SNPs) seem to influence many levels of antigen-specific T-cell responses, including peptide-MHC binding, TCR recognition as well as effector functionality and the phenotype of the antigen-specific T-cells.

- A high proportion of *M.tb*-specific CD8+ T-cells express a precursor-like phenotype based on expression of the cell-surface markers CD45RA and CCR7. This might be due to cells that belong to a compartment of antigen-experienced memory cells expressing both precursor markers (CD45RA/CCR7) and ‘stem-cell markers’ (CD95/c-kit).

6 FUTURE PERSPECTIVES

We have developed novel MHC class I allotypes including previously not commercially available alleles (e.g. HLA-A*30:01, B*58:01 and C*07:01) with the hope that these might be useful not only in association with *M.tb* but also in other disease settings (infectious as well as malignant). Some alleles have previously been connected with protection or association with certain diseases. Expression of HLA-A*30:01 has, for example, been associated with increased risk of type 1 diabetes [171] as well as with severity of *plasmodium falciparum* mediated malaria [172], B*58:01 has been associated with HIV elite controllers [173] as well as for increased risk of certain drug hypersensibility reactions [174]. HLA-C*07 alleles have, on the other hand, been associated with predisposition for Graves' disease [175]. Very little is reported on the peptide repertoire of this allele and it would in general be interesting and important to discover additional C*07:01 restricted immunogenic epitopes in different disease settings and also to determine its peptide binding motif and subsequently identify and analyze additional antigen-specific T-cells.

Only a small fraction of the identified *M.tb*-derived binding epitopes identified in these studies were validated by multimer analysis as actually being CD8+ T-cell epitopes. Therefore, we have identified a reservoir of putative epitopes that could be selected for *M.tb* multimer construction, based on the binding characteristics (affinity and dissociation rate) determined within this study. Such multimers could then be used in future studies in which identification of *M.tb*-specific T-cells could provide additional value. It would also help to visualize a very broad array of T-cell responses restricted by a high number of different MHC class I molecules.

Regarding the 62 *M.tb*-specific multimers used in these studies, additional verification needs to be made in larger patient cohorts. Yet, we hope to be able to validate the use of *M.tb*-specific MHC class I multimers in studies of the specific immune response in *M.tb*-infected patients, and to link these markers with clinical endpoints. Examples include gauging vaccine take of novel anti-*M.tb* vaccine candidates and testing novel diagnostic markers (e.g. ESAT-6 responses, independent of cytokine production) in well defined cohorts.

The immunogenicities of glycosyl transferases and cyclopropane fatty acid synthase were previously discovered by our group in the context of MHC class II presentation as well as for antibody recognition [128, 129], and we have in these studies also been able to demonstrate CD8+ T-cell recognition. It is of importance to further characterize the ability of these antigens to elicit a strong CD8+ T-cell response, since they are not secreted (like the majority of the previously characterized *M.tb*-derived antigens) but intracellularly retained. In the future, we would like to address other issues concerning these *M.tb*-derived proteins, e.g. expression patterns and their potential use as vaccine candidates.

Other questions that arose during this thesis that we would like to examine include:

- To decipher if the allele-specific recognition, phenotypic and cytolytic expression pattern identified in these studies in association with *M.tb* antigens are specific for this disease, or if it could be extrapolated to other infectious disease settings as well.
- To further characterize the antigen-specific CD8+ T-cells with a precursor like phenotype based on expression of the cell-surface markers CD45RA, CCR7, CD95 and c-kit. It would also be of interest to study why those obviously antigen-experienced cells express these markers. What is the impact of these variations in vaccinated individuals that encounter an infection with an *M.tb* variant and what are their functions; is there an association with the biologically and clinically relevant endpoints (i.e. long-term immune memory formation)?
- To validate the multimers expressing the altered TB10.4-derived epitopes in different population cohorts and further characterize the subsets of specific CD8+ T-cells recognizing the original and altered epitopes.

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