Characterization of the cytokine profile in adults with latent and active tuberculosis from a high endemic country

And

On the role of the cytotoxic protein granulysin in childhood tuberculosis

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„Der Mensch hat dreierlei Wege, klug zu handeln: Erstens durch Nachdenken, das ist der Edelste, zweitens durch Nachahmen, das ist der Leichteste, und drittens durch Erfahrung, das ist der Bitterste.“

Konfuzius

Table of contents

Summary

Characterization of the cytokine profile in adults with latent and active tuberculosis from a high endemic country

Tuberculosis (TB) is a global health problem with ~2 billion infected people worldwide. The vast majority of infected individuals is able to control TB, while only ~10% develop active disease. The immunologic correlates determining the protection against reactivation of the latent form of active TB remain elusive. The adaptive immune system plays an important role in the response against *Mycobacterium tuberculosis* (*M. tuberculosis*), especially $CD4^+$ T cells are crucial for efficient containment of the pathogen. Since polyfunctional $CD4^+$ T cells have been associated with protection against various pathogens, the question was raised if higher frequencies of polyfunctional $CD4^+$ T cells can be linked to protection against reactivation of active TB.

To address this the T_H1 T cell cytokine profile of active TB patients was analyzed and compared with healthy latently infected individuals (LTBI). Furthermore TB patients were followed up under anti-microbial therapy to monitor changes in the cytokine pattern expressed by $CD4^+$ T cells. Hereby, for the first time, the simultaneous expression of four T_H1 cytokines, IFNγ, TNFα, IL-2 and GM-CSF, was investigated using multi color flow cytometry. After antigen-specific stimulation multifunctional memory T cells ($CD45RO^+$) co-expressing IFNγ, TNFα, IL-2 and GM-CSF were strongly represented in both treated and untreated TB patients. Interestingly, this proportion of polyfunctional memory T cells was also found in LTBI. After the first two months of drug treatment the proportion of antigen-specific polyfunctional T cells was significantly increased, indicating a positive impact of these cells during therapy.

To gain detailed information about the potential of $CD4^+$ T cells to produce cytokines we incubated PBMCs with a superantigen. In this case the profile was significantly different between these two groups and it changed during therapy. While the expression of IFNγ was significantly lower in $CD4^+$ T cell of TB patients in comparison to LTBI, the expression of TNFα, IL-2 and GM-CSF showed significant higher frequencies in memory T cells of TB patients.

To conclude, upon antigen stimulation, polyfunctional memory T cells are found in TB patients pre- and post therapy as well as in LTBI. A difference in the frequency between active TB patients and LTBI could not be detected and therefore a correlation with protection against reactivation from the latent to the active form of TB cannot be drawn.

On the role of the cytotoxic protein granulysin in childhood tuberculosis Childhood tuberculosis (TB) remains a major cause of child mortality worldwide with ca. one million new cases annually. Notably, children have an increased risk of rapidly progressing to active TB disease. The situation is further complicated by higher incidences of extrapulmonary TB. Childhood TB diagnosis remains insufficient not the least because of the *paucibacillary*

nature of infant TB, which results in a high proportion of false sputum-smear negative diagnoses. Therefore novel approaches are urgently needed. Because of the central role of T lymphocytes in host defense against TB, immune diagnosis should focus on these cells. The second part of this thesis describes $CD4^+$ cytotoxic T lymphocytes (CTL) as a possible new candidate for diagnosis. The focus was hereby on the expression of the cytotoxic protein granulysin. Granulysin has been shown to attack *M. tuberculosis* extracellular as well as within macrophages in combination with perforin.

To investigate the expression of granulysin by $CD4^+$ T cells a newly described 7d *in vitro* assay was used. Stimulation lead to a strong induction of antigen-specific $CD4^+$ $CD45RO^+$ memory T cells which was absent in healthy controls. Simultaneously significant induction of granulysin in TB experienced children could be detected. Proliferating memory T cells could be identified as the main source. Further experiments showed that these cells express a central- and effector memory like phenotype and analysis of children after drug therapy revealed granulysin expressing $CD4^+$ T cells as very long lived and partially IFNγ positive. Phenotyping of these cells indicated the upregulation of several T_H1 and activation marker. Granulysin expressing T cells were also positive for perforin and granzyme B, two further important cytotoxic molecules. Generation of granulysin positive $CD4^+$ T cell lines demonstrated the simultaneous induction of transcription factors of T_H1, T_H2 and T_H17 subsets upon antigen stimulation. Also, it could be shown that in $CD4^+$ CTLs the transcription factor EOMES correlates with granulysin expression and can be induced independently of T-Bet. Further, granulysin expression could be associated with the reduction of bacterial burden in *M. tuberculosis*-infected target cells.

In summary, the work presented in this thesis describes the analysis of granulysin expression by $CD4^+$ T cells after 7d *in vitro* assay as possible new tool to diagnose TB infection in children. The analysis of granulysin expression at different time points after anti-microbial drug therapy suggests these cells as possible read out for vaccine studies and granulysin expression could correlate with treatment outcome.

1 Introduction

1.1 Immune system

The mammalian immune system has evolved as a means of host defence against invading pathogens and microorganisms. In order to counter the wide spectrum of invading organisms i.e. viruses, bacteria, fungi, protozoa and helminths, the immune system comprises complex and diverse protection mechanisms. All cells of the immune system derive from pluripotent stem cells in the bone marrow. A subset of precursor cells develops into lymphocytes in the bone marrow and/or in the thymus. These cells then migrate into the secondary lymphoid organs, such as the spleen, lymph nodes, as well as small masses of lymphoid tissue such as Peyer's patches and selected regions of mucosal surfaces.

After overcoming the physical barriers provided by the epidermis and mucosa, pathogens face two different arms of immunity, the innate and adaptive immune systems. Although these systems differ in many ways, they are functionally intertwined to a great extend [1,2,3]

1.1.1 Innate immune system

The innate immune system consists of an immune defense apparatus, which lacks immunologic memory. It is initiated directly after contact with foreign antigens. The response of the innate immune system is triggered by a set of pattern recognition receptors that can identify molecular motifs shared by large groups of microorganisms. These receptors are germ-line encoded and contain a genetically predetermined specificity, respectively. Thus these receptors are limited in their variety. After recognition of various products of fungal, bacterial, viral or parasitic origin they elicit a fast and efficient response [4] .

One of the best-known classes of innate immune receptors are the toll-like receptors (TLRs). To date, ten different TLRs have been described [5] . All of them differ from each other in ligand specificity, expression pattern and presumably in the target genes whose expression they can induce. Among pattern recognition receptors (PRRs), TLR-2 recognizes the largest array of ligands e.g. peptidoglycan and bacterial lipoproteins. It forms a heterodimer with TLR-6, which increases the repertoire of ligand specificity [6] . Lipopolysaccharides (LPS) from Gram-negative bacteria are recognized by TLR-4. TLR-5 detects bacterial flagellin, while TLR-9 has high affinity for unmethylated CpG DNA of bacteria [7,8,9] . TLR-3 has been shown to bind to double-stranded RNA (dsRNA) as it is expressed after certain viral infections. In the context of *Mycobacterium tuberculosis* (*M. tuberculosis*) infection, roles respectively for TLR-2 and TLR-4 have been suggested [10] . With the 19kDa lipoprotein of *M. tuberculosis* one ligand has been identified, at least for TLR2 [11] . However, TLRs aren't the only PRRs on the

surface of innate immune cells. Other classes include the nucleotide oligomerization domain (NOD)-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs) [12,13] . The variety of PRRs expressed by innate immune cells allows rather rapid elimination of an enormous range of microbes although the cells lack antigen specificity. As a consequence many pathogens get cleared without the intervention of the adaptive immune system. Activation of the adaptive immune system depends upon many effector mechanisms initiated by the innate system [14] .

PPR mediated secretion of inflammatory cytokines leads to further recruitment of innate immune cells to the site of infection. Recognition of pathogens leads to different immune defense mechanisms like phagocytosis, secretion of mediators or release of cytotoxic granules. The most efficient members of the innate immune cells are neutrophils, macrophages and dendritic cells (DCs). Neutrophils constitutively patrol the body and make up the first cluster of cells that cross the blood vessel wall to enter inflammatory sites. While these cells are primarily phagocytic, a new defense mechanism of neutrophils has been described recently. Upon activation, neutrophils release granular proteins and chromatin that together form extracellular fibers that bind Gram-positive and Gram–negative bacteria. These neutrophils extracellular traps (NETs) can degrade virulence factors and kill bacteria [15] . The second class of innate immune cells are macrophages. These cells are also phagocytotic and can activate several bactericidal mechanisms. In addition they are professional antigen presenting cells (APCs) that present processed fractions of pathogens to cells of the adaptive immune system. Macrophages mature continuously from monocytes that migrate into tissue and exhibit receptors that enable them to identify pathogens. The scavenger receptor, for example recognizes negatively charged lipids such as lipoteichoic acids (LTA), which are cell-wall components of Gram-positive bacteria [16] . Another one is CD14, a receptor for bacterial lipopolysaccharide (LPS) [17] . The mannose receptor recognizes certain sugar molecules found on the surface of several bacteria and some viruses, including HIV [18,19] . The third cell type and most potent activator of adaptive immune cells are DCs. While they are also able to phagocytose pathogens, their main focus lies on antigen uptake, processing and presentation in the lymph nodes to cells of the adaptive immune system. The often mentioned phagocytosis is an active process that plays a crucial role in *M. tuberculosis* infection (see 1.6.1). Upon binding the pathogen gets surrounded by the membrane of the phagocyte and gets internalized in a membrane-bound vesicle called phagosome. Macrophages and neutrophils possess granules called lysosomes that contain enzymes, proteins and peptides that can mediate antimicrobial response. After the engulfment of a pathogen, the mature phagosome fuses with one or more lysosomes to generate a phagolysosome in which the antimicrobial content is released to destroy the pathogen [14] .

A special role of the innate systems is played by natural killer cells (NK cells). These cells get activated by interferons and macrophage-derived cytokines to add to the primary defense against certain intracellular infections. NK cells are triggered by invariant receptors that recognize components of infected cell surfaces to release cytotoxic granules. These contain

effector proteins that penetrate the target cell membrane and induce programmed cell death [20,21] . NK cells also possess a second set of receptors that block its cytotoxic activation. These inhibitory receptors are specific for major histocompatibility complex (MHC) class I alleles. Many pathogens have developed strategies to reduce the number of MHC molecules on the surface which display pathogen-derived peptides to T cells. NK cells are able to recognize this alterations in MHC class I expression and kill these cells directly or induce apoptosis in these infected cells [22] .

As mentioned above DCs play a major role in connecting the innate and adaptive immune systems. Immature DCs constantly use endocytosis to engulf soluble extracellular antigens as well as apoptotic materials [23] . In addition inflammatory signals such as interferon gamma (IFNγ) or TLR signalling are required for activation of the DC and its maturation to a professional APC. The activated DC then presents MHC-peptide complex to T lymphocytes and induces cell proliferation and differentiation to effector functions, which initiates the adaptive immune response.

1.1.2 Adaptive immune system

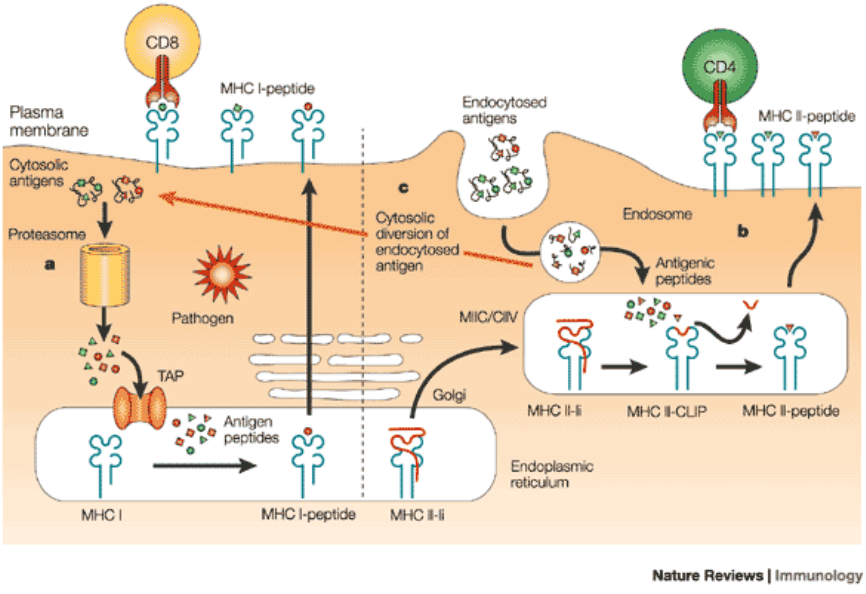
Two major cell types constitute the adaptive immunity, namely B and T cells.

B cells develop in the bone marrow and recognize soluble antigens directly through the B cell receptor (BCR). An activated B cell forms a plasma cell. Plasma cells then secrete antibodies, immunoglobulins (Ig) possessing the same antigen specificity. The secretion of antibodies, which bind pathogens or their toxic products, is the main effector function of B cells in adaptive immunity [14] . Antibodies have different ways to interfere with an infection. The simplest way is by binding directly to the pathogen and thereby blocking its access to the target host cell. A second way is by opsonisation, which predisposes the pathogen to phagocytosis [24] . The third function of antibodies is to activate a system of plasma proteins known as complement. It can directly destroy certain bacteria by pore formation.

In contrast to B cells, T cells rely on the interaction of their T cell receptor (TCR) with cells bearing the antigen. Stem cells that continuously migrate from the bone marrow to the thymus develop into T cells. Two surface molecules of T cells, namely CD4 and CD8 are of particular importance during T cell development. Both molecules, along with the CD3 group of molecules form an essential part of the T cell receptor (TCR) complex. CD4 binds to an invariant part of the MHC class II molecule, whereas CD8 binds to an invariant part of the MHC class I molecule [2] . Early in their development in the thymus, immature T cells express simultaneously CD4 and CD8. If they have an appropriate T cell receptor, these double-positive T cells will potentially recognize a self-antigen-derived peptide on either MHC class I or II. Subsequently, expression of one of the molecules is lost and cells become single-positive for either CD8 or CD4 T cells with distinct effector functions [25] . Classically the two different MHC classes present peptides of two different origins. MHC class I

molecules present cytosolic antigens that have been degraded by the proteasome and transported into the lumen of the endoplasmatic reticulum (ER) by the transporter associated with antigen processing (TAP) [26] . Inside the ER suitable peptides are loaded onto the MHC-I molecule to generate MHC-peptide complexes. The complex is then transported to the cell surface and can be recognized by an appropriate $CD8^+$ T cell. On the contrary, MHC class II complexes are not loaded in the ER directly but arrive in the lumen of the cell in the MIIC/CIIV compartment that fuses with endosomes that contain endocytosed antigens. After loading of an appropriate peptide into the groove of the MHC-II, the complex gets transported to the surface of the APC for interaction with a $CD4^+$ T cell [27] (Scheme 1).

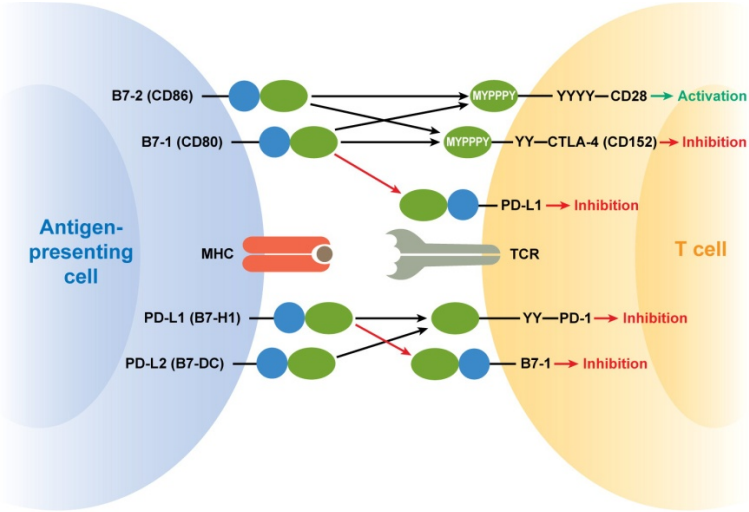
The general scheme of the presentation of cytosolic antigens to $CD8^+$ T cells via MHC-I and exogenous antigens via MHC-II to $CD4^+$ T cells does not hold true at all times. For DCs it has been shown that exogenous peptides that have been derived from other cells can be presented by its own MHC-I pathway. This process is called cross-presentation [28,29] . This reverse presentation is also important in the immune response against *M. tuberculosis*, which is internalized by phagocytes. While most antigens from *M. tuberculosis* are presented via MHC-II to $CD4^+$ T cell that provide most of the immune response, cross presentation to $CD8^+$ T cells takes place, as well [23] . This is mostly done by apoptotic vesicles harbouring mycobacterial antigens that are taken up by uninfected APCs and then presented via MHC-I to $CD8^+$ T cells.



Scheme 1: Antigen presentation via MHC class I and II [30]

1.2 T cell activation and co-stimulation

T cell receptors (TCRs) are associated with the CD3 complex of molecules, which transmit activation signals after the binding of the TCR to the MHC:peptide on the antigen-presenting cell. As mentioned earlier T cells are restricted in their ability to recognize either MHC class II ($CD4^+$) or MHC class I ($CD8^+$). Additional signals are required to further activate the T cell. One such signal is provided by the cross-linking of CD28 on the surface of T cells with CD80 (B7-1) and CD86 (B7-2) molecules on the surface of the APC [31] . Further activation signals are provided by CD154 (CD40 ligand) which binds to CD40 on the APC [32] . Some signals i.e. CD28, CD40L and ICOS lead to proliferation of the designated T cell [33] . But there are also receptors that lead to the opposite effect and hinder cells from further expansion. Cytotoxic T lymphocyte antigen 4 (CTLA-4) expression has been shown to be upregulated on activated T cells and counterbalances the CD28-mediated signals, thus preventing overstimulation of the lymphoid system [34,35,36] . Another marker found on activated T cells is programmed death 1 (PD-1). Upon activation, T cells undergo a dramatic expansion in number and develop functions to deal with the invading pathogen. For the removal of these effector cells, inhibition of the T cell response is needed. This is achieved by the expression of PD-1 on the surface of these cells. The binding of PD-1 to PD-L1 on the APC, then leads to a down-modulation of T cell responses [37,38] (Scheme 2). A third signal that is needed for activation is delivered through cytokines. The cytokine milieu released during activation is crucial for T cell development especially in the $CD4^+$ compartment. In general, the combination of all three signals TCR:MHC-peptide binding, CD28:CD80/CD86 cross-linking and cytokine co-stimulation leads to the activation and proliferation of naïve T cells resulting in the generation of antigen-specific effector T cells that act during the acute phase of T cell driven immune response. Additionally, it also leads to the formation of memory T cells, which will be further explained in the following sections.



Scheme 2: Overview of co-signals between T cell and APC [37]

1.3 e, effector and memory T cells

1.3.1 Naive T cells

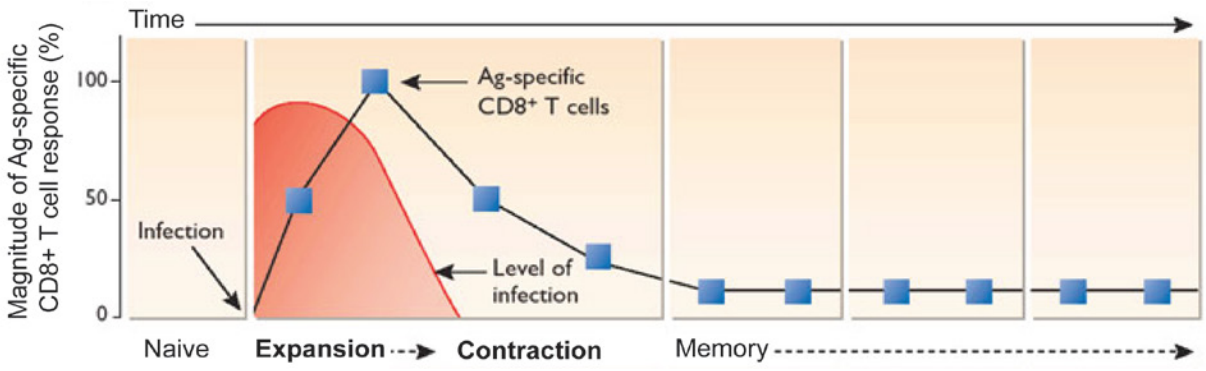
Prior to contact with an antigen, naive T cells converge in the secondary lymphoid tissues and migrate from one to the other via blood or lymph [39,40] . In contrast to the spleen the entry into lymph nodes from the bloodstream through the high endothelial venules (HEV) is highly specific. The luminal surface of the HEV expresses several ligands including addressin, PNAd and SLC chemokine [41,42] . These ligands are recognized by two lymph node homing receptors on the T cell, namely CD62L and CCR7 [43] . These markers can be used to evaluate the differentiation status of effector and memory T cells. Naive T cells constantly migrate and leave the lymphoid organs to patrol the entire human body. These T cells are long-lived but rely on two survival signals, linkage to self-peptide/MHC complexes on DCs, mainly in the T cells zones of lymph nodes and the cytokine IL-7 [44,45,46] .

1.3.2 Effector T cells

After initial contact with the APC $CD4^+$ naive T cells differentiate into effector cells. These cells then undergo multiple rounds of proliferation and polarize in correspondence with the cytokine milieu provided by the surrounding cells. When an antigen-specific T cell interacts with the APC, a tight synapse develops. This is termed as the immunological synapse [47,48] . This leads to rapid clustering of TCR molecules binding to peptide/MHC complexes on the APC. The triggering of CD3 and TCR is supported by either CD4 or CD8 and other co-stimulatory molecules on the T cells [49] . This paves the way for cytokine secretion and following differentiation into either cytotoxic $CD8^+$ T cells or in the case of $CD4^+$ T cells, into one of the following phenotypes T_H1, T_H2, T_H17 and T_{reg}, which will be discussed in more detail in Section 1.4. In a typical infection, the rapid increase in numbers of antigen-specific effector T cells allows fast elimination of the pathogen. After this event the high frequencies of effector cells become redundant and get cleared in minimal time.

1.3.3 Memory T cells

Although most effector cells disappear after the eradication of the pathogen, some cells survive though the contraction phase and become long-lived memory T cells (Scheme 3). It has been shown in the acute infection models of lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenesis*, that 5 to 15 % of virus-specific T cells remain after the peak of $CD8^+$ T cell response and develop into memory T cells [50,51] .



Scheme 3: Ag-specific CD8$^+$ T cell response after acute infection. Modified after [52]

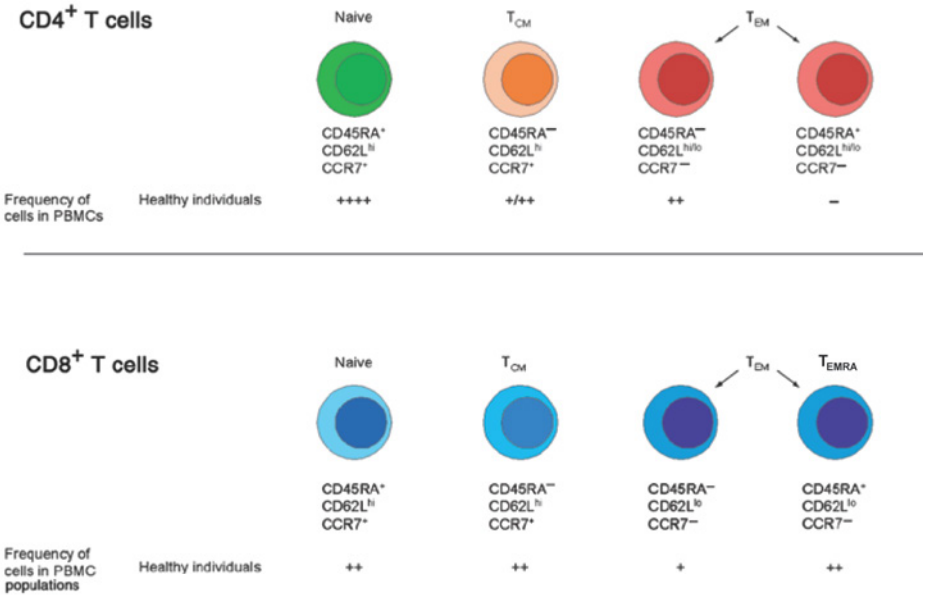
The cytokine IL-7 appears to play a crucial role in the transition of effector cells into memory T cells. There is evidence showing that absence of IL-7 leads to a lack of CD4$^+$ and CD8$^+$ T cells differentiating into memory T cells after infection [53,54] . In the case of CD8$^+$ memory T cells in addition to IL-7 the presence of CD4$^+$ T cells is also crucial for generation and maintenance [55] .

Memory T cells are characterised by long-term persistence and their ability to express effector functions rapidly after a secondary infection. In accordance with the migration and effector properties, two different T cell memory subpopulations have been identified [56,57] . The first population can be identified by the constitutive expression of the surface markers CD62L and CCR7, and is called central memory T cell (T_{cm}). The expression of these two receptors enables T_{cm} cells to recirculate through secondary lymphoid organs. The second population is called effector memory T cells (T_{em}) and lacks the expression of CCR7 and is also predominantly negative for CD62L. Subsequently these cells are found in non-lymphoid tissue [56] . Another marker to differentiate naive from memory T cells is the leukocyte common antigen (CD45). While naïve T cells express the isoform CD45RA, antigen-experienced T cells substitute CD45RA with the second isoform CD45RO [56,58] (Scheme 4).

Following TCR triggering, T_{cm} mainly produce IL-2 and after proliferation, they efficiently differentiate into effector cells and produce large amounts of IFNγ or IL-4 [59] . On the contrary T_{em} produce effector molecules quite rapidly and produce IFNγ, IL-4 and IL-5. CD8$^+$ T cells also express perforin within hours after antigenic stimulation. The relative proportions of T_{cm} and T_{em} in the blood vary between CD4$^+$ and CD8$^+$ T cells. While CD4$^+$ memory T cells are predominantly T_{cm}, in CD8$^+$ T cells the T_{em} subset is prominent. Both populations are long-lived and in antigen-primed individuals antigen-specific T_{cm} and T_{em} can be detected up to 10 years [56] . Although both populations show high responsiveness to antigen stimulation the expansion potential decreases from T_{cm} to T_{em} [60] . The decreasing potential to proliferate and to survive could be correlated with a decrease in the telomere length and an increased susceptibility to apoptosis [61] . In summary, T_{cm} show higher expansion potential and are able to patrol through secondary lymphoid organs and after further proliferation steps these cells can produce effector molecules like IFNγ or IL-4. On the

contrary T_{em} express immediately after TCR triggering various inflammatory chemokines in order to move into inflamed tissues and also cytokines for effector functions.

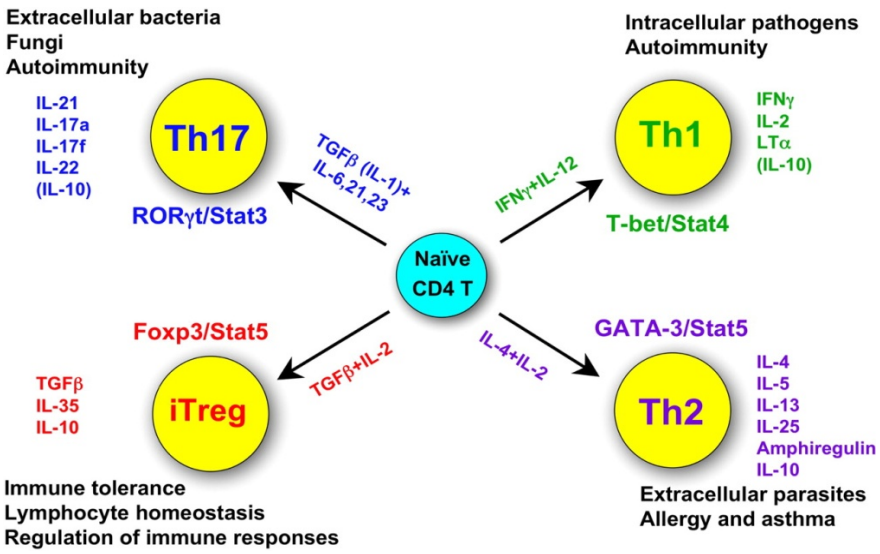
While for $CD4^+$ T cells the expression CD45RA seems to be almost exclusively on naïve T cells, there is a small subset of human memory T cells existing as $CD8^+CCR7^-CD45RA^+$ ($CD8^+$ T_{EMRA}). These cells play a role in the immune response to human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) [62,63] . $CD8^+$ T_{EMRA} have also been shown to be clonally expanded in childhood tuberculosis (TB) [64] (Scheme 4).



Scheme 4: Subsets of memory T cells. Modified after [65]

1.4 CD4+ T cell populations

Besides the co-stimulatory signal, the surrounding cytokine mileu produced by cells of the innate immune system upon infection is crucial for the polarization of $CD4^+$ T cells [66] . A fundamental function of T helper (T_H) cells it to regulate B-cell development and support cytotoxic $CD8^+$ T cells. The T_H1 and T_H2 lineages have been long considered to be the two destinct developmental routes pursued by the $CD4^+$ T cell [67,68,69] . Recently, further lineages with destinct cytokine patterns and effector funtions have been described, showing complexity and varity within the $CD4^+$ T cell compartment. The necessary cytokine milieu for differentiaton, important transcription factors as well characteristic cytokines and chemokine receptors for the different lineages will be discussed and are summarised in Schemes 5 and 6.



Scheme 5: Summary of CD4 T helper fates. Modified after (1)

1.4.1 T-helper cells type 1 (T_H1) lineage

The T_H1 cells are induced by IL-12 or IFNγ during the early phase of infection. IL-12 is a heterodimeric cytokine and is typically secreted by activated DCs that have been exposed to bacterial ligands. Through Stat4, IL12 is the key inducer of T_H1 cells since it represses the expression of GATA-3, a crucial transcription factor for T_H2 commitment and induces the expression of T-bet (Scheme 6). T-bet has been demonstrated to be the main regulator of T_H1 development. It is dependent on Stat1, the major transducer of IFNγ signalling [70] . T-$bet^{-/-}$ cells have severe defects in T_H1 differentiation but still produce some IFNγ due to the expression of Eomesodermin (Eomes) [71,72] . Eomes, another T-box family member, which is critical for IFNγ production by $CD8^+$ T cells, is upregulated during T_H1 differentiation and

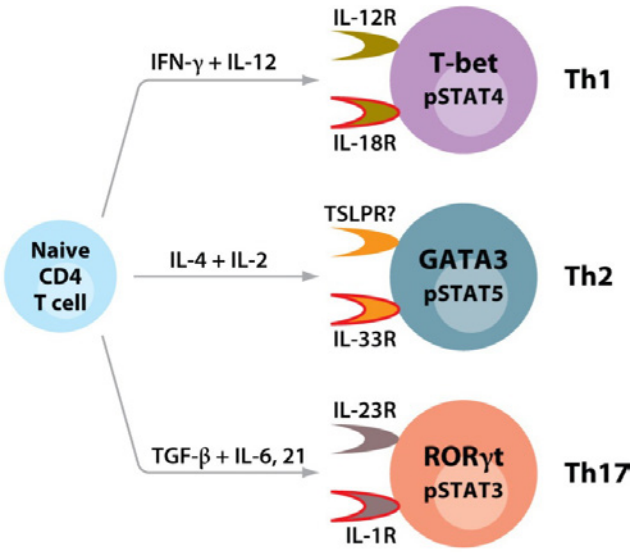
may be involved in IFNγ secretion by $CD4^+$ T cells as well. IFNγ secretion is characteristic of the T_H1 cytokine profile. It is important for activating macrophages to increase their microbicidal activity [73] . It is also essential for the regulation of B-cell proliferation and immunoglobulin class switching [74] . Furthermore, T_H1 are IL-2, lymphotoxin alpha (LTα) and TNFα producers. IL-2 serves as a growth factor for T cells in the periphery and is supporting the production of further effector cytokines, activity of cytotoxic T lymphocytes (CTL) and generation of T_H1 memory [75,76] . Another important cytokine produced is the granulocyte-macrophage colony stimulating factor (GM-CSF). This molecule enhances microbicidal activity, oxidative metabolism and phagocytotic activity of neutrophils and macrophages [77] . GM-CSF is essential for the growth and development of progenitors of granulocytes and macrophages. It also improves the cytotoxic functions of these cells. More cytokines are associated with T_H1 reflecting the complexity of T cell-mediated immune response. Although chemokine receptors are not strictly limited to one characteristic T_H-lineage, some receptors such as CXCR3 and CCR5 show a strong association with T_H1 T cells [78,79,80] .

1.4.2 T-helper cells type 2 (T_H2) lineage

As opposed to T_H1 cells, T_H2 cells promote the humoral immune response and are mainly involved in host defense against extracellular parasites [81,82] . For generation of T_H2 cells, the existence of IL-4 and IL-2 is necessary [83,84,85] . Mice that lack IL-4, IL-4 receptor or Stat6, the downstream signalling molecule for the IL-4 receptor, fail to develop T_H2 cells in response to most stimuli [86,87,88,89,90] . If exogenous IL-4 is not provided, naïve $CD4^+$ T cells can produce limited amounts of this cytokine upon TCR-mediated GATA3 transcription and IL-2-mediated Stat5 activation [91] (Scheme 6). T_H2 differentiation is totally abolished *in vitro* and *in vivo* in the absence of the transcription factor GATA3 [92,93] . Deletion of GATA3 from fully differentiated T_H2 cells completely blocks the production of the characteristic cytokines IL-5 and IL-13. As already mentioned IL-4 is the positive feedback cytokine for T_H2 cell differentiation and is the main mediator of IgE class switching in B cells [94] . IL-5 on the other hand plays a crucial role in recruiting eosinophils, which are important for the cellular immune response against parasites [95] . The cytokine IL-13 is the effector molecule in the expulsion of helminths [96] . Apart from IL-33 receptor alpha (IL-33Rα), the chemokine receptor CCR3, CCR4, CCR8 and CRTh2 are expressed on T_H2 T cells [78,97,98,99] .

1.4.3 T-helper cells type 17 (T_H17) lineage

T_H17 cells mediate the immune response against extracellular bacteria as well as fungi, albeit contributing to the induction of many organ-specific autoimmune diseases [100] . Three different groups showed that T_H17 cells could be generated *in vitro* from naïve progenitor cells by TCR stimulation in the presence of IL-6 and transforming growth factor beta (TGF-β) [101,102,103] . Further studies in human cells could show that T_H17 differentiation consists of 3 stages: a differentiation stage, requiring TGF-β and IL-6, an amplification stage mediated by IL-21 and a stabilization stage with the help of IL-23 [104,105,106,107,108] . It is worth mentioning that all three cytokines IL-6, IL-21 and IL-23 activate Stat3 (Scheme 6). The transcription factor ROR-related orphan receptor gamma (RORγT) has been identified as the main regulator of T_H17 cells [109] . Using reporter and knockout mice it was demonstrated that T_H17 cells exist constitutively in the intestinal lamina propria and that their development is severely impaired in the absence of RORγT. Upon activation T_H17 cells produce IL-17a, IL-17f, IL-22 and IL-21 [110,111] . IL-17a and IL-17f both use the receptor IL-17RA, although IL-17a binds with greater affinity [112] . IL-17a can induce the inflammatory cytokine IL-6 as well as the chemokine IL-8, and thus has an important role in the induction of inflammatory immune responses [113,114] . Both IL-17a and IL-17f recruit and activate neutrophils during the immune response against extracellular bacteria and fungi. IL-21, serves in a feedback response like IFNγ in the case of T_H1 and IL-4 in the case of T_H2 cells [106,107,115] . The production of IL-22 is facilitated by the aryl hydrocarbon receptor which is highly expressed on T_H17 cells [116] . It participates in dermal infection, but also supports host immune defense against *Klebsiella pneumonia* and *Citrobacter rodentium* and protects hepatocytes during acute liver inflammation [117,118,119,120] . Furthermore, T_H17 cells can be characterised by high expression of IL-23 receptor (IL-23R) substantial amounts of IL-1 receptor 1 (IL-1R1) and co-expression of CCR6 and CCR4 [102,105,121] (Scheme 6)

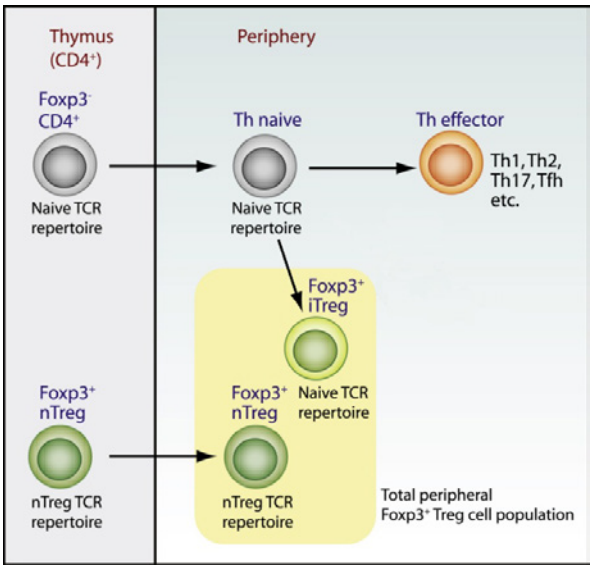


Scheme 6: Cytokine milieu for T_H1, T_H2 and T_H17 development and characteristic receptors, transcription factors and signal transducers and activators of transcription (STAT) for the different lineages. Modified after [122]

1.4.4 Natural and adaptive regulatory T cells

Regulatory T cells (Treg) play a crucial role in maintaining self-tolerance and regulating immune responses [123] . Recent findings have shown that Treg can be separated into two different subpopulations. The first population is called "natural Treg (nTreg) cells" and exists as thymic-derived forkhead box P3 (Foxp3) positive Treg cells [124,125,126,127] . In recent years, it became evident that $Foxp3^+$ Treg cells can also be generated outside the thymus under different conditions, thus earning the term "adaptive Treg (iTreg) cells". These cells derive from non-regulatory T cells that acquire Foxp3 expression and regulatory functions [128,129,130] (Scheme 7). In mice, these cells show similar regulatory functions *in vitro* and *in vivo* [131] . However, iTreg cells in humans have thus far failed to demonstrate activity in a functional *in vitro* assay [132] . TGFβ plays a major role in iTreg differentiation and is important for nTreg development [133,134] . IL-2-mediated Stat5 activation together with TGFβ are required for the induction of Foxp3 expression, and thus for survival and function of Treg cells also after differentiation [135,136] . As already mentioned Foxp3 is the main transcriptional regulator of Treg cells [137,138] . Continuous expression of Foxp3 is required to maintain the suppressive activity of these cells [139] . Over expression of this factor in conventional T cells leads to the acquisition of a Treg phenotype [137] . Treg cells produce the suppressive cytokines TGFβ, IL-10 and IL-35. While TGFβ plays a greater role in the generation of iTreg cells and as a mediator of suppression, IL-10 is critical in Treg-mediated prevention and cure of inflammatory bowel disease [140,141,142] . IL-35 is also expressed by Treg cells and required for maximal suppressive activity [143] . nTreg cells express IL-2 receptor alpha (CD25), which is also expressed by conventional T cells upon activation although to lesser extent and more transiently [123] . Treg cells also express CTLA-4, GITR

and Folr4. However, these markers can only be used to distinguish Treg cells from naïve conventional T cells since these proteins are also expressed by the latter upon activation.



Scheme 7: Thymic and peripheral generation of Foxp3$^+$ T_{reg} cells. Modified after [144]

1.5 Cytotoxic T lymphocytes

The development of CTL responses is necessary for the control of a variety of bacterial and viral infections [145] . These cells are mainly $CD8^+$ T cells but several studies have shown the existence of $CD4^+$ CTLs [146,147,148,149,150,151] . Experiments have shown that, while damage caused by antibodies and complement on target cells is restricted to the plasma membrane, lymphocytes cause an overall disintegration of the cell, including the nucleus [152,153] . It could be demonstrated that target cells play an active role in their own destruction that is initiated by the CTLs [154] . Lymphocyte-mediated killing can be confined to two distinct pathways, the Fas-Fas ligand pathway and via the exocytosis pathway [155] .

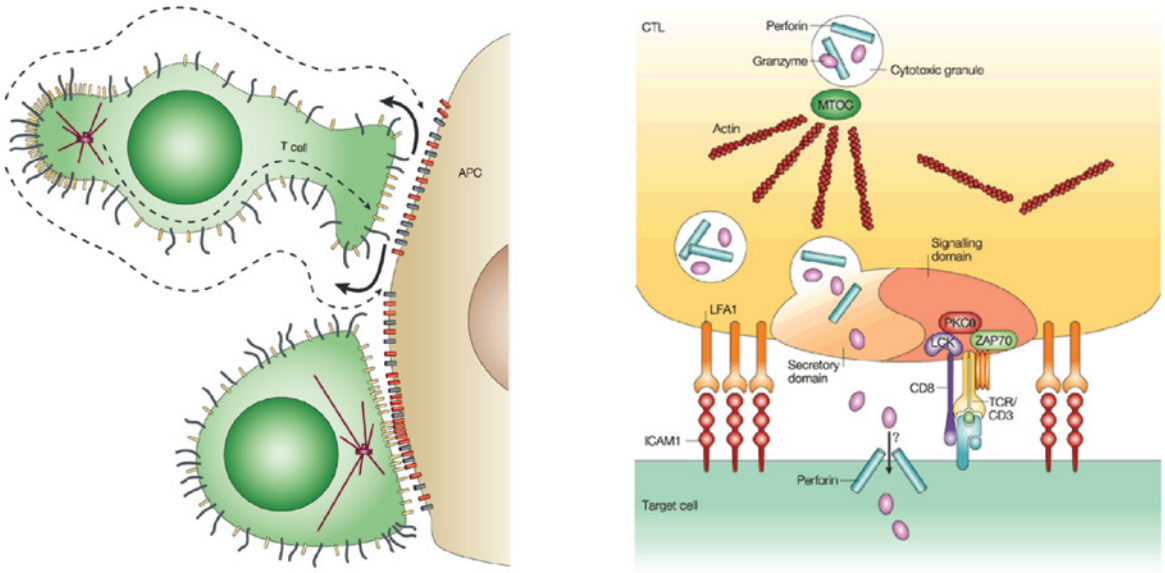
1.5.1 The Fas-Fas ligand linkage-mediated pathway

Such induced apoptosis by CTLs is important in the control of constantly stimulated T cells, and in promoting tolerance to self-antigens [156] . While target cells are positive for the death receptor Fas, effector CTLs and NK cells express the corresponding ligand, FasL. Thus, these cells are more susceptible to apoptosis mediated by this pathway [157,158] . Fas belongs to the tumor necrosis factor receptor (TNFR)-I type family and after linkage to FasL it leads to recruitment of FAS-associated death domain (FADD). In turn, FADD recruits procaspase 8 or 10, which undergo activation [159] . Caspase 8 interacts with procaspase 3, 6 or 7 hence activating them resulting in cleavage of cellular DNA. Caspase 8 can also hydrolize Bid, which damages the mitochondrial outer layer and trigger cytochrome-C release which also leads to morphological changes e.g. breakdown of the cytoskeleton and the nuclear membrane [160,161,162] .

1.5.2 Granule-dependent exocytosis pathway

This is the major pathway used by CTLs and NK cells to induce killing of cells [163] . In NK cells, granules are preformed and thus, can kill target cells within minutes of the first stimulation of activation receptors. Since NK cells belong to the innate immune system, they respond rapidly but antigen unspecific [164] . In contrast, naïve $CD4^+$ and $CD8^+$ CTL precursors have no cytotoxic activity and require an activation time of 1-3 days for optimal activity. This activation process and proliferation requires TCR-stimulated induction of cytokine receptors e.g. IL-2 and IL-6 which then induce the expression of granule components. The activated cells are then ready to interact with the adjecent antigen-presenting cell. During exocytosis, the microtubule-organizing centre (MTOC) orchestrates the movement of the granules towards the point of contact with the target cell [165,166]

(Scheme 8). In the area between the target and the CTL, a well-organized immunological synapse is assembled [48,167] . In the central region, the TCR-CD3 complex, CD4 or CD8 co-receptor and associated signalling molecules can be found. Larger molecules like CD2 and leukocyte function-associated antigen 1 (LFA1) surround this complex to stabilize the synapse (Scheme 8).



Scheme 8: After initial contact between TCR and MHC:peptide complex and the formation of the immunological synapse (left), granule proteins are released through the exocytic domain of the synapse (right) [168,169] .

The lytic molecules stored in granules that induce apoptosis are perforin, granzymes and granulysin.

1.5.2.1 Perforin

The importance of perforin has been evaluated in animal models and human diseases [170,171] . Patients with mutations in the perforin gene show an impaired immune response to intracellular pathogens. However, the function and mechanism of perforin remains unclear [172] . It could be shown that perforin is able to form cylindrical pores on the target cell membrane via polymerization in the presence of Ca^{2+} [173,174,175,176] . The pores can enable granzymes and granulysin to passively travel through the target cell membrane. It also leads to ion exchange, which consequently leads to osmotic disbalance and ultimately cell death [175] . Despite being the most accepted hypothesis about the role of perforin, there is only little experimental evidence to support this. On the contrary it has been shown that Granzyme-B is introduced into the target cell by binding to the mannose-6-phosphate

receptor in the absence of perforin [177] . However, the presence of perforin is necessary for the induction of apoptosis. Current evidence indicate that perforin is not essential for the entry of the proteases into the target cells but rather for cytolysis [156,178] .

1.5.2.2 Granzymes

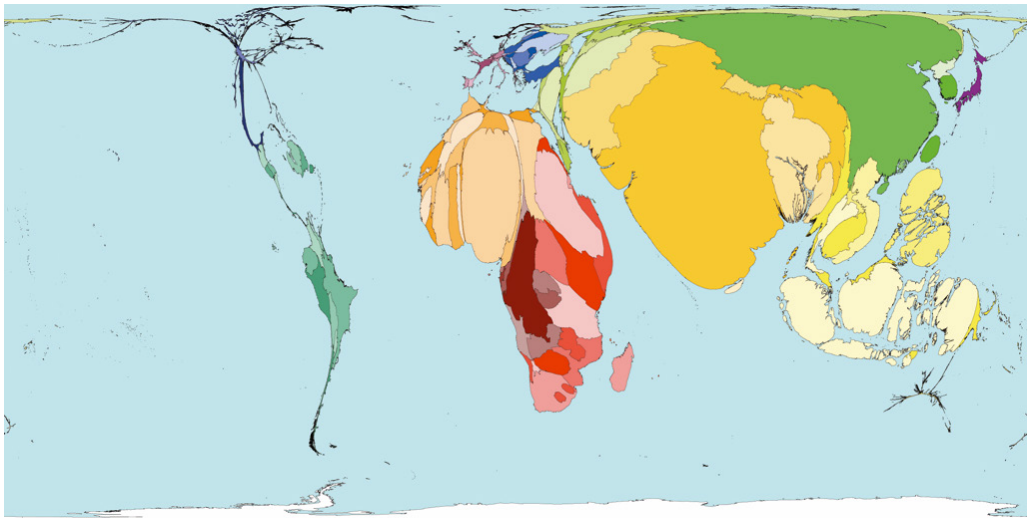
The most abundant proteins in the lytic granules are Granzyme-A (Grz-A) and –B (Grz-B) [179] . Grz-A induces caspase-independent apoptosis and activates a slow cell death by cleavage of single-stranded DNA and hydrolyzation of proteins containing basic amino acids such as arginine or lysine [180] . Grz-B induces apoptosis through two different pathways. Firstly, it activates caspase-3 and other caspases, which promote the fragmentation of DNA or of the cytoskeleton [181,182] . Since the complete inhibition of caspase activity does not lead to impaired Grz-B-mediated cell death, a second pathway was proposed [183,184,185] . Some authors suggest that Grz-B preferentially induces apoptosis by alteration of the mitochondrial membrane instead of caspase-activation, but this remains controversial [186,187] .

1.5.2.3 Granulysin

Granulysin is a cytolytic molecule that is expressed from 3 to 5 days on, after initial activation of the CTL [188] . It is found in human CTLs, but till now no homologous molecule in the mouse could be described. It is cytolytic against tumors and microbes, including gram-positive and –negative bacteria and a major antimicrobial against *M. tuberculosis*, by altering the bacterial membrane to increase lysis [189,190] . Granulysin also leads to cell death by damaging the cell wall based on negative charges, disrupting the transmembrane potential in mitochondria, and inducing release of cytochrome-C [191] . The function of granulysin is not only as a cytotoxic molecule, but also as a chemo-attractant and pro-inflammatory activator [192] .

1.6 M. tuberculosis

M. tuberculosis is the main causative agent of human TB and kills nearly 2 million people every year with the highest prevalence in the developing world. An estimated 2 billion people worldwide are latently infected with nine to ten million new cases every year [193,194] (Scheme 9). Despite the global threat of TB morbidity and mortality, scientific breakthroughs in the field of TB research are rare. The only vaccine available, Bacille-Calmette-Guérin (BCG), does not protect adults against pulmonary TB and only few discoveries in the field of anti-TB drugs have taken place in the last 40 years.



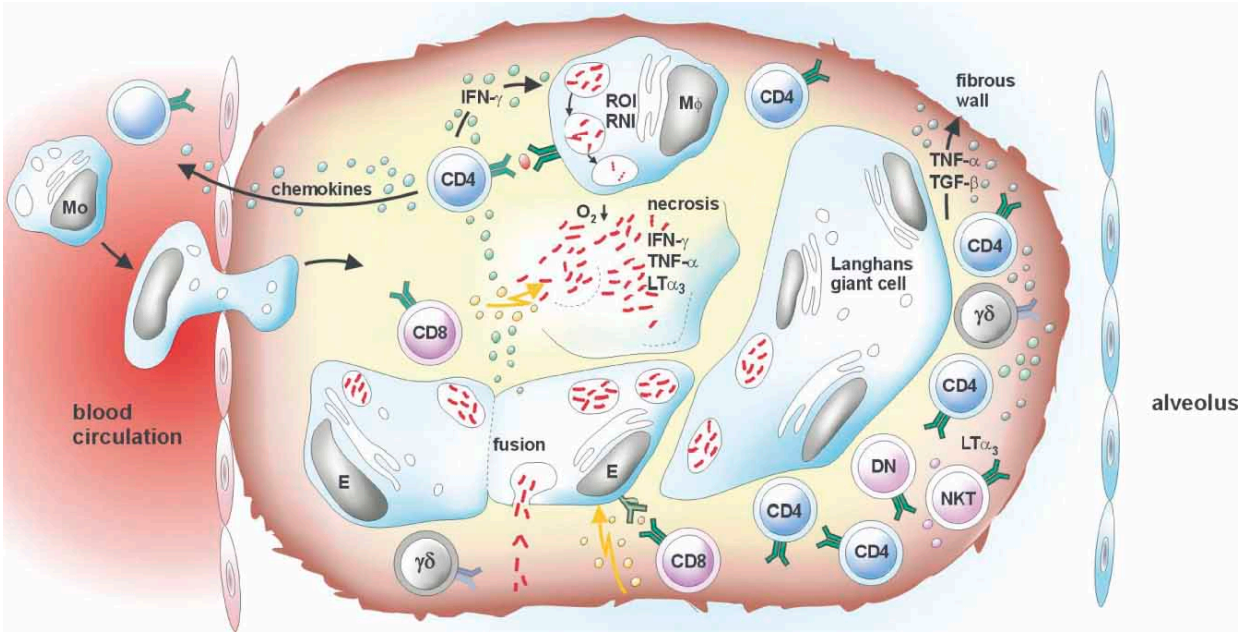
Scheme 9: Territories are sized in proportion to the absolute number of people who died from tuberculosis in one year. © Copyright 2006 SASI Group (University of Sheffield) and Mark Newman (University of Michigan).

1.6.1 Epidemiology and disease

On the March 24th 1882, Robert Koch, who received the Nobel prize in 1905, discovered *M. tuberculosis* as the etiologic agent of TB [195] . Descriptions of the disease itself can be found in ancient texts [196] . One can hypothesize that the genus Mycobacterium originated more than 150 million years ago [197] . The pathogen is slow growing with a doubling time of up to 48 hours. It has been classified as an acid-fast bacterium, due to its ability to be stained by only certain dyes, and is coated with a thick cell wall. This unique cell wall contains mycolic acids which are connected via arabino-galactan polysaccharide to the conventional peptidoglycan layer [198] . The genome of *M. tuberculosis* has a size of 4.41 Mb and harbours about 4000 protein-coding genes [199] .

Infection with *M. tuberculosis* generally occurs via inhalation of droplets containing the pathogen through close contact with a patient with active pulmonary TB. In most cases this

leads to an infection in the lung, but can also affect other organs. In the alveolar space, the bacteria encounter alveolar macrophages and DCs that engulf the *M. tuberculosis*. Although the precise uptake mechanism *in vivo* remains to be established, one of the most important receptors *in vitro* is complement receptor type 3 (CR3) [200,201] . Once internalized, *M. tuberculosis* is encapsulated in the phagosome. Within the early phagosome, the bacteria block phagosome acidification and actively interfere with phagolysosomal fusion by immune evasion mechanisms [202] . In addition, the bacteria are also able to persist and proliferate within the phagosome. The infected cells transport the bacteria into the lung parenchyma. The production of TNFα and inflammatory chemokines from the infected macrophages drives the recruitment of neutrophils, NK cells, $CD4^+$ and $CD8^+$ T cells, each of which produce their own set of chemokines and cytokines that lead to the remodelling of the infection site into a structure called granuloma [203,204,205] . The formation of a stable granuloma is responsible for the immune containment during the latent, or subclinical, period of the infection (Scheme 10).

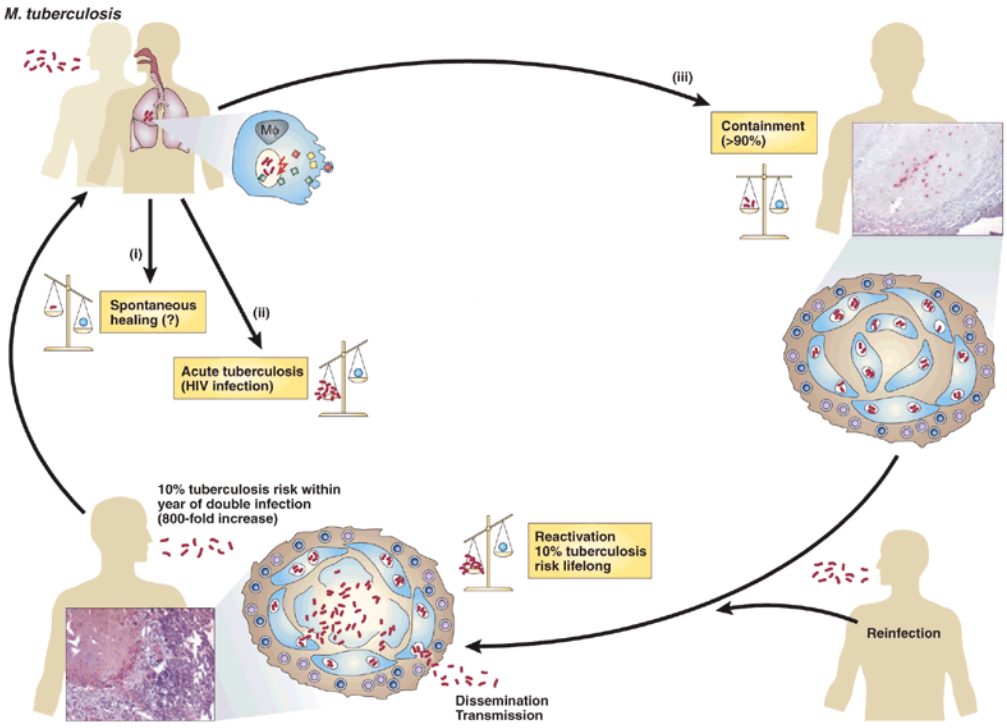


Scheme 10: Classical granuloma structures and proposed local immune response [204]

1.6.2 Latency versus active disease

The vast majority of *M. tuberculosis* infected individuals will not develop active TB but remain latently infected for the duration of their lives (Scheme 11). Reactivation only occurs in 5 to 10% of infected persons and can be triggered by immunosuppression due to age, corticosteroids, malnutrition or other factors [206] . Very importantly co-infection with HIV increases the risk of developing active pulmonary TB by several 100-fold [207] . A striking feature of *M. tuberculosis* is its ability to survive under hypoxic and nutrient-poor conditions

by shifting to a dormant life stage. This is characterised by alternative energy catabolism and a thickening of the cell wall [199,208,209] . By using an *in vitro* starvation model, 48 differentially regulated genes have been identified [210,211] . Among this set of genes, a transcription factor, namely Rv3133c could be identified and associated with the activation of all 48 genes. Deletion of this dormancy survival regulator (DosR) leads to loss of persistence in hypoxic conditions [212,213] . In contrast to dormancy, resuscitation promoting factors (Rpf) have been associated with the reactivation of the bacteria from a dormant stage to active replication [214,215,216] . The very mechanisms facilitating resuscitation of *M. tuberculosis* and subsequent disease reactivation remain elusive.



Scheme 11: Infection cycle of M. tuberculosis. Modified after [207] .

1.6.3 Diagnosis of *M. tuberculosis*

For over 100 years, the standard diagnostic test for *M. tuberculosis* infection has been the tuberculin skin test (TST). It was discovered by Robert Koch in 1890 and proposed as a possible treatment for TB, but experiments failed due to overwhelming immune response that caused several deaths. Nevertheless it remained useful as a way to diagnose mycobacterial infections in humans [217] . In 1934, Dr. Florence Siebert developed a method to purify the tuberculin and formulated a protein precipitate of antigens from metabolically active *M. tuberculosis*, namely purified protein derivatives (PPD). Currently, PPD is used intradermally in the Mantoux technique to diagnose TB [218] . A negative TST does not

necessarily exclude infection with *M. tuberculosis*, since up to 25% of people with active TB are nonreactive to the Mantoux test [219] . Another disadvantage of the TST is the compromised specificity due to numerous shared antigens with the BCG vaccine and non-tuberculous mycobacteria (NTM) [220] . In addition to the TST, microscopic examination of acid fast-stained sputum, with Ziehl-Neelsen (ZN) is one of the easiest, most inexpensive and rapid methods used in most developing countries. The culture method is even more sensitive than microscopy and is used additionally in most resource-limited countries [221] . A modification of this technique is the liquid media support growth with an increased recovery of positive cultures [222] . Furthermore, chest radiography can identify calcified lesions in the parenchyma of the lungs indicating a granuloma. IFNγ release assays (IGRA) are a new set of diagnostics using *M. tuberculosis*-specific antigens and reduce the risk of false positivity due to cross-reaction with other mycobacterial strains. Currently, two IGRA formats have been officially approved in many countries. These are the Quantiferon – TB Gold® and the T-Spot-TB® diagnostic tests. Both systems rely on host reaction to infection by measuring IFNγ produced by T-cell responses to the *M. tuberculosis*-specific antigens 6kDa early secreted antigenic target (ESAT6) and 10kDa culture filtrate protein (CFP-10). These antigens are encoded by the region of difference-1 (RD-1), which is a region in the genome of *M. tuberculosis* but absent in BCG and most other mycobacteria [217] . While the Quantiferon – TB Gold® is based on an IFNγ enzyme-linked immunosorbent assay (ELISA), the T-Spot-TB® employs an *ex vivo* enzyme-linked immunospot assay. IGRAs have several important advantages over the TST. The testing requires only one visit to the diagnostic centre and since the assay is *ex vivo*, there is no danger of a boosting effect when tested again. Also, results can be obtained within one day. But in line with the TST, this test cannot distinguish between latent and active TB.

1.6.4 Vaccines and biomarkers

The vaccine Bacille-Calmette-Guérin (BCG), which is an attenuated strain of *Mycobacterium bovis*, remains the only approved vaccination against TB. As already mentioned BCG can protect newborns from severe forms of childhood TB, like TB meningitis or miliary TB, but has no protective effect in adults [223] . Thus the development of new anti-TB vaccines is urgently needed and promising candidates have entered different stages of clinical testing [193] (Table 1). Basically, six different approaches are being pursued with the objective to establish either a new prime vaccine or to find a possibility to boost the protective efficacy of BCG. The use of a recombinant BCG strain, generation of attenuated *M. tuberculosis* vaccine strains, subunit vaccines, viral vector delivery systems and DNA vaccination.

Table 1: The most advanced vaccine candidates against TB. Modified after [224]

Candidate	Type	Current status	Reference
Mtb 72 F fusion protein in AS02A	Subunit (protein/adjuvant)	Clinical phase I trial in healthy uninfected volunteers ongoing	[225]
Ag85-Esat-6 fusion protein in IC31	Subunit (protein/adjuvant)	Clinical phase I trial started 2005	[226]
Aeras-402 Adenovirus	Subunit (protein/adjuvant)	Clinical phase I trial completed in 2008	[227]
r-MVA-Ag85	Subunit (protein/adjuvant)	Clinical phase I trial in BCG-vaccinated and unvaccinated healthy uninfected volunteers completed	[228]
r-BCG-Ag85	Viable	Clinical phase I trial in healthy uninfected volunteers completed	[229]
r-BCGΔure:Hly	Viable	Clinical phase I trial completed in healthy uninfected TST^+ and TST^-	[230]

Biomarkers are defined as an indicator of a physiological or pathological process or pharmacological response to a therapeutic intervention and are urgently needed in the battle against TB [193,231] . These biomarkers are not only needed to monitor the protective efficacy of a new vaccine candidate. Scientists are also searching for a biomarker with prognostic value for the outcome of infection as well as therapeutic markers to monitor the success of drug treatment. Different approaches are taken in the search for biomarkers, including transcriptomics, metabolomics or immune markers. In the past it was proposed that the release of IFNγ by antigen-specific T cells could serve as a marker of protection. This has been shown to not hold true and that the measurement of a single cytokine, like IFNγ, is insufficient to predict protection against infection with *M. tuberculosis* [232,233] . Recent studies conducted in mice with several pathogens suggested a protective role for polyfunctional CD4 T cells expressing multiple cytokines (IFNγ, TNFα and IL-2) and have been used as feedback of immunogenicity in ongoing TB vaccine trials [76,227,234,235] .

1.6.5 Childhood tuberculosis

Childhood TB remains a leading cause of infant mortality worldwide. While intense scientific and clinical research efforts have focused on TB in adults, childhood TB has been relatively neglected. Nevertheless with roughly one million cases per year estimated globally infantile TB is a public-health emergency, particularly in developing countries [236,237,238,239] .

Children are at a much higher risk of progressing to active disease than adults, with the highest prevalence among children under two years of age and the lowest in children aged between five and 10 years [240,241] . Also, children have a higher risk of not only progression, but also of extra pulmonary dissemination and death [242] .

Due to its paucibacillary nature, diagnosis of childhood TB remains a great challenge. The use of the Mantoux skin test (TST) in children results in very poor specificity, especially in countries where BCG vaccine is given at birth and NTMs are endemic. In addition most children with active TB are sputum smear negative. Less than 10 to 15% of children with proven TB exhibit sputum smear positivity for AFB [243] . IGRAs could offer a more specific alternative [244,245] . However as IGRAs are quite expensive, ethical concerns about venipuncture in healthy children and uncertainty about the association between a positive result and later development of active disease, they could not yet become standard diagnostic tools in high burden countries [239] . Hence the most determining factors to investigate a possible TB in children are: compatible signs and symptoms, X-ray suggestive for TB, and likelihood of infection with *M. tuberculosis* [246] .

The diagnosis of TB remains the biggest challenge in children and better and more reliable tests are needed. This is especially important in children, since the rates of progression and dissemination are highly increased in comparison to adults.

1.7 Adaptive immune response to *M. tuberculosis*-infection

During the initial contact, infected macrophages transport mycobacteria into the draining lymph nodes, where presumably secreted antigens like ESAT6 and CFP-10 are presented to local $CD4^+$ T cells [207,247,248] . It has been shown that this process occurs rather slowly and no T cell activation takes place within the first 9 days following aerosol infection in mice [249] . This allows the bacteria to grow and manipulate the response to the infection. Once the T cells are activated, they differentiate into effector cells and migrate to the lung [250] . The most important and prominent cytokines as well as the T cell populations that provide these proteins are discussed in the following section.

1.7.1 T-cell cytokines in response to *M. tuberculosis*

IFNγ is crucial for the T cell response in mycobacterial disease. In the mouse model this has been demonstrated by the dramatically altered course of infection in IFNγ knock-out mice while human individuals with defects in the IFNγ or the IFNγ-receptor gene are more susceptible to severe TB [251,252,253] . IFNγ is expressed by both $CD4^+$ and $CD8^+$ T cells in TB, but can also be secreted by infected macrophages in an IL-12-dependent manner [254,255] . Although IFNγ production alone is insufficient to control *M. tuberculosis* infection, it is required for protection against this pathogen. However, IFNγ is produced by healthy PPD^+ individuals as well as those suffering from active TB, suggesting that this cytokine may be an unreliable immune correlate of protection [256] .

The cytokine IL-2 is also very important since it is required for secondary expansion of memory T cells [257] . A deficiency in the IL-2 induced T cell proliferation has been observed in patients with active TB, yet the treatment of adults suffering from the infection with recombinant IL-2 did not lead to an improvement. This underlines the complexity of this cytokine network [258,259,260,261] .

GM-CSF, a cytokine expressed by multiple immune cells including T cells, has been suggested to be potentially important in anti-mycobacterial immunity. Mice deficient for GM-CSF show reduced inflammation and T_H1 response in the lung, fail to control *M. tuberculosis* infection and rapidly succumb to severe necrosis [262] . Interestingly, the over-expression of GM-CSF does not reverse the effect, indicating that a fine regulation of expression is pivotal for protection. *M. tuberculosis*-specific $CD4^+$ T cells that express GM-CSF have been detected in children with active and latent TB and in response to a novel vaccine candidate. Taken together, this strengthens the hypothesis of the anti-mycobacterial effect of this cytokine [235,263] .

The involvement of TNFα in the immune and pathological response in TB is required for the control of acute *M. tuberculosis* infection [264,265] . Mice deficient for TNFα rapidly died with higher bacterial burden compared to controls. Deletion of TNFα during the chronic phase of infection leads to breakdown of the granuloma and an enhanced pro-inflammatory response [266] . TNFα also affects cell migration to the tissue and influences the expression of adhesion molecules as well as chemokines and chemokine receptors. In summary, the role of TNFα in the immune response to *M. tuberculosis* is crucial and multifaceted. This is proven by the observation that patients undergoing treatment with anti-TNFα antibody for rheumatoid arthritis develop reactivation from latent TB [267] .

In contrast to IFNγ and TNFα, IL-10 is considered as an anti-inflammatory cytokine produced by T cells during *M. tuberculosis* infection. It leads to downregulation of IL-12, produced by the macrophages, which subsequently decreases the IFNγ expression by T cells. It directly limits T_H1 immunity and exacerbates disease [268] .

The role of the T_H2 cytokine IL-4 in immune defense remains controversial. Since *M. tuberculosis* is a strong inducer of IL-12 the T_H1 response is favoured in infected hosts. Nevertheless, some reports document the existence of T_H2 responses in TB, but in PBMC from TB patients, no such phenomenon can be observed. While IFNγ expression is elevated in human granulomas, only little IL-4 RNA could be detected in lymph nodes [269,270,271] .

1.7.2 $CD4^+$ T cell subsets involved in the immune response to *M. tuberculosis*

Since *M. tuberculosis* resides primarily in vacuoles within macrophages, the presentation of microbial antigens via MHC class II to $CD4^+$ T cells is the main route of immune recognition. Studies in mice without $CD4^+$ T cells have shown the importance of this subset for the control of infection [272,273,274,275] . In humans the rising number of HIV patients co-infected with *M. tuberculosis* also provides evidence for the importance of $CD4^+$ T cells. HIV patients with latent TB have 8-10% annual risk of developing active TB compared to a 10% lifetime risk in subjects without HIV co-infection [276] . The primary function of $CD4^+$ T cells the immune defense is believed to be the production of IFNγ and other cytokines for recruitment and activation of macrophages, which can lead to the control of infection. $CD4^+$ T cells also provide help to $CD8^+$ T cells through the secretion of IL-2.

$CD4^+$ T cells activated by *M. tuberculosis* antigens can also differentiate in CTLs and help the infected macrophages control the intracellular bacterial load [277,278,279,280,281] . Along the lines of cytotoxic effector functions, $CD4^+$ CTLs express granzymes, Fas-L, granulysin and perforin [282] . As already mentioned granulysin in particular expresses potent bactericidal activity against *M. tuberculosis* [189] . Even single peptides derived from granulysin demonstrate bactericidal activity against *M. tuberculosis* [283] .

As described earlier, a subset of functional T cells produces IL-17 (T_H17) and these cells can be observed in the mouse model as well as humans exposed to TB [284] . After blocking of IL-17 during a high-dose challenge in mice, neutrophils recruitment was hindered and led to an alteration of the early inflammation response [285] . Whether these cells are protective or damaging is yet to be investigated.

Regulatory T cells (Tregs) expand in the lung during *M. tuberculosis* infection and their deletion leads to improvement in the immune response [286] . The depletion of Treg also leads to an increased number of IFNγ producing T cells *in vitro* suggesting a suppressive role for Treg of T_H1 response in TB [287] .

Despite all this knowledge about cellular mechanism counteracting against *M. tuberculosis* that were accumulated over the past centuries, a person is dying of TB every 20 seconds. This reminds us of our responsibility as researcher to better ways to diagnose, prevent and treat this devastating disease [288] .

1.8 Aims of the study

This thesis focuses on two different aims, which are closely related and both investigating the T cell response in anti-microbial immunity.

Characterisation of polyfunctional T cells in healthy latently TB infected (LTBI) and TB patients

TB together with malaria and AIDS remains the major cause of death and morbidity globally and is the deadliest bacterial infection with almost 2 million deaths every year [194] . With two billion healthy latently infected people worldwide, a correlate for protection against a possible reactivation is urgently needed [193] . It has been shown that $CD4^+$ T cells are crucial for the protective immune response against *M. tuberculosis* and that T_H1 cytokines are essential to initiate and sustain the containment of the pathogen [289] . Therefore this thesis investigated the possible use of polyfunctional $CD4^+$ T cells, expressing multiple T_H1 cytokines, as a protective marker against reactivation of the latent form of active TB. We also further aimed to characterise the changes in the cytokine expression of IFNγ, TNFα, IL-2 and GM-CSF under anti-microbial therapy. To fulfil these goals the following objectives were pursued:

- Installation of a new field side in a TB clinic in a high endemic township of Cape Town, South Africa.
- Recruitment of a total of 50 healthy household contacts, that live in close contact to a diagnosed active TB patient, and 50 TB patients before therapy that were followed up under anti-microbial therapy.
- Characterization of cytokine profile in both study groups and longitudinal changes under therapy.

In summary this study gives new insight into functional differences of polyfunctional $CD4^+$ T cells in LTBI and active TB patients in a highly endemic set-up and elucidates the role and behaviour of multi-cytokine secreting cells during anti-TB drug therapy.

Role of granulysin expressing $CD4^+$ CTLs in childhood tuberculosis

Due to its paucibacillary nature, diagnosis of TB in children remains very difficult and happens mostly through evaluation of probabilities by the paediatrician instead of functional assays. Even though CTLs are classically $CD8^+$ T cells, several studies report the involvement of $CD4^+$ CTLs in the immune defense against *M. tuberculosis*. We questioned if the measurement of the cytotoxic granule protein granulysin could be a new way to diagnose TB and how long after therapy granulysin expressing $CD4^+$ T cells can be detected. For that we recruited children with active or latent TB. We then measured granulysin expression in $CD4^+$ T cells by using a recently described 7d *in vitro* assay [290] . To further characterise the molecular mechanisms in $CD4^+$ CTLs in response to *M. tuberculosis*-antigens, we generated granulysin expressing and deficient T cell lines. By comparing T cell lines of these two groups we investigated the expression of transcription factors and cytokines.

2 Methods

2.1 Study population of adults from high endemic country

Peripheral blood (40 ml) was obtained from 54 LTBI and 55 TB patients out of the community of SAREPTA, Cape Town, South Africa. All participants were of African descent. All participants were BCG vaccinated as assessed by a scar over the deltoid region. Human immunodeficiency virus (HIV) – testing (Abbot) was performed at recruitment and HIV positive participants were excluded from the study. All study subjects signed written informed consent for participation. TB patients were self-reporting, untreated cases with a first episode of TB and were all acid-fast bacilli positive on two smears. If possible index cases were again bled one week after start of therapy, two months and one week after end of treatment. All LTBI had recorded household exposure to a smear-positive adult pulmonary TB index case that was diagnosed not more than two months before recruitment of the house hold contact (HHC). Tuberculin skin testing (TST) of all LTBI was performed using two tuberculin units of *M. tuberculosis* purified protein derivative (PPD) RT23 for *in vitro* use (Statens Serum Institute) injected intradermally immediately after bleeding. Indurations were read between 48 and 72 hours following test administration. All included HHC were negative for acid-fast bacilli in the sputum on two smears and showed no abnormalities on the chest X-ray which was done on recruitment of all contacts. Ethical approval for the study was obtained from the Committee for Human Research of the University of Stellenbosch. Donor characteristics are summarized in Table 1 (see section 3.1.1).

2.2 Study population for childhood tuberculosis

Children were enrolled at the paediatric pneumology and immunology department of the Charité, Berlin, Germany. Children were diagnosed based on tuberculin skin test (TST), bacteriologic diagnosis, imaging and IGRA. The intervention cut-off for the TST was used as suggested in [291] . As IGRA, Quantiferon® TB-Gold In-Tube was used according to manufacturer's instructions and a positive result was defined as >0.35 IU/mL. Differentiation between latent and active TB was based on diagnostic algorithm also described in [291] . Children that underwent antimicrobial therapy were treated with Isoniazid (INH), Rifampizin (RMP), Pyrazinamide (PZA), Ethambutol (EMB) or combinations of these first-line antituberculosis drugs. All included children were negatively tested for drug-resistance. After end of therapy children underwent X-Ray and were tested for sputum positivity when positive before treatment. Healthy controls were children admitted to the hospital for non-TB related diseases. All donors gave informed consent. The study was approved by the local

ethics committee [EA2/0128/4]. Donor characteristics are summarized in Table 3 (see section 3.2.1).

2.3 Cell culture handling

All cell procedures were performed under sterile condition using a laminar flow safety cabinet. Cells were always resuspended in medium and cultured in cell culture plates in a CO_2 Incubator set at a temperature of 37°C and 5% CO_2. Cells were counted using an Inverted Laboratory Microscope and a counting chamber.

2.4 Isolation of PBMC from human blood

For isolation of peripheral blood mononuclear cells (PBMC), blood was collected using Vacutainer (BD Bioscience) coated with Sodium-Heparin. The blood was diluted 1:1 in PBS and layered over 15ml lymphocyte separation medium (Ficoll) in a 50ml tubes. Ficoll is a hydrophilic polysaccharide (p=1.07g/ml) that allows the separation of PBMCs by density gradient centrifugation. After the layering tubes were centrifuges at 1100xg for 30min at room temperature (RT) without brake. The supernatant containing plasma was carefully removed and the layer of PBMCs located at the interface of the gradient was recovered and transferred in a new 50ml tube. The tube was filled up with PBS and the sample was centrifuged at 800xg for 10 min. at 4°C. The supernatant was discarded and the washing step was repeated. The pellet was resuspended in an appropriate amount of buffer and counted.

2.5 Intracellular staining after short-term stimulation assay

Isolated PBMCs were reconstituted in RPMI-1640 (Invitrogen) supplemented with 10% AB Rh-positive heat-inactivated human serum (Sigma-Aldrich), 100U/ml penicillin, 100µg/ml streptomycin (PAA), 1mM L-glutamine (PAA) and 10mM HEPES (PAA) (cRPMI) and $2x10^5$ PBMCs were transferred in 96-well and brought to a final volume of 200µl. Cells were cultured at 37°C / 5% CO_2 with medium alone as a background control or with 5µg/ml Staphylococcus enterotoxin B (SEB, Sigma-Aldrich), 10µg/ml purified protein derivative (PPD) from *M. tuberculosis* (Statens Serum Institute) or 10µg/ml recombinant *M. tuberculosis* protein early secreted protein-6 (ESAT6) and culture filtrate protein-10 (CFP-10) for 16h. After 4h of incubation 10µg/ml Brefeldin A (Sigma-Aldrich) was added which inhibited the secretion of produced cytokines by the Golgi apparatus. Subsequently, the cells were centrifuged for 6 minutes at 800xg and after removal of the supernatant 100µl Cytofix/Cytoperm (BD Bioscience) for fixation and permeabilization were added and

incubated at 4°C for 30 minutes. Treatment with 150µl Cytoperm/wash (BD Bioscience) was performed twice before staining. Antibodies used for the staining were used in different combinations and a complete list is shown in Apendix 6.3. After staining cells were incubated at 4°C for 45 minutes and subsequently cells were washed twice with Cytoperm/wash and finally resuspended in phosphate-buffered saline (PBS, Gibco) containing 10% fetal calf serum (FCS, Invitrogen) and analyzed using a FACS-LSRII (BD Bioscience).

2.6 Intracellular CD107a/b staining

For staining with lysosomal associated membrane protein-1 and -2 (LAMP-1/-2 or CD107a/b) the standard protocol for intracellular staining had to be modified based on staining protocol described in Betts et al. [292] . In summary, cells were incubated and stimulated as described above in short term stimulation, but in contrast after 4h not only Brefeldin A (Sigma-Aldrich) but also the antibody for CD107a/b (BD Bioscience) and Golgi-Stop containing Monensin (BD Bioscience) at a concentration of 10µg/ml were added. Monensin also inhibits the secretion of cytokines by the Golgi apparatus but also neutralizes the pH in endosomes and lysosomes [293] . After incubation of additional 12h, cells were fixed, permeabilized and stained for other markers as described before.

2.7 7d *in vitro* restimulation assay

Isolated PBMCs were resuspended in cRPMI and 2×10^5 PBMC per well were seeded in 96-well round bottom plates (NUNC) in 200µL medium. Cells were cultured at 37°C / 5% CO_2 with medium alone or together with 10µg/ml purified protein derivative (PPD) from *M. tuberculosis* (Statens Serum Institute), 5µg/ml recombinant ESAT6-CFP10 fusion-protein (courtesy of T.H.M. Ottenhoff, Leiden University), TcF antigen from *Trypanomosoma cruzi* (T. cruzi) (Prospec), recombinant protein Pp65 from human Cytomegalovirus (HCMV, Prospec) or recombinant protein EBNA-1 form Epstein-Barr-virus (EBV, Prospec) for 7d. A second restimulation with the respective antigen was performed at day 6. In addition 10µg/ml of the secretion inhibitor Brefeldin A (Sigma-Aldrich) was added 12h before analysis to avoid release of cytokines by the Golgi apparatus. Subsequently, cells were fixed and permeabilized using Cytofix/Cytoperm (100 µl) (BD Biosciences) for 30min at 4°C. Treatment with Cytoperm/wash (150µl) (BD Biosciences) was applied twice before staining with fluorochrome-labeled antibody mixtures (mAb). After staining for 45min at 4°C, cells were washed twice in Cytoperm/wash, resuspended in phosphate-buffered saline (PBS, Gibco) containing 10% fetal calf serum (FCS, Invitrogen) and analyzed using a FACS-LSRII (BD Biosciences).

2.8 Magnetic cell separation (MACS®)

The MACS® separation technology (Miltenyi) is based on the labeling of cell surface molecules with antibodies coupled to magnetic beads. Cell suspension was subsequently applied on a MACS separation column (Miltenyi) which is placed inside a strong magnet using a MACS® Separator (Miltenyi). In this high-gradient magnetic field cells that are labeled with magnetic antibodies are remaining in the column while unlabeled cells can be collected as negative fraction in the flow through. After removal of the column from the magnetic field positively selected cells can be eluted from the column. The MACS® technology is a simple and fast method to enrich cells with a grade of purity and apparently a low impact on the sorted cells.

2.8.1 Isolation of monocytes

Monocytes were enriched and separated using the Monocyte Isolation Kit II (Miltenyi). It is an indirect labeling system which leaves the monocytes untouched. Non-monocytes are labeled using a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A. In a second step cell suspension was incubated with anti-biotin beads which label all non-monocytes magnetically. After transferred on a MACS® separator (Miltenyi) monocytes could be found in the flow through with no need of direct labeling.

2.9 Proliferation assays

To measure the proliferation of T cells prior to stimulation cell suspensions were stained using a carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE). CFDA-SE is a non-fluorescent dye that passively diffuses into cells. Once it is in the cell it gets cleaved by intracellular esterases to the highly fluorescent form carboxyfluorescein succinimidyl ester (CFSE). This reacts with intracellular amines to form a fluorescent conjugate that is well retained. These conjugates are inherited to the daughter cell equally and therefore the mean fluorescence intensity is halved with every proliferation step. This loss of fluorescence can be displayed in a FACS as distinct peaks. The fluorescence from CFSE can be detected in the same channel as FITC.

Up to $1x10^7$ PBMCS were incubated with 5 µM CFDA-SE (Invitrogen) in 1ml pre-warmed PBS/0.1%BSA per $1x10^6$ cells for 10 min at 37°C. Cells were than quenched by adding 5 times of ice-cold medium and incubated on ice for additional five minutes. After centrifugation the pellet was washed in fresh media two times and cells were used for stimulation assays.

2.10 Generation of granulysin expressing T cell lines

Peripheral blood mononuclear cells were obtained from an adult TST+ subject by centrifugation over Ficoll-Paque (Biochrom AG) and cultured in RPMI 1640 (Gibco), supplemented with 10% heat-inactivated human AB serum (Sigma-Aldrich) stimulated with PPD (Staten Serum Institute) according to the 7d *in vitro* assay described above. After stimulation cells were pooled and stained with CD3 APC, CD4 APC-Cy7, CD8 PerCp-Cy5.5, CD25 Pe-Cy7, CXCR3 FITC (all BD Bioscience). Cells showing a $CD3^+$, $CD4^+$, $CD8^-$, $CD25^+$, $CXCR3^+$ phenotype were sorted using a BD FACS ARIA 2 (Becton Dickinson). A serial dilution of 0.3, 0.5 and 1 cell per well in a 96-well plate (Nunc) was performed and cells were co-cultivated with 1x105 autologous irradiated PBMCs (28 grays). PPD (10 µg/ml) and recombinant IL-2 (Active Bioscience) (40 U/ml) were added. Well that showed visible growth were further cultivated with irradiated PBMCs, Phytohemaglutinin (PHA) (Invitrogen) (2,5 µg/ml) and IL-2.

2.11 Quantitative real-time polymerase chain reaction

After 5d stimulation cells were lysed in TRIZOL (Invitrogen). Total RNA was reverse transcribed using random hexamers and Superscript reverse transcriptase (Invitrogen) as per manufacture instructions. QPCRs were performed using gene-specific primers (Qiagen). Fold changes compared to unstimulated controls were calculated using the ΔΔCt method [294] . Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene.

2.12 Assessment of cytolytic activity of T cell lines in monocytes

Autologous PBMCs were isolated as already described.

Monocyte isolation and infection with mycobacteria: Monocytes were isolated by Monocyte Isolation Kit II (Miltenyi) (see 2.2.7.1) and $1x10^4$ monoyctes were plated per well in a round-bottom, 96-well plate (Nunc) in RPMI-1640 (Invitrogen) supplemented with 7.5% AB Rh-positive heat-inactivated human serum (Sigma-Aldrich), 1mM L-glutamine (PAA) and 10mM HEPES (PAA). Monocytes were infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 5. Infection took place at 37°C and 5% CO_2 for 24h.

Co-cultivation of infected monocytes with T cell lines: Different T cell lines were added at a target:effector ratio of 1:10. Plates were incubated for additional 24h at 37°C and 5% CO_2.

Assessment of colony forming units (CFU): After 24h cells were lysed with 100µl of 0,1% Triton-X100 in PBS. 50µl of different dilutions of lysed cell suspension (1:100; 1:1000;

1:10000; 1:100000) were plated on 7H11 agar plates. Plates were incubated at 37°C and CFU were counted after at least 3 weeks.

2.13 Assessment of cytolytic activity of T cell lines in macrophages

Generation of autologous macrophages and infection with mycobacteria: Isolated monocytes (see above) were matured to macrophages by incubating cells in RPMI-1640 (Invitrogen) supplemented with 7.5% AB Rh-positive heat-inactivated human serum (Sigma-Aldrich), 1mM L-glutamine (PAA) and 10mM HEPES (PAA) and macrophage colony-stimulating factor (50ng/ml) (PeproTech) for five days at 37°C and 5% CO_2 in cell culture flasks (Corning). Macrophages were harvested and counted. $1x10^4$ macrophages were plated per well in a round-bottom, 96-well plate (Nunc) in RPMI-1640 (Invitrogen) supplemented with 7.5% AB Rh-positive heat-inactivated human serum (Sigma-Aldrich), 1mM L-glutamine (PAA) and 10mM HEPES (PAA) and rested for 24 hours. Afterwards, macrophages were infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of five. 1µCi of [^{3}H]-Uracil (Perkin Elmer) was added to each well and incorporation was allowed. Infection and incorporation took place at 37°C and 5% CO_2 for 24h.

Co-cultivation of infected macrophages with T cell lines: Different T cell lines were added at a target:effector ratio of 1:10. Plates were incubated for additional 24h at 37°C and 5% CO_2. Afterwards the plates were fixed, harvested and scintillation counts measured.

3 Results

3.1 Characterization of the cytokine profile in adults with latent and active tuberculosis from a high endemic country

3.1.1 Study design

Previous work in the mouse model has provided evidence for a potential association of polyfunctional memory T cells with the protection against intracellular pathogens. This has been shown by different groups in viral, fungal and bacterial infections [76,295,296] . Based on these findings we addressed the question whether polyfunctional T cells could be a protective marker against reactivation of the latent form of TB. We established a field site in Cape Town, South Africa, recruited TB patients and followed them up during anti-TB drug therapy. Using multi color flow cytometry for the measurement of intracellular cytokines we compared our findings with results obtained from LTBI. The characteristics of donors included in this study are listed in the following table 2.

Table 2: Donor characteristics

	LTBI	TB patients
Total number	40	45
Male (%)	17 (42)	28 (62)
Female (%)	23 (58)	17 (38)
Age range years (Median)	15 – 57 (28)	16 – 58 (31)

3.1.2 Cytokine response in latent and active TB patients under therapy

To evaluate the role of multi-cytokine expression by $CD4^+$ T cells in the protection against active TB we used a previously described 16h *in vitro* short-term stimulation assay and modified it for PBMC stimulation [297] . Freshly isolated PBMCs were incubated in medium containing different stimulating antigens. Unstimulated PBMCs in medium were used as control. To inhibit the release of cytokines, a secretion inhibitor was added after 4h of stimulation. Due to the short duration of stimulation, only antigen-experienced T cells (memory T cells) express cytokines after stimulation [263] .

An example of the gating strategy used in the first set of experiments is shown in Figure 1A. Lymphocytes were selected based on their size (FSC) and granularity (SSC) and further subdivided using the CD4 and CD8 T cell markers. Then the expression of the cytokines IFNγ, TNFα, Interleukin 2 (IL-2) and GM-CSF in $CD4^+$ T cells was measured. For antigen-specific stimulation the purified protein derivative (PPD) from *M. tuberculosis* and the immuno-dominant early secreted antigenic target 6 kDa (ESAT-6) were used. To ensure the ability of the cells to express cytokines staphylococcal enterotoxin B (SEB) was used as a positive control. SEB bridges the constant region of the major histocompatibility complex (MHC) II molecule on the antigen presenting cell (APC) to the Vβ region of the T cell receptor (TCR) regardless of a match in the D- and J-region and the TCRα-chain [298] .

First the general expression of all four measured cytokines (IFNγ, TNFα, IL-2 and GM-CSF) produced by $CD4^+$ T cells after different stimulations were measured. By comparing the cytokine response after PPD stimulation no differences could be detected between LTBI and patients with active TB prior to therapy for any of the four measured cytokines (Fig. 1B). After the first week of therapy the expression of TNFα increased and was significantly higher in comparison to TB patients without treatment. After two months of treatment the PPD-specific expression of IFNγ and GM-CSF were significantly higher compared to TB patients that had not started therapy. This effect diminished after completion of therapy. The expression of IL-2 remained stable during therapy and did not differ from the LTBI group.

Also stimulation with the recombinant protein ESAT6, no differences in IFNγ expression between LTBI and patients with active TB could be measured. Further, no changes in frequency could be monitored under therapy (Fig. 1C). TNFα expression significantly decreased after the end of therapy when compared to the study groups before and one week after start of therapy. For IL-2 the same pattern observed after PPD stimulation held true for ESAT-6 stimulation. The only cytokine that could be shown to differ in its expression between LTBI and TB patients before therapy was GM-CSF.

In the last set of stimulations the cells were treated with SEB, which served as a positive control and was used to show the maximum potential of the $CD4^+$ T cells to express all four cytokines (Fig 1D). T cells from LTBI expressed significantly higher levels of IFNγ compared to cells from TB patients before therapy. Even though no significant differences for the antigen-specific expression of IL-2 could be detected, T cells from donors that completed their therapy showed a higher potential to express IL-2 in comparison to patients who received therapy for one week and two months, respectively. The stimulation with SEB did not lead to differences between any of the study groups in the context of GM-CSF and TNFα expression. To ensure, that the difference in IFNγ expression after mitogen stimulation was not limited to the stimulation with SEB we performed a QuantiFeron - TB Gold® testing in a group of TB patients and LTBI (Fig. 2). The results are consistent with the data acquired using ICS. IFNγ concentrations measured in the supernatants revealed no differences between study groups after antigen stimulation. However as it was shown after SEB stimulation using ICS, the IFNγ levels were significantly higher in LTBI after mitogen stimulation.

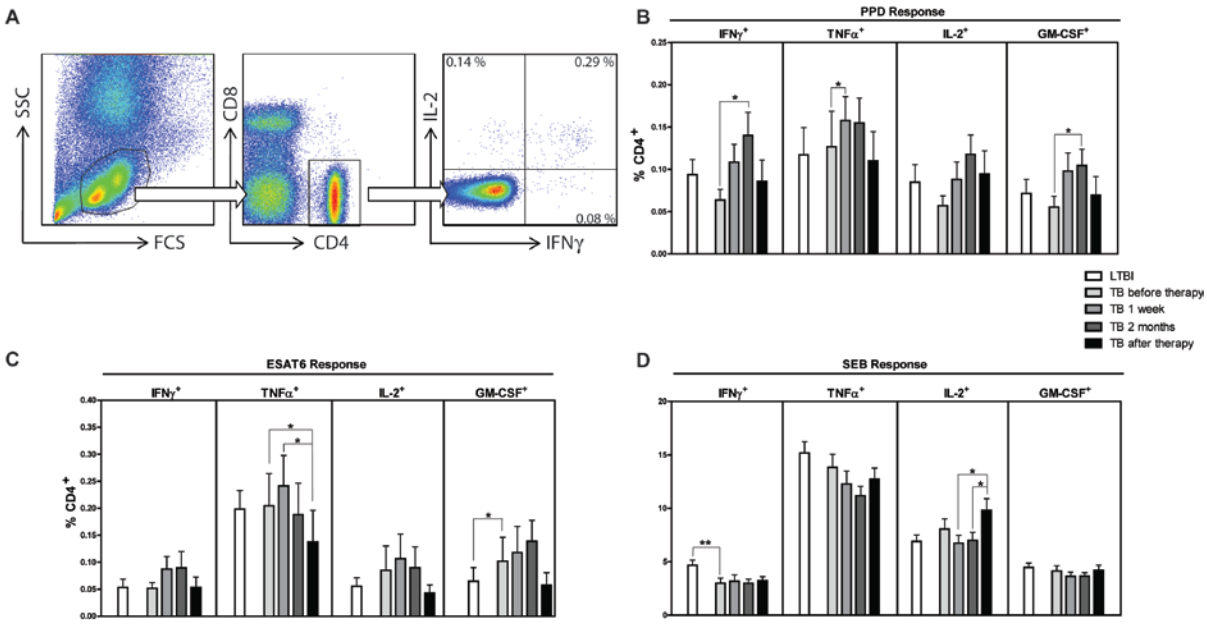


Figure 1: **Differences in cytokine expression of CD4$^+$ T cells after 16h short term *in vitro* stimulation.**

A. Representative dot plot showing the gating strategy for lymphocytes due to characteristic size (forward scatter, FCS) and granularity (side scatter, SSC). In a second gating step lymphocytes were subdivided using the expression of CD4 and CD8 on the surface of T cells. Next CD4$^+$ T cells were analyzed for the expression of the different cytokines. The third plot shows the expression of IFNγ and IL-2 for a representative donor after PPD stimulation. PBMCs from 44 LTBI (white bar), 42 patients with active pulmonary TB before therapy (light grey bar), 37 TB patients after one week of therapy (grey bar), 33 TB patients after two months of therapy (dark grey bar) and 25 TB patients after end of therapy (black bar) were stimulated with **B.** PPD, **C.** ESAT-6 and **D.** SEB. Individual background values of unstimulated controls were subtracted. Bars indicate arithmetic mean and the error bars represent the standard error. Nominal two-sided *p*-values for the Mann-Whitney U-test are shown as follows: * $p < 0.05$, ** $p < 0.01$.

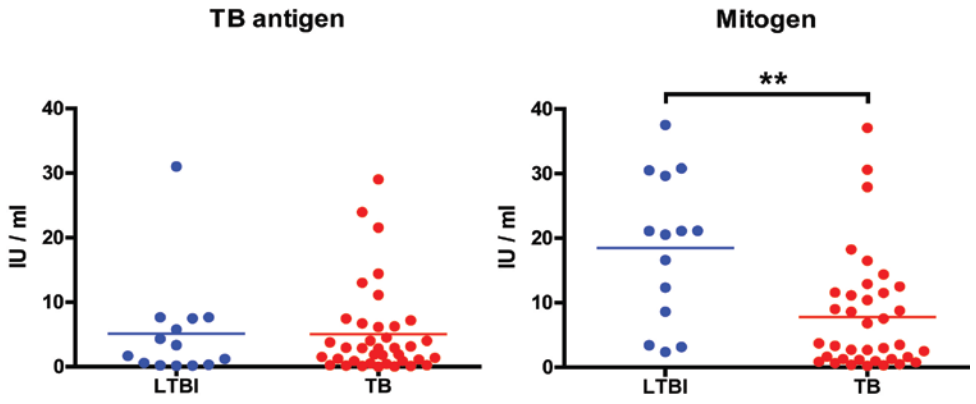


Figure 2: Quantiferon – TB Gold® In-tube results.

Whole blood from 37 TB patients (TB) and 14 LTBI were incubated according to manufacturer's instructions. IFNγ measurement in Quantiferon supernatants were done with the IFNγ enzyme linked immunosorbent assay (ELISA). Individual background values of unstimulated controls were subtracted. Means are shown and significance between different patient groups and healthy controls are indicated by asterisks (Mann-Whitney-U test ** $p < 0.01$).

3.1.3 Characterization of multi-functional CD4+ T cells from patients undergoing therapy and in comparison to LTBI

To investigate whether T cell responses differ in terms of polyfunctionality in LTBI and patients with active TB under therapy, the simultaneous expression pattern of the four measured cytokines was analyzed. In a first post-acquisition analysis the $CD4^+$ T cells were grouped by their ability to express either all four cytokines (4+), three different cytokines (3+), two (2+) or were singly expressing one of the four cytokines (1+) regardless of their expression pattern (Fig. 3).

After PPD stimulation a proportion of $CD4^+$ T cells that were expressing all four cytokines could be detected even though the frequencies did not differ between LTBI and TB patients and did not change during therapy (Fig. 3A). Polyfunctional T cells frequencies that expressed three cytokines were increased after two months of therapy when compared to patients that had not received therapy. The most disparities in frequencies could be observed in the group of double and single positive $CD4^+$ T cells. While the proportion of T cells expressing two cytokines were significantly increased during the first two months of therapy in comparison to TB patients before therapy, the frequency of single positive T cells in contrast decreased during therapy.

In the case of stimulation with the *M. tuberculosis* protein ESAT-6 a decrease in the proportion of highly multi-functional T cells (4+) could be observed after end of therapy. Simultaneously higher frequencies of T cells expressed only one of the measured cytokines (1+) (Fig. 3B).

After SEB stimulation the proportion of four-cytokine-producers (4+) was significantly different in LTBI and TB patients and increased after end of therapy. In contrast the majority of $CD4^+$ T cells showed a single cytokine expression pattern. This subset was significantly reduced after two months of therapy when compared to TB patients before therapy (Fig. 3C).

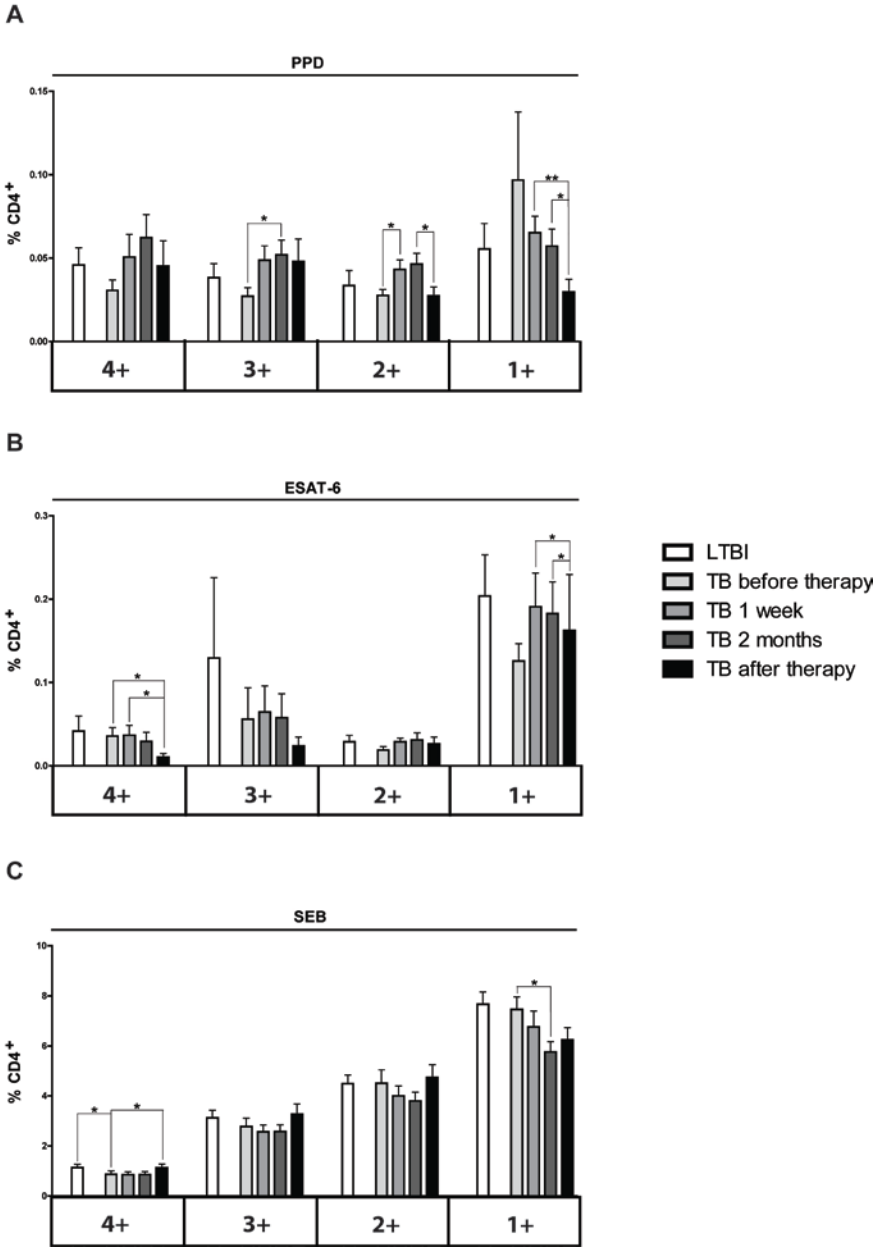


Figure 3: Cytokine profile of $CD4^+$ T cells after 16h short term *in vitro* stimulation.

PBMCs from 44 LTBI (white bar), 42 patients with active pulmonary TB before therapy (light grey bar), 37 TB patients after one week of therapy (grey bar), 33 TB patients after two months of therapy (dark grey bar) and 25 TB patients after end of therapy (black bar) were stimulated with **A.** PPD, **B.** ESAT-6 or **C.** SEB. Individual background values of unstimulated controls were subtracted. First group (4+) shows the frequency of $CD4^+$ T cells that express all 4 cytokines ($IFN\gamma^+/TNF\alpha^+/IL\text{-}2^+/GM\text{-}CSF^+$). Second group represents the proportion of all possible combinations of triple expression (e.g. $IFN\gamma^+/TNF\alpha^-/IL\text{-}2^+/GM\text{-}CSF^+$). Third group shows the sum of all 6 combinations of double positive $CD4^+$ T cells (e.g. $IFN\gamma^+/TNF\alpha^-/IL\text{-}2^-/GM\text{-}CSF^+$). The last column shows the proportion of single-positive $CD4^+$ T cells (e.g. $IFN\gamma^+/TNF\alpha^-/IL\text{-}2^-/GM\text{-}CSF^-$). Bars indicate arithmetic mean and the error bars represent the standard error. Nominal two-sided *p*-values for the Mann-Whitney U-test are shown as follows: * $p < 0.05$, ** $p < 0.01$.

To elucidate the changes in the cytokine profile after PPD stimulation in more detail, all different combinations of the four cytokines were analyzed (Fig. 4).

It could be shown that the proportion of $CD4^+$ T cells that expressed the three cytokines IFNγ, TNFα and IL-2 were increased after two months of therapy and stayed elevated after end of therapy (Fig. 4B). Also the frequency of T cells co-expressing IFNγ, TNFα and GM-CSF increased under therapy but unlike the first group this effect could not be seen after end of therapy.

In Fig. 4C the four, out of the six possible combinations of double positive T cells, which showed significant differences, are displayed. IFNγ and TNFα co-expressing cells were significantly increased after one week of therapy in comparison to patients after therapy. Same held true for TNFα and GM-CSF expressing $CD4^+$ T cells. The frequency of TNFα + IL-2 and the IL-2 + GM-CSF co-expressing T cells showed a peak two months after start of therapy.

The frequency of IFNγ single-positive cells did not change under therapy and was not differently expressed in LTBI (Fig. 4D). The proportion of T cells exclusively expressing TNFα showed its peak after one week of therapy. A similar effect could be shown for GM-CSF single-positive T cells. Only IL-2 single positive T cells showed a significant difference in the proportion between LTBI and TB patients before therapy.

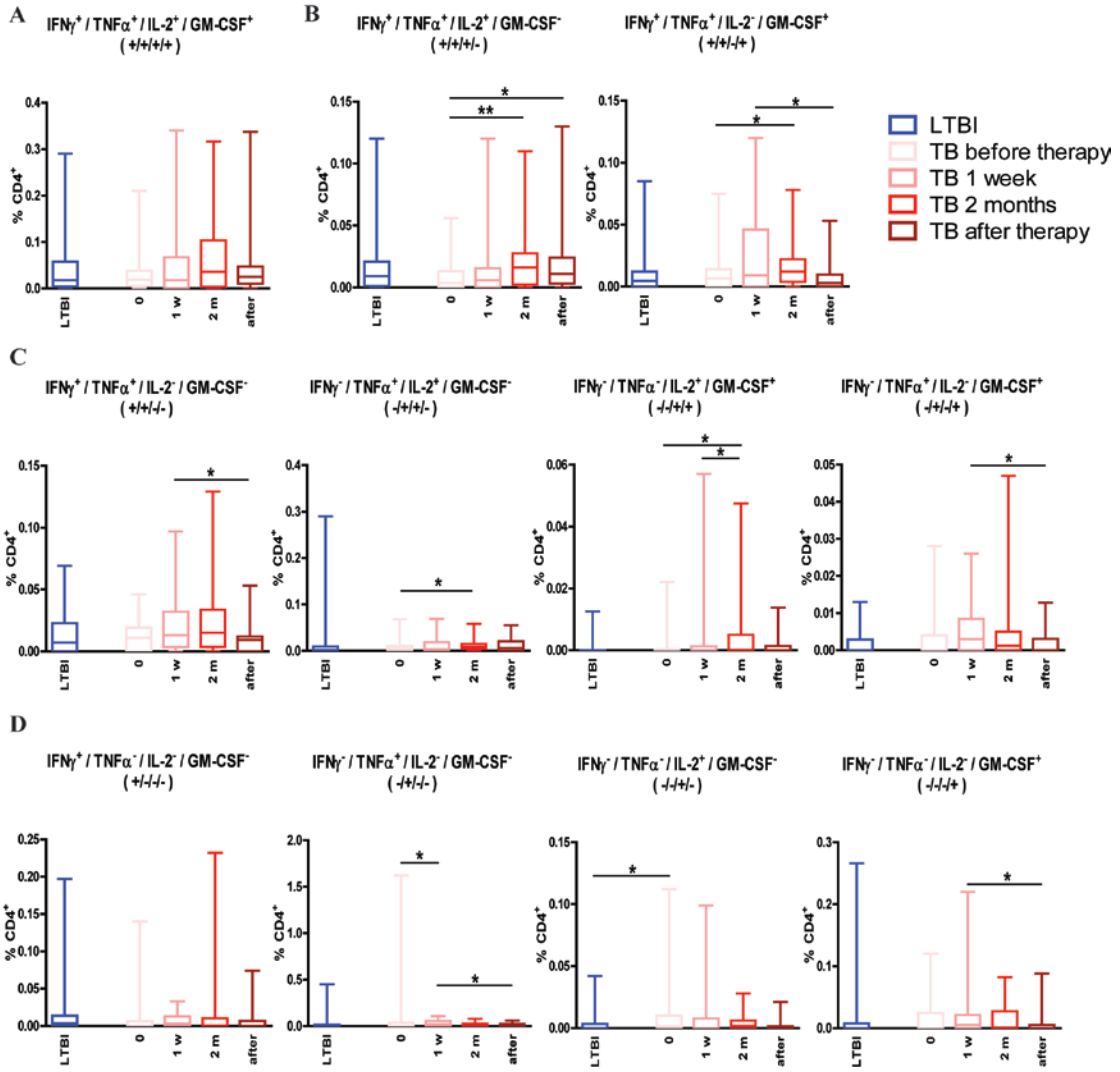


Figure 4: Detailed CD4$^+$ T cell cytokine profile after 16h PPD stimulation.

PBMCs from 44 LTBI (blue / LTBI), 42 patients with active pulmonary TB before therapy (light red / 0), 37 TB patients after one week of therapy (pink /1 w), 33 TB patients after two months of therapy (red / 2 m) and 25 TB patients after end of therapy (dark red / after) were stimulated. Individual background values of unstimulated controls were subtracted. **A.** Proportion of CD4$^+$ T cells expressing all four cytokines **B.** shows the 2 different combinations of triple positive T-cells that showed significant differences. **C.** Frequencies of double-positive CD4$^+$ T cells **D.** Frequencies of single cytokine expressing T cells. The horizontal line indicates the arithmetic median and the box represents the interquartile range of each dataset. The length of the whisker displays overall range of each dataset. Nominal two-sided *p*-values for the Mann-Whitney U-test are shown as follows: * $p < 0.05$, ** $p < 0.01$.

3.1.4 Analysis of CD4+ memory T cells from patients undergoing treatment and in comparison to LTBI

To further characterize the cytokine profile an additional marker was used to subdivide the CD4$^+$ T cells into naive and memory cells. It has been previously shown that CD45RO can be used as a marker to differentiate between these two subsets [59] . It was demonstrated by us that the majority of cytokine expression after 16h short term *in vitro* stimulation with *M. tuberculosis* antigens can be found in the central memory (T_{cm}) and effector memory (T_{em})

compartment, which are both positive for CD45RO [263] . Therefore, we focused in the next step exclusively on $CD4^+$ $CD45RO^+$ memory T cells, which increased frequencies of antigen-specific T cells (Fig. 4A).

Similar to the $CD4^+$ T cell analysis (Fig. 1) first the expression of all four cytokines by $CD4^+$ $CD45RO^+$ T cells was analyzed, regardless of their pattern (Fig. 5). After stimulation with PPD only IL-2 was higher expressed by $CD4^+$ memory T cells after therapy when compared to one week after start of treatment (Fig. 5B). After stimulation with ESAT-6 the expression of IFNγ and GM-CSF differed between LTBI and TB patients that were not treated (Fig. 5C). In the last stimulation with the superantigen SEB it could be shown that the expression of TNFα was higher expressed in TB patients compared to LTBI. During treatment TNFα expression was reduced, which was recovered after end of therapy. Also the IL-2 expression of $CD4^+$ memory T cells increased when compared between two months and end of therapy. For all three cytokines (TNFα, IL-2 and GM-CSF) a significant lower frequency was observed in LTBI in comparison to TB patients before therapy (Fig. 5D).

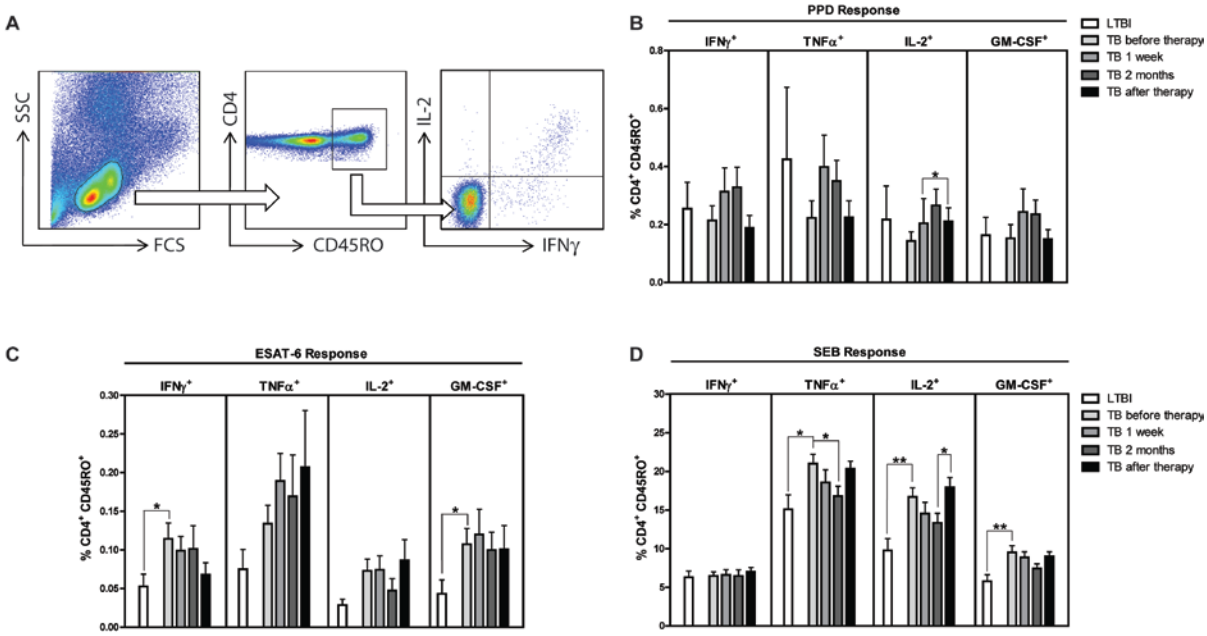


Figure 5: Differences in cytokine expression of $CD4^+$ $CD45RO^+$ memory T cells after 16h short term *in vitro* stimulation.

A. Representative dot plot showing the gating strategy for lymphocytes according to size (forward scatter, FCS) and granularity (side scatter, SSC). In a second gating step CD4 T cells were selected based on surface expression of this marker (not shown). Then $CD4^+$ memory cells were chosen using the expression of CD4 and CD45RO (second plot). Next the $CD4^+$ $CD45RO^+$ T cells were analyzed for the expression of the different cytokines. In the third dot plot the expression of IFN□ and IL-2 for a representative donor after PPD stimulation is shown. PBMCs from 16 LTBI (white bar), 25 patients with active pulmonary TB before therapy (light grey bar), 24 TB patients after one week of therapy (grey bar), 29 TB patients after two months of therapy (dark grey bar) and 26 TB patients after end of therapy (black bar) were stimulated with **B.** PPD, **C.** ESAT-6 and **D.** SEB. Individual background values of unstimulated controls were subtracted. Bars indicate arithmetic mean and the error bars represent the standard error. Nominal two-sided *p*-values for the Mann-Whitney U-test are shown as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.1.5 Characterization of multi-functional memory CD4+ T cells from patients undergoing therapy and in comparison to LTBI

Next we analyzed the distribution of multi-functional T cells within the CD45RO$^+$ memory compartment. Therefore, the different patterns of co- or single-expression of the four measured cytokines (IFNγ, TNFα, IL-2 and GM-CSF) were analyzed and grouped accordingly (Fig. 6).

The population of memory T cells that co expressed all four cytokines after PPD stimulation did not change significantly during therapy (Fig. 6A). Same held true for the triple-positive population. The two cytokine expressing memory T cells showed a significant reduction when compared between the two months time point and after therapy.

Stimulation with ESAT-6 showed a decrease in the proportion of quadruple positive memory T cells after end of treatment compared to patients before anti-microbial therapy (Fig. 6B).

When stimulated with SEB, the combination of triple positive memory T cells was increased in TB patients compared to LTBI. This frequency decreased under the first two months of treatment and increased again after end of therapy. Also the population of single expressing CD4$^+$ memory T cells was more strongly induced in TB patients when compared to LTBI and decreased during therapy, but in contrast the frequency remained lower after end of therapy (Fig. 6C).

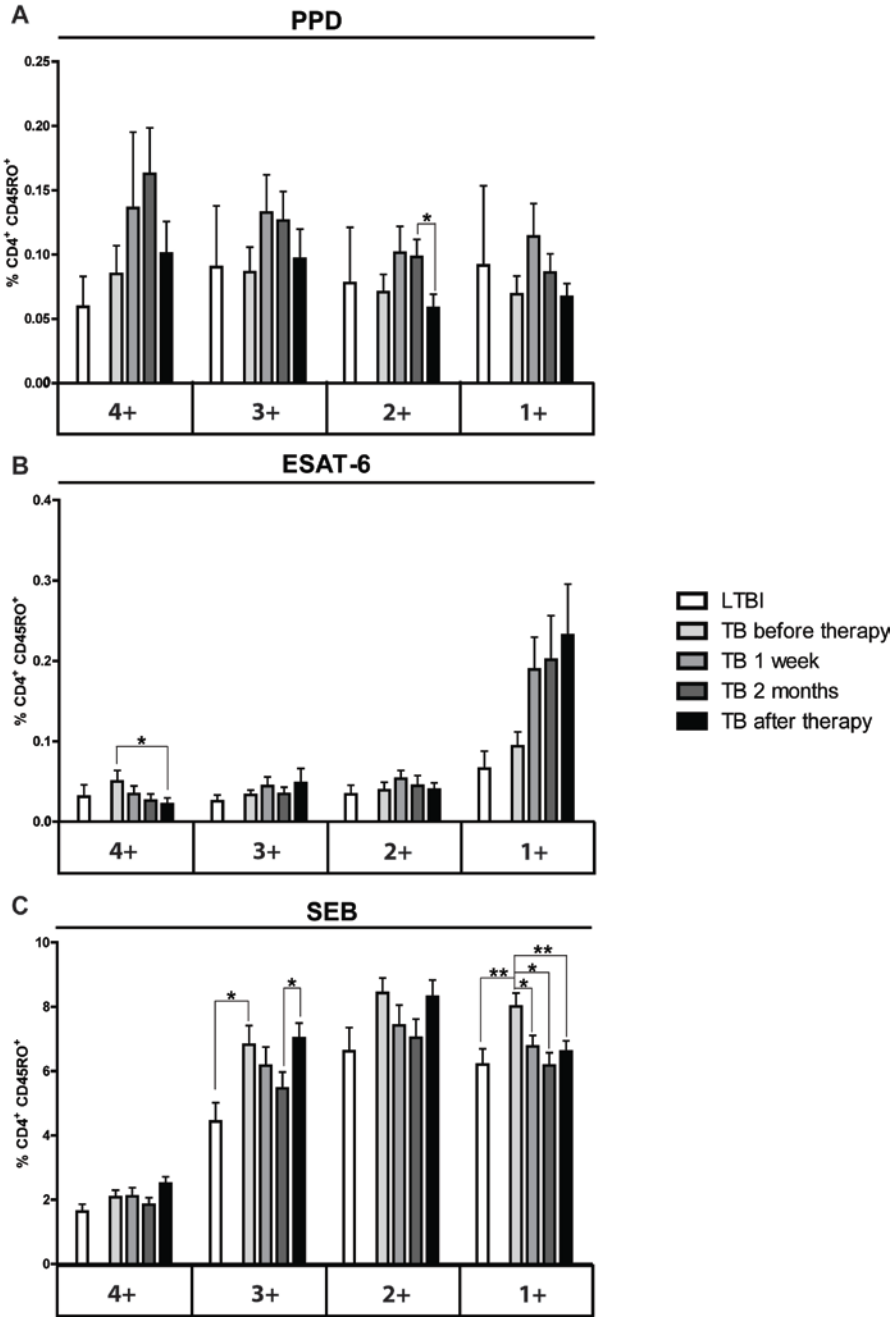


Figure 6: Cytokine profile of CD4$^+$ CD45RO$^+$ memory T cells after 16h short term *in vitro* stimulation.

PBMCs from 16 LTBI (white bar), 25 patients with active pulmonary TB before therapy (light grey bar), 24 TB patients after one week of therapy (grey bar), 29 TB patients after two months of therapy (dark grey bar) and 26 TB patients after end of therapy (black bar) were stimulated with **A.** PPD **B.** ESAT-6 and **C.** SEB. Individual background values of unstimulated controls were subtracted. First group (4+) shows the fraction of CD4$^+$ T cells that express all 4 cytokines (IFN$\square^+$/TNFα$^+$/IL-2$^+$/GM-CSF$^+$). Second group represents the proportion of all possible combinations of triple expression (e. g. IFN$\square^+$/TNFα$^-$/IL-2$^+$/GM-CSF$^+$). Third group shows the sum of all 6 combinations of double positive CD4$^+$ T cells (e. g. IFN$\square^+$/TNFα$^-$/IL-2$^-$/GM-CSF$^+$). The last column shows the proportion of single-positive CD4$^+$ T cells (e. g. IFN$\square^+$/TNFα$^-$/IL-2$^-$/GM-CSF$^-$). Bars indicate arithmetic mean and the error bars represent the standard error. Nominal two-sided *p*-values for the Mann-Whitney U-test are shown as follows: * $p < 0.05$, ** $p < 0.01$.

3.1.6 Co-stimulation with IL-7 induces higher cytokine expression and leads to increased multi-functionality

The cytokine IL-7 is primarily known for its role in the development and homeostasis of memory T and B cells and their survival [299,300] . The sources *in vivo* are keratinocytes, thymic cells as well as adherent bone marrow stroma cells [301,302,303] . The associated membrane bound receptor (CD127) is a heterodimer of a alpha-chain, that is exclusive for IL-7R and a common gamma chain, that is shared with the receptors of IL-2, IL-4, IL-9 and IL-15 [304] . It can be found on the surface of activated and naive T cells, B cells and human endothelial cells [305] . It has been shown that the stimulation with IL-7 enhanced the production of T_H1 cytokines, mainly IFNγ and TNFα, and increased proliferation [306,307] . Our aim was to study the responsiveness to low-dose IL-7 (5 ng/ml) used for co-incubation with PPD and its impact on the polyfunctionality of $CD4^+$ T cells.

The simultaneous stimulation with PPD and IL-7 led to a significant increase of all four measured cytokines in LTBI compared to stimulation without IL-7 (Fig. 7A). In TB patients with active TB an increase in IFNγ, IL-2 and GM-CSF positive T cells could be shown. After one week of therapy this effect diminished and except for TNFα after two months no expression differences under and after therapy could be observed. To answer the question if IL-7 led to a stronger induction of multifunctional T cells the contribution of IL-7 co-incubation to the different T cell subsets was investigated and it could be shown that only the proportion of T cells that express all four cytokines and the populations expressing IFNγ, TNFα and GM-CSF respectively increased significantly (Fig. 7B). Next, the cytokine expression after co-incubation with IL-7 was compared during therapy and in comparison to LTBI (Fig. 7C). The expression of TNFα and IL-2 increased after two months of therapy compared to patients without treatment.

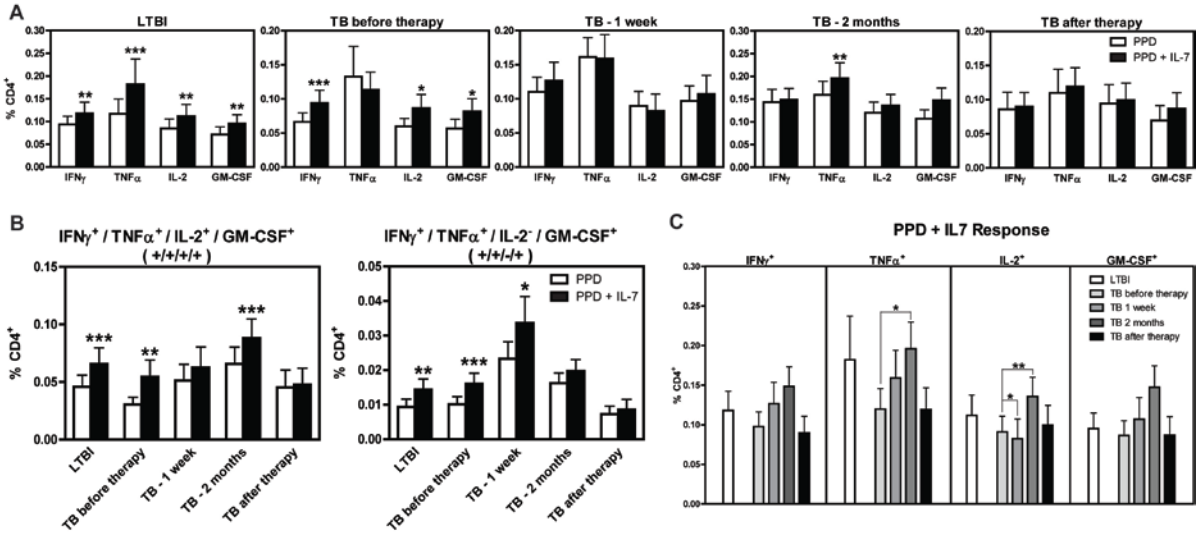


Figure 7: Analysis of cytokine expression and polyfunctional T cells after stimulation with PPD in the presence or absence of IL-7.

A. PBMCs from 44 LTBI, 40 patients with active pulmonary TB before therapy, 36 TB patients after one week of therapy, 33 TB patients after two months of therapy and 25 TB patients after end of therapy were stimulated with PPD with (black bar) or without (white bars) IL-7 (5µg/ml). Individual background values of unstimulated controls or cells incubated only with IL-7 were subtracted. **B.** First graph shows the fraction of $CD4^+$ T cells that express all four cytokines ($IFN\gamma^+/TNF\alpha^+/IL\text{-}2^+/GM\text{-}CSF^+$) for all five different study groups. Second graph represents the proportion of triple positive T cells ($IFN\gamma^+/TNF\alpha^+/IL\text{-}2\text{-}/GM\text{-}CSF^+$). **C,** PBMCs from 44 LTBI (white bar), 40 patients with active pulmonary TB before therapy (light grey bar), 36 TB patients after one week of therapy (grey bar), 33 TB patients after two months of therapy (dark grey bar) and 25 TB patients after end of therapy (black bar) were simultaneously stimulated with PPD and IL-7. The percentage of IFNγ (first bracket), TNFα (second), IL-2 (third) and GM-CSF (fourth) are shown. Bars indicate the arithmetic mean and error bars above represent the standard error. *P*-values for the Wilcoxon signed rank test, used in **A** and **B**, are shown as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$. Nominal two-sided *p*-values for the Mann-Whitney U-test, used in **C**, are shown as follows: * $p < 0.05$, ** $p < 0.01$.

3.1.7 Impact of T_H2 and T_H17 on cytokine response during treatment and in comparison to LTBI

So far we focused on 4 cytokines associated with T_H1 responses. In a second panel of staining we investigated the impact of two additional subsets of $CD4^+$ T cells, T_H2 and T_H17, by staining for their characteristic cytokines.

The role of humoral immunity and therefore T_H2 cells in the immune response to *M. tuberculosis* is controversial. T_H2 cells are characterized by IL-4, but not IFNγ secretion, which is important for the up-regulation of antibody production [308] . The role of IL-4 expression in patients infected with *M. tuberculosis* remains controversial. Some groups reported higher levels of IL-4 in TB, while others could not find any differences [260,269,309] .

The frequencies of antigen-specific T_H2 $CD4^+$ T cells after stimulation with PPD and ESAT6 were both below detection limits and could therefore not be compared between the single study groups (data not shown). Frequencies of IL-4 positive T cells between 1 – 2 % of all

$CD4^+$ T cells could be observed after stimulation with SEB. The frequencies did not differ between LTBI and patients with active TB. Moreover, no changes of expression could be seen in patients undergoing treatment (Fig. 8).

The newly described $CD4^+$ T cell lineage, T_H17, was first associated with protection against extracellular bacteria and fungi. But later it was linked with protection against intracellular pathogens like *Salmonella enterica* [310,311] . While IL-17 itself seems to play an important role in TB, the main source appears to be gamma-delta T cells [285,312,313] . Recent studies associated T_H17 cells with immune response to TB as well [314,315] . In the experimental setup used in this study almost no IL-17 expressing T cells were observed after stimulation with PPD or ESAT-6 (data not shown). The treatment with SEB led to a frequency of around 1% in the $CD4^+$ T cell compartment of IL-17 expressing T cells in all study groups (Fig. 8). Again no changes during the treatment could be observed.

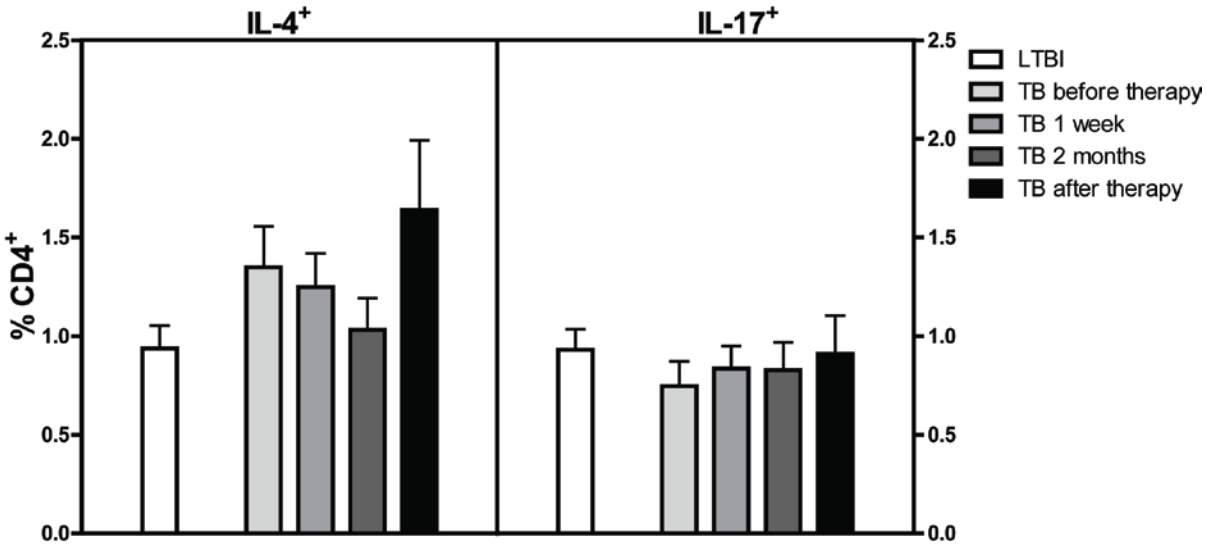


Figure 8: Cytokine expression of IL-4 and IL-17 in $CD4^+$ T cells after 16h SEB - stimulation.

PBMCs from 40 LTBI (white), 28 patients with active pulmonary TB before therapy (light grey), 25 TB patients after one week of therapy (grey), 25 TB patients after two months of therapy (dark grey) and 20 TB patients after end of therapy (black) were stimulated. Individual background values of unstimulated controls were subtracted. Bars indicate arithmetic mean and error bars above represent the standard error.

3.1.8 Concluding remarks on the cytokine profile of $CD4^+$ T cells from TB patients and LTBI

In sum, we investigated the cytokine expression of IFNγ, TNFα, IL-2 and GM-CSF by $CD4^+$ T cells from LTBI and TB patients under therapy. We could demonstrate, that GM-CSF as the only tested cytokine is increased in TB patients after stimulation with the *M. tuberculosis* protein ESAT6 and identified several changes in the cytokine expression of all four cytokines in patients during therapy. Stimulation with SEB revealed a reduced ability of T cells from TB

patients to express IFNγ compared to LTBI. Measurement of polyfunctionality showed a subset of antigen-specific T cells from TB patients and LTBI, which co-expressed GM-CSF in combination with IFNγ, IL-2 and TNFα. These experiments also demonstrated an increase of multi-cytokine expressing T cells and a simultaneous decrease in single positive T cells under anti-microbial therapy. In addition we also measured cytokine expression of IL-17 and IL-4, which did not undergo any changes under therapy. In our study we were also able to demonstrate that co-cultivation with low dose IL-7 leads to an increase of all four measured cytokines in LTBI and of three cytokines in patients with active TB. Co-stimulation with IL-7 also improved polyfunctionality of T cells from both study groups.

3.2 The role of the cytotoxic protein granulysin in childhood tuberculosis

Cytolytic T lymphocytes (CTLs) are known to play an essential role in the immune response to intracellular bacteria such as Listeriae, Chlamydiae and also Mycobacteria *[281,316,317]* . CTLs use two major pathways to kill target cells. The first one is by expressing FAS ligand (FAS-L) on their surface. When bound to FAS on the surface of the target cell FAS-L leads to activation-induced cell death. The second way of cell-mediated cytotoxicity is via granule exocytosis into the immunologic synapse between the CTL and the target cell [164] . Effector proteins contained within cytotoxic granules include perforin, granzymes and granulysin [169] . Granulysin has been shown to have a wide range of antimicrobial activities against different kinds of bacteria, parasites and fungi [189] . Even though CTLs are typically found in the $CD8^+$ T cell compartment there have been several reports of $CD4^+$ T cells with cytotoxic capacities in the immune response to *M. tuberculosis* [282,318,319] . In our study we focused on the role of granulysin expressing $CD4^+$ T cells in the immune response to childhood TB by using a newly described long term *in vitro* restimulation assay [290] .

3.2.1 Granulysin expression is upregulated in latently infected children and children with active TB after antigen stimulation

Since Schuck et al. [290] demonstrated, that by using a 7d *in vitro* assay it is possible to aggravate the frequency of antigen-specific CD4 T cells, we raised the question if this assay could be used to detect CTLs in children with latent or active TB. To investigate this, in a first validation experiment the induction of $CD4^+$ memory T cells after antigen stimulation in healthy, TB infected children and children infected with NTMs was monitored. The characteristics of donors included in this study are listed in the Table 3. As described before, the surface expression of CD45RO was used to differentiate naive from memory T cells. In children that have been infected with *M. tuberculosis* a strong induction of memory cells could be seen (Fig. 9A). Without stimulation only children with active TB before treatment showed a significant higher proportion of $CD4^+$ memory T cells compared to healthy children (Fig 9B). In all patient groups stimulation with PPD led to significant increase in the frequency of memory T cells compared to healthy controls. This held also true for children with *M. tuberculosis* infection after stimulation with ESAT6-CFP10 regardless of therapy. To investigate if this increase is simply due to the differentiation of naive cells to memory cells or specific induction of antigen-specific memory T cells a proliferation assay was performed. It could be demonstrated that stimulation with PPD led to a strong proliferation of T cells only within the memory compartment (Fig. 9C).

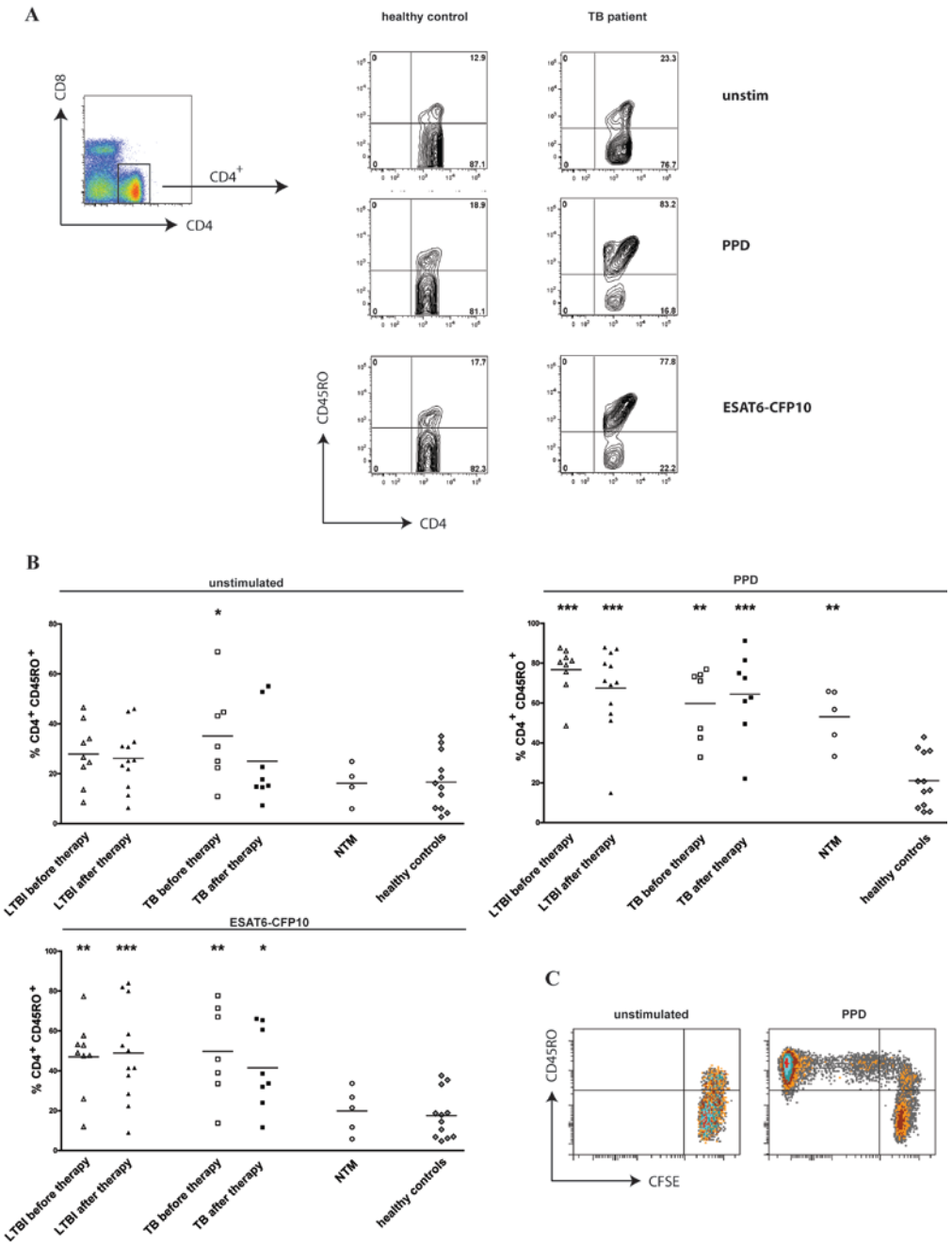


Figure 9: Antigen-specific increase of CD45RO$^+$ memory T cells after 7d *in vitro* assay.

A Representative density plot showing CD4 and CD8 staining of lymphocytes (right), CD4$^+$ T cells were further gated for their distribution of $CD45RO_{low}$ vs. $_{high}$ after left unstimulated, stimulated with PPD or with ESAT6-CFP10. A representative example for a patient with active TB (left) and a healthy control (right) is shown. **B.** Percentage of CD4$^+$ CD45RO$^+$ memory T cells without stimulation (left), with PPD (right) or ESAT6-CFP10 (bottom left) from 9 LTBI before therapy (clear triangles), 12 LTBI after therapy (black triangles), 7 patients with active TB before therapy (clear squares), 8 patients with active TB after treatment (black squares), 4 patients with NTM infection (clear circle) and 12 healthy controls (grey diamonds). Means are shown for each group and significance between different patient groups versus healthy controls are indicated by asterisks (Mann-Whitney-U test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **C.** Representative proliferation dot plot of CD4$^+$ T cells of a patient with active TB after incubating in media (unstimulated) and after stimulation with PPD.

After having demonstrated the potential value of a 7d *in vitro* restimulation assay to induce antigen-specific memory T cell growth in children with TB, we investigated the induction of CTLs by measuring the expression of granulysin. An example of the gating strategy and the induction of granulysin after antigen stimulation are displayed in Figure 10A. In a next step children that were diagnosed with TB regardless of therapy were compared to children with a NTM infection and healthy controls (Fig. 10B). For both, TB ($p < 0.001$) and NTM ($p < 0.01$) infected children a significant induction of granulysin in antigen-experienced T cells could be detected after being stimulated with PPD. As expected, only in children with TB granulysin expressing T cells could be significantly induced ($p < 0.001$) after stimulation with ESAT6-CFP10. Age-dependent differences in granulysin expression after PPD or ESAT6-CFP10 stimulation could not be detected. Stimulation with Ag85A or B did not reveal significant differences between the three study groups. To ensure the antigen specificity recombinant proteins from *T. cruzi* (Chagas), human cytomegalovirus (HCMV) and Epstein Barr Virus (EBV) were used. As expected the stimulation with the *T. cruzi* antigen did not result in detectable granulysin expression. In the group of TB patients as well as in the healthy controls granulysin induction could be shown after stimulation with EBV and HCMV antigen in some of the participants. In a next step we grouped children with *M. tuberculosis* infection in consideration of their clinical status (Fig. 10C). Both children with active and latent infection showed a significant increase in granulysin expressing memory T cells after PPD and ESAT6-CFP10 stimulation. The same induction could be detected in children that underwent antimicrobial therapy.

Table 3: Donor characteristics

	LTBI	LTBI after therapy	active TB patients	TB patients after therapy	NTM-infected children	healthy controls
Total number	**9**	**17**	**7**	**9**	**5**	**13**
Male	5	9	3	6	4	9
Female	4	8	4	3	1	3
Age range years (Median)	2 – 17 (13)	1 – 15 (9)	1 –16 (14)	1 – 16 (6)	2 – 13 (7)	1 – 15 (9)

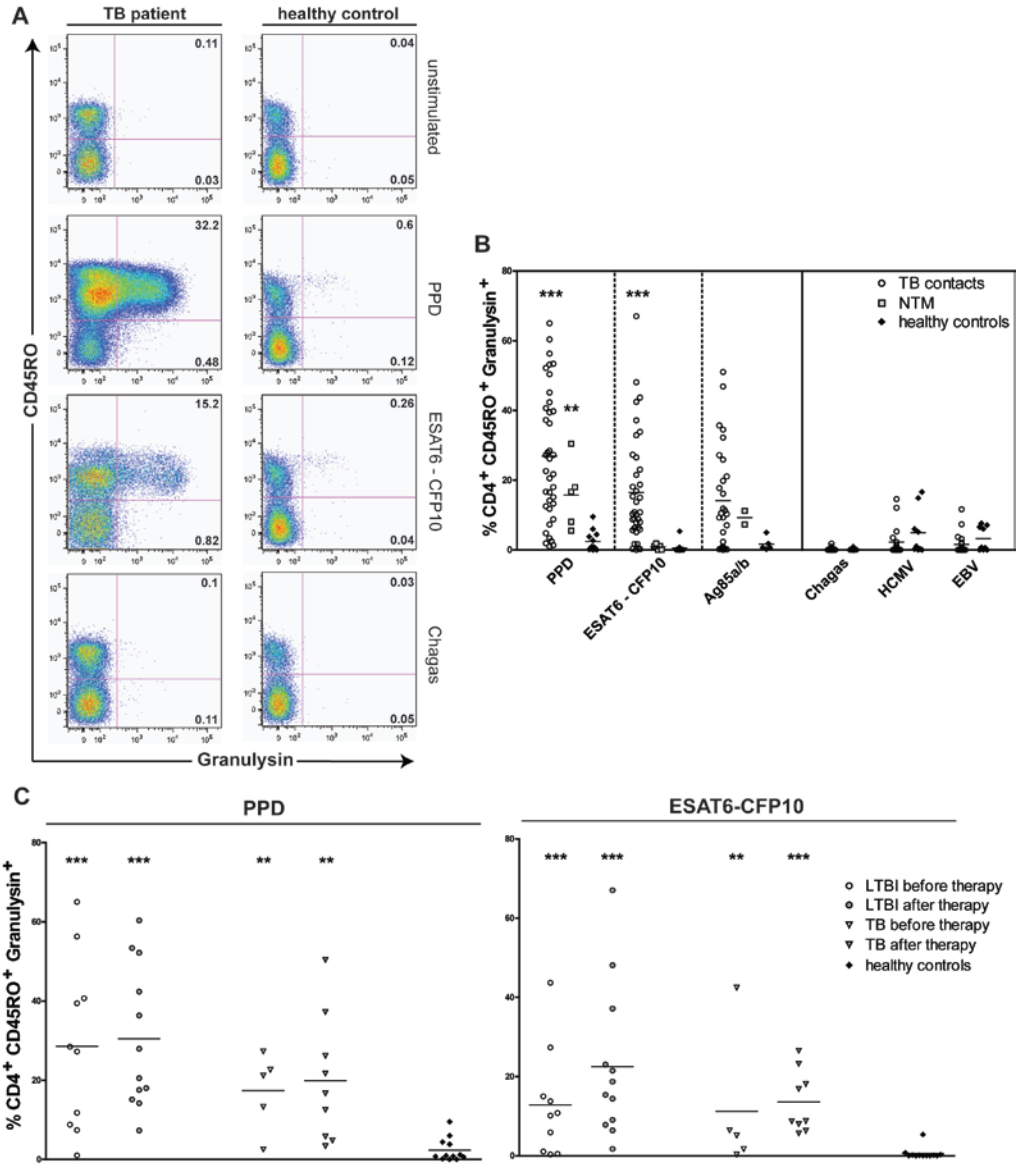


Figure 10: Antigen-specific induction of granulysin in CD4+ CD45RO+ memory T cells after 7d in vitro assay.

A Representative pseudo-color plot showing expression of CD45RO and Granulysin of $CD4^+$ T cells from patients with active TB (left) and a healthy control (right) after 7d restimulation assay. As described before, PBMCs were incubated in medium alone (unstimulated), stimulated with PPD, ESAT6-CFP10 or recombinant *T. cruzi* TcF Antigen (Chagas). **B.** Granulysin expression is depicted for each stimulus as percentage of $CD4^+$ $CD45RO^+$ memory T cells. Frequencies of 38 TB experienced patients (LTBI and TB patients before and after therapy) (circles), 5 NTM infection (squares), 12 healthy controls (diamonds) are shown. As stimulus PPD, ESAT6-CFP10, Ag85a/b, recombinant T. cruzi TcF Antigen (Chagas), recombinant cytomegalo-virus antigen Pp65 (HCMV) and recombinant Epstein-Barr-Virus antigen EBNA-1 (EBV) were used as antigens. **C.** TB experienced patients were subdivided in different groups. Percentage of granulysin expressing $CD4^+$ $CD45RO^+$ memory T cells from 10 LTBI before therapy (clear circles), 12 LTBI after therapy (grey circles), 5 patients with active TB (clear triangles), 9 TB patients after therapy (grey triangles) and 12 healthy controls (black diamonds) are shown. Individual background values of unstimulated controls were subtracted. Means are shown and significance between different patient groups versus healthy controls are indicated by asterisks (Mann-Whitney-U test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2.2 Granulysin expressing cells show central and effector memory-like phenotype

In previous experiments we have demonstrated, that CD45RO$^+$ memory T cells contribute to the vast majority of granulysin expression after antigen stimulation. To provide evidence, that the high ratio of granulysin positive cells is due to proliferation of antigen specific memory T cells, a proliferation assay was performed. Isolated PBMCs from children infected with *M. tuberculosis* were incubated with CFSE and analyzed with the previously described 7d *in vitro* assay. With the help of flow cytometry this sequential loss of fluorescence can be displayed as distinct peaks. As demonstrated in Fig. 11, stimulation with PPD and ESAT6-CFP10 led to strong proliferation of CD45RO$^+$ memory T cells and these cells were the main producers of granulysin after antigen stimulation. As a positive control Phytohemagglutinin (PHA) was used, which induced proliferation but almost no induction of the cytotoxic protein.

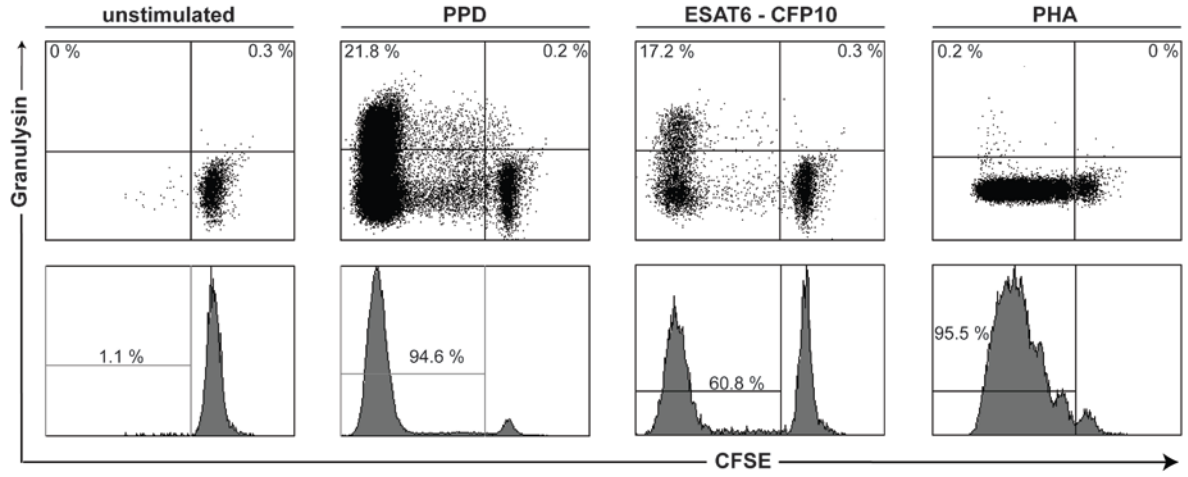


Figure 11: Antigen-specific induction of granulysin exclusively in proliferating CD45RO$^+$ memory T cells after 7d *in vitro* assay.

Representative dot plots (top) and histograms (bottom) of TB infected children show frequencies of $CFSE_{high}$ and $CFSE_{low}$ populations and percentages of CD4$^+$ CD45RO$^+$ T cells expressing granulysin for each stimulus. As positive control (far right) cells were incubated with 5µg/ml Phytohemagglutinin (PHA). Results shown are representative of six independent experiments.

To further characterise the phenotype of the granulysin expressing T cells in children, we used the two surface markers CD45RA and CCR7, which can be used to differentiate not only naïve from memory T cells, but also to subdivide the memory T cell compartment into central memory T cells (T_{cm}) and effector memory T cells (T_{em}) [59] . These two subpopulations harbour different effector functions. While T_{cm} mostly produce IL-2, T_{em} are characterized by rapid expression of effector functions and IFNγ expression. Both populations are negative for the expression of CD45RA but differ for CCR7. While T_{cm} can be stained for CCR7 on the surface, T_{em} are negative for this lymph node homing marker. Naive

T cells express both proteins on their surface. The memory phenotype of granulysin expressing memory $CD4^+$ T cells, to our knowledge, has not been investigated so far. Co-staining for CD45RA, CCR7 and granulysin was performed on samples from children with *M. tuberculosis* infection and, granulysin expressing cells were grouped accordingly to the expression of these two surface markers (Fig. 12). As expected from previous experiments using CD45RO to differentiate, almost no granulysin expressing cells could be detected in the naive T cell compartment. Interestingly, the proportion of effector- and central-memory like T cells that expressed granulysin was almost equal after PPD stimulation. After stimulation with recombinant ESAT6-CFP10 protein significantly more effector memory-like T cells expressed granulysin compared to central memory like T cells.

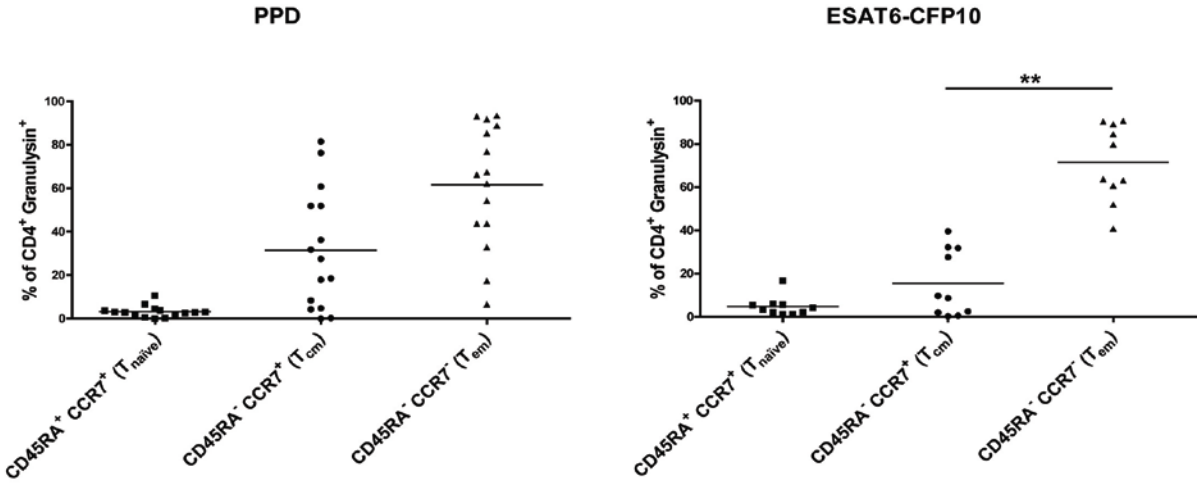


Figure 12: Characterization of the memory phenotype of granulysin expressing $CD4^+$ T cells in children.

PBMCs of children with TB were stimulated in a previously described 7d *in vitro* assay with PPD (left) or ESAT6-CFP10 (right). Subsequently, cells were stained for granulysin, CD45RA and CCR7 and granulysin positive cells were grouped based on distribution of the surface markers. Means are shown for each group and significance between T_{cm} and T_{em} are indicated by asterisks (Wilcoxon signed rank test ** $p < 0.01$).

3.2.3 Granulysin expression is highly inducible in patients that completed therapy

Since we could detect similar frequencies of granulysin expressing T cells in the memory compartment of children that had been treated for an active or latent form of *M. tuberculosis* infection when compared to untreated children, we wanted to know how long after the completion of antimicrobial treatment granulysin expressing T cells could be found. As a comparison we included IFNγ, which is used in the diagnosis of *M. tuberculosis* infection in children (see Introduction) and has been shown in animal models to be crucial for the control of *M. tuberculosis* [206,320,321] . Since no significant differences between the study groups of treated children with an active TB and treated children that had been latently

infected could be detected, we combined both groups to increase the total number of patients for this analysis.

While during the first 20 months after treatment no significant difference in IFNγ and granulysin expression after stimulation with PPD or ESAT6-CFP10 could be detected, a differential induction of these two proteins could be detected at a later time point for both stimulations (Fig. 13A). After PPD stimulation granulysin expression could be seen by in average 36%±16 of $CD4^+$ $CD45RO^+$ T cells, at the same time the frequencies of IFNγ expressing T cells decreased to 10%±5 in children that had finished treatment more than 20 months ago. Same holds true for ESAT6-CFP10 stimulation where we could detect differences in granulysin expression of $CD4^+$ $CD45RO^+$ T cells compared to IFNγ expressing T cells with an average frequency of 22%±21 and 6%±7, respectively.

Since the simultaneous staining for IFNγ and granulysin revealed particular co-expression of both molecules (data not shown), we questioned how the frequencies of the three possible subpopulations behave over time (Fig. 13B). While the frequency of memory T cells only expressing IFNγ remained stable over time, the proportion of T cells co-expressing IFNγ and granulysin was declining. The majority of T cells expressing granulysin only and the contribution of this subpopulation to the total response increased significantly over the measured period of time. These data indicate a shift from double-positive T cells towards long-lived single granulysin expressing T cells over time after treatment.

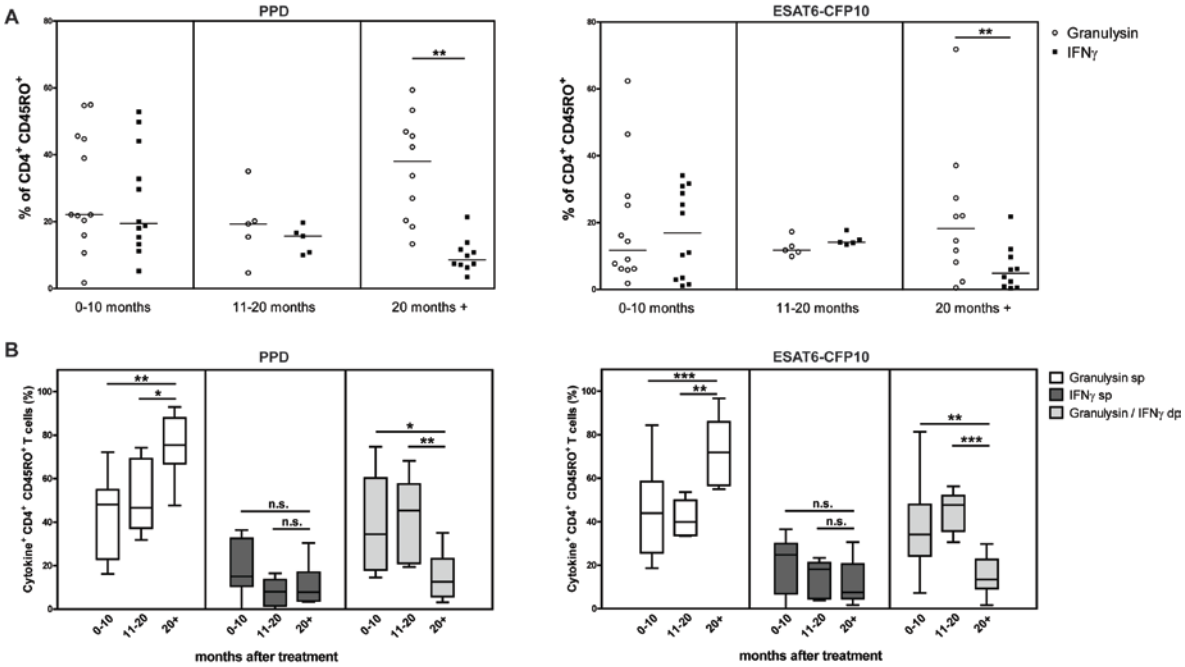


Figure 13: Antigen-specific induction of granulysin and/or IFNγ in CD45RO$^+$ memory T cells of treated patients after different time points.

A. Expression of granulysin (white circles) and IFNγ (black squares) of CD4$^+$ CD45RO$^+$ memory T cells in PBMCs of treated children after different time points upon stimulation with PPD (left) or recombinant ESAT6-CFP10 (right). Individual background values of unstimulated controls were subtracted. Medians are shown for each group and significance is indicated by asterisks (Wilcoxon signed rank test ** $p < 0.01$). **B.** Graph represents percentage of total CD4$^+$ CD45RO$^+$ memory T cell response expressing granulysin alone (Granulysin sp (white bar)), IFNγ alone (IFNγ sp (grey bar)) or both (Granulysin/IFNγ dp (light grey bars)) after stimulation with PPD (left) or recombinant ESAT6-CFP10 (right). The median is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. Significance between different groups are indicated by asterisks (Mann-Whitney-U test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2.4 Phenotyping of antigen-specific granulysin expressing CD4$^+$ T cells

To further understand the characteristics of granulysin positive T cells in children, we stained for a set of surface and intracellular markers of activation and cytotoxicity (Fig. 14). Stenger et al. [322] have demonstrated that CD4$^+$ T cells of PPD$^+$ adults, when stimulated with a crude cell wall extract of *M. tuberculosis*, express high levels of chemokine (C-X-C motif) receptor 3 (CXCR3) and chemokine (C-C motif) receptor 5 (CCR5). We could confirm these findings for CXCR3 in children not only after stimulation with the protein derivate of *M. tuberculosis* (PPD) but also with a single recombinant fusion protein (ESAT6-CFP10). The expression of CCR5 on the other hand was not significantly up-regulated on cells expressing granulysin. It was also reported that the expression of CD154 (CD40L) could be used to specifically identify antigen-induced CD4$^+$ T helper cells [323] . This finding could also be demonstrated in granulysin expressing T cells. In the context of T cell activation markers we also measured for the expression of CTLA-4, which is induced after TCR ligation and CD28 costimulation [324] and HLA-DR, which has been described to distinguish activated T cells from resting ones [325] . Another marker that has been described as being only present on

activated T cells is IL-2 receptor (CD25) [326] . The induction of all three markers was also present in granulysin positive memory T cells (data for CD25 not shown). Since granulysin is only one of several cytotoxic markers of human CTLs, we investigated the presence of further proteins associated with cytolytic activity. As mentioned, CD107a/b (Lysosomal-associated membrane protein -1/ -2) can be used as a marker of degranulation [292] . Granzymes are found abundantly in lytic granules and induce apoptosis by a caspase-dependent or –independent pathway [156] . The other cytotoxic granule, whose expression we measured, was perforin. After release by exocytosis perforin forms pores in the cell membrane of the target cell and allows granzymes and granulysin to enter the cell [175] . All three markers associated with cytotoxicity and degranulation, CD107a/b, granzyme B and Perforin were significantly upregulated in granulysin positive $CD4^+$ memory T cells in children.

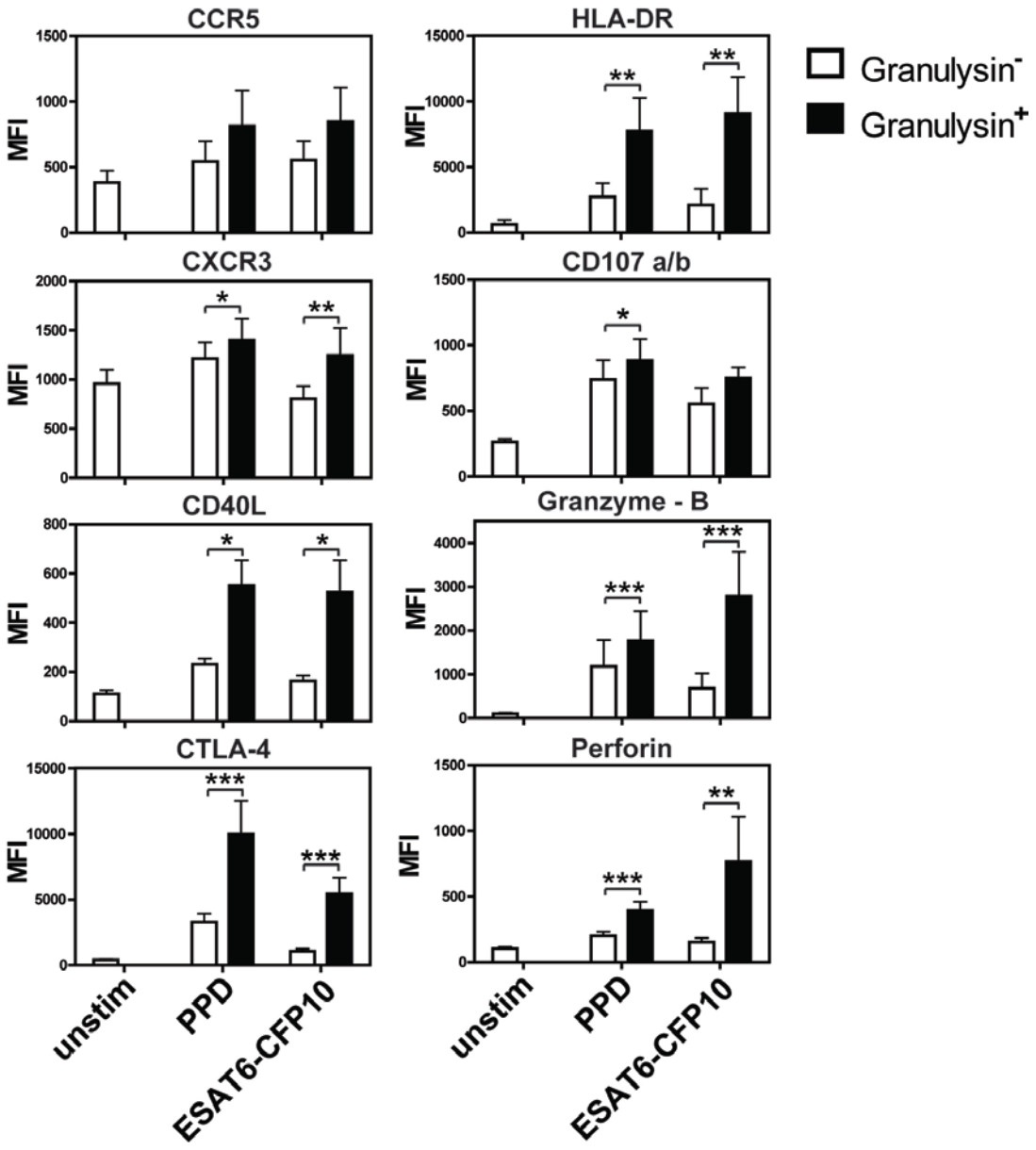


Figure 14: Measurement of the median fluorescence intensity (MFI) of different surface and cytotoxic marker in the granulysin expressing CD4$^+$ CD45RO$^+$ memory T cell compartment.

2x10^5 PBMCs from patients infected with *M. tuberculosis* were stimulated with PPD, recombinant ESAT6-CFP10 or left unstimulated using the previously described 7d *in vitro* assay. Median fluorescence intensity of shown markers was measured in CD4$^+$ CD45RO$^+$ memory T cells and compared between cells expressing granulysin upon stimulation (black bar) and the granulysin-negative population (white bar). Background levels of expression are shown in the unstimulated controls. Due to limitations in patient material different numbers of patients were included in the analysis (CCR5 n=9; CXCR3 n=10; CD40L n=7; CTLA-4 n=15; HLA-DR n=8; CD107 a/b n=12; granzyme-B n=21; Perforin n=12). Bars indicate arithmetic mean and the error bars above represent standard error. Significance between granulysin positive and negative T cells is indicated by asterisks (Wilcoxon signed rank test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2.5 Granulysin expressing T cell lines show non-polarized phenotype

One of the critical limitations of intracellular flow cytometry assays is the need of fixation and permeabilization of the cells prior to measurement (see Methods section). This makes it impossible, for example, to sort out T cells expressing granulysin antigen-specifically for further functional characterization. To overcome this problem we designed a panel of surface markers for fluorescence activated cell sorting, which allowed us to sort these cells without any need of fixation/permeabilization. The recovered cells could then be used for further testing.

After stimulating PBMCs with PPD using the previously described 7d *in vitro* assay, cells were stained without fixation and permeabilization and collected via a cell sorter. Due to the necessity of repeated bleedings over several weeks for re-stimulation and further assays PBMCs from an adult LTBI were used. Beforehand, the expression of surface markers by granulysin positive T cells of adults, were tested and led to consistent results compared to children with TB (data not shown). Lymphocytes were gated by their properties of size and granularity and target cells were identified by the combination of the markers: $CD3^+$ $CD4^+$ $CD8^-$ $CD25^+$ $CXCR3^+$. To estimate the expected ratio of granulysin expressing cells, in parallel, a small proportion of the cells were fixed and permeabilized for a normal intracellular cytokine staining (ICS). This could show that around 40% of all memory T cells sorted by this method are positive for granulysin (data not shown). Subsequently a serial dilution of 0.3/0.5/1 cell per well was performed. Out of 120 wells for each dilution, one cell line could be expanded out of the 0.3 cell per well dilution (0.8%), eight out of the 0.5 cell per well (6.7 %) and 17 out of the one cell per well dilution (14.1%). After further expansion a total of 14 T cell lines were checked for their antigen-specificity and their ability to express cytokines after stimulation with PPD for 5 days (Fig. 15). A stimulation period of 5 days showed best induction of granulysin expression in T cell lines (data not shown). As a polyclonal stimulation, cells were separately stimulated with PMA and Ionomycin on day four. Lines were grouped according to their median fluorescence intensity (MFI) after PPD stimulation. The lines responding by high granulysin (MFI over 750) expression after 5d stimulation with PPD (line 2, 5, 14, 16, 17) were grouped as “granulysin high”, T cell lines showing a very low expression (MFI lower than 750) of granulysin upon stimulation with PPD (line 1, 3, 10, 12, 15, 18, 19, 21, 25) were named “granulysin low”. In addition to PPD for antigen-specificity and PMA + Ionomycin as a polyclonal control, cells were also incubated with TcF antigen from *T. cruzi* to check the reactivity of the T cell lines towards an unrelated antigen. In a last experiment, T cell lines were left unstimulated to measure the background levels of cytokine expression produced by these lines.

This could show that, except for one line in the group of granulysin high expressing lines, all tested T cell lines showed low background expression of granulysin. As an exception T cell line 16 showed even without stimulation a very high expression of granulysin. The stimulation with the TcF antigen from *T. cruzi* led to almost no increase of granulysin

expression. The T cell line 16, which already showed high background induction, was also highly positive after stimulation with TcF, but in contrary to the stimulation with PPD the expression level after Tcf stimulation did not increase compared to the unstimulated control. Therefore no unspecific induction to a non-related antigen could be detected for any of the T cell lines. Polyclonal stimulation with PMA and Ionomycin led to no further induction of granulysin expression in any of the T cell lines. This data goes in line with the findings of the 7d *in vitro* assay, where also no granulysin induction in $CD4^+$ $CD45RO^+$ memory T cells could be detected after PMA and Ionomycin stimulation (Fig. 10). Simultaneous measurement of IFNγ after PMA and Ionomycin stimulation demonstrated a strong induction of IFNγ expression in most T cell lines, indicating that T cell lines are generally able to respond to polyclonal stimulation (data not shown). No changes in granulysin expression after stimulation could be detected, indicating no interference by the feeder cells.

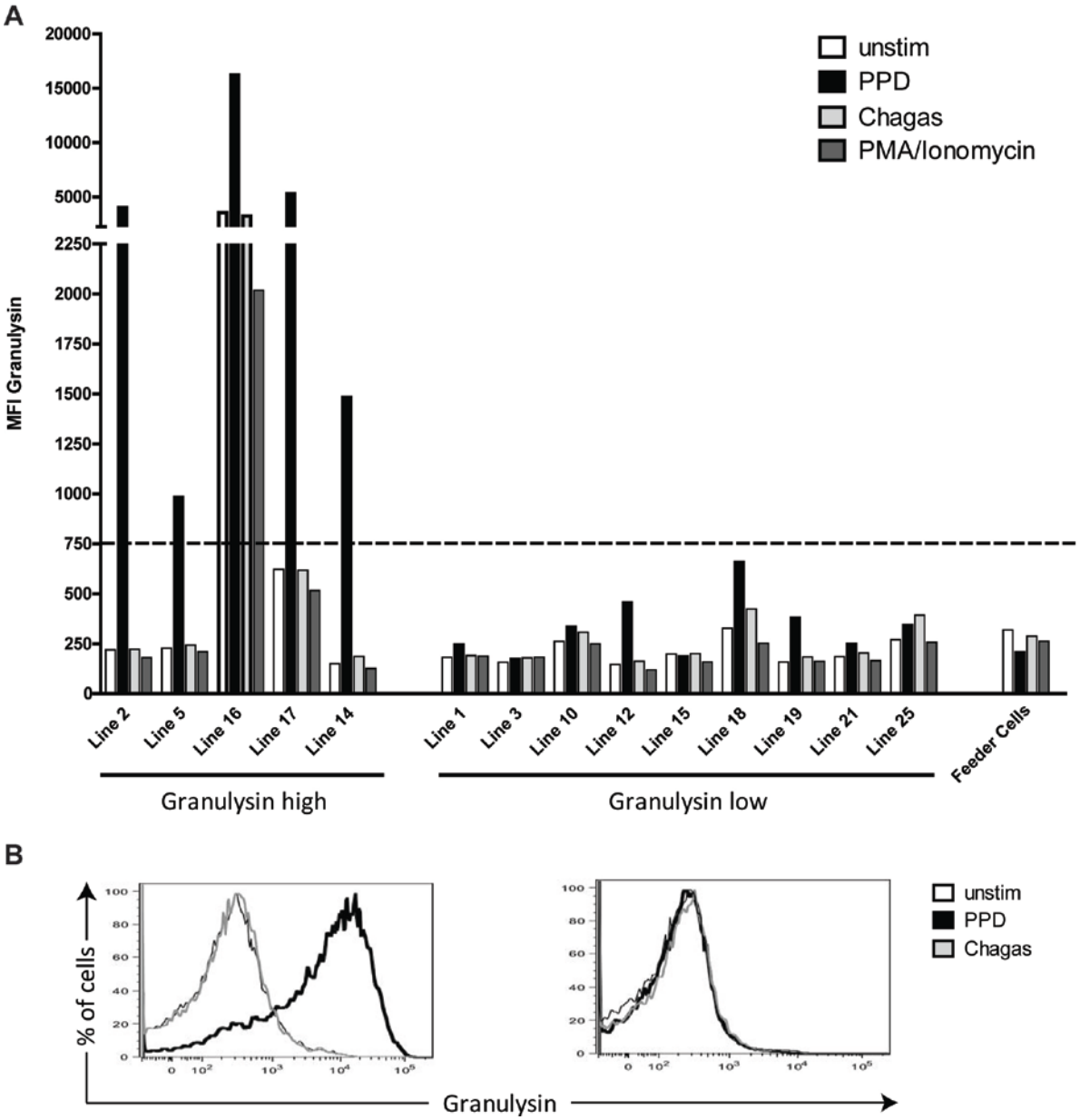


Figure 15: Median fluorescence intensity (MFI) of T cell lines after 5 day *in vitro* stimulation.

A. 1x10^5 T cells from 14 different T cell lines were either left unstimulated or incubated with PPD (10µg/ml), TcF antigen from *T. cruzi* (5µg/ml) for five days or PMA (10ng/ml) + Ionomycin (1µg/ml) on day four. 1x10^5 irradiated autologous PBMCs (feeder cells) (30 gray) from the same donor were added as antigen presenting cells. MFI of granulysin expression of CD4$^+$ CD45RO$^+$ memory T cells are shown. T cell lines with a MFI above 750 (dotted line) upon PPD stimulation were classified as "granulysin high", otherwise T cell lines were grouped as "granulysin low". Dotted line indicates MFI threshold for grouping. Expression of cytokines by feeder cells alone are shown as well (right). **B.** Representative MFI histogram for granulysin expression of CD4$^+$ CD45RO$^+$ T cells without stimulation or after stimulation with PPD (10µg/ml) or TcF Antigen from *T. cruzi* (5µg/ml) for 5 days. Histograms display line 17 (granulysin high, left) and line 1 (granulysin low, right).

Till now we were only able to detect expression on the protein level. After the establishment of T cell lines we could not only investigate cytokines on mRNA expression level, but also activation of transcription factors. This could be done using the quantitative real-time polymerase-chain-reaction (qPCR) technology.

Due to limitations in growth of the T cell line, for this set of experiments two lines were chosen that showed high expression of granulysin after PPD stimulation, namely T cell line 2 and 16, and one line with low frequency of granulysin-positive cells (line 1). Cells were left unstimulated, stimulated with PPD for five days or polyclonal stimulated with PMA and Ionomycin on day four In all cases irradiated autologous PBMCs were used as antigen presenting cells (APC).

For QPCR different clusters of primers were used. The first cluster included primers representing genes associated with CTLs. EOMES as well as T-bet are members of the T-box family of transcription factors and play an important role in T-cell biology [71,327] . So far both have been described to be required for normal CTL differentiation in $CD8^+$ T cells. Lack of both transcription factors leads to an induction of interleukin-17-secreting lineage [328,329] . It also has been demonstrated that EOMES upregulates the transcription of perforin and promotes differentiation of effector cytolytic $CD8^+$ T cells [330] . Moreover it was suggested that the expression of EOMES is restricted to $CD8^+$ and NK cells and therefore only little is known about the role of EOMES in $CD4^+$ T cell development [331] . It is expressed in very low levels in naïve $CD4^+$ T cells but seems to be able to compensate T-bet deficiency for IFNγ and T_H1 development in mice [332] . The role of this transcription factor in the context of $CD4^+$ CTLs has not been investigated yet. In contrast the role of T-Bet in $CD4^+$ T cells has been investigated quite intensively. It is considered the master regulator of the differentiation into the T_H1 lineage, which is characterized by IFNγ and TNFα expression [333,334] . As the main transcription factor of T_H2 cells the expression of trans-acting T cell-specific transcription factor GATA-3 (GATA-3) was investigated. Simultaneously, the transcription level of IL-4, one major cytokine of T_H2 T cells, was measured. GATA-3 has been shown to promote T_H2 responses by induction of cytokines like IL-4, -5 and -13, induction of growth of the compartment of T_H2 cells and also inhibition of T_H1 differentiation [335] . Quite recently retinoic acid-related orphan receptor gamma t (RoRγt) was identified as the responsible transcription factor for T_H17 T cell differentiation [336] . Using our setup, transcription level of this factor was reviewed as well as the cytokine IL-17 itself. Finally we checked IL-10 transcription, since it was previously described to be expressed in T_H1 and T_H2 T cell clones [337] .

Results are shown in Table 4 and summarized as a heat map in Figure 16.

As expected, both transcription factor EOMES and T-Bet were highly upregulated in T cell line 2 and 16. In these two lines that showed high expression of granulysin in the FACS experiment, EOMES was upregulated by 12- and 15-fold, T-bet expression was differently increased by 17-fold in line 2 and by six-fold in line 16. Consequently the expression of granulysin, perforin and IFNγ was upregulated with respect to the unstimulated control. For

granulysin, this goes in line with the data collected using flow cytometry. In T cell line 1, which was used to represent the group of lines with low expression of granulysin after PPD stimulation in FACS, no up-regulation of the transcription factors EOMES and T-Bet could be detected. In contrast granulysin, perforin and IFNγ transcripts were slightly induced.

Like T-bet and EOMES, GATA-3 was highly up-regulated in the T cell lines 2 and 16 by 9.6- and 12.1-fold respectively and remained unchanged in the case of line 1. This was accompanied by an up-regulation of expression of IL-4 and IL-13 in the "granulysin high" T cell lines. In line 1 an increase of IL-4 gene transcription of five-fold could be demonstrated as well.

The measured transcription of RoRγt revealed an up-regulation of this factor in T cell line 2 and 16. The associated cytokine IL-17 was up-regulated in line 2 by 4.1-fold and down-regulated by 6.6-fold in line 16. In T cell line 1 both genes underwent no changes in expression after PPD stimulation.

The measurement of IL-10 showed a down-regulation on transcriptional level in all tested lines ranging from 42.2-fold (line 2) to 4.3-fold (line 1).

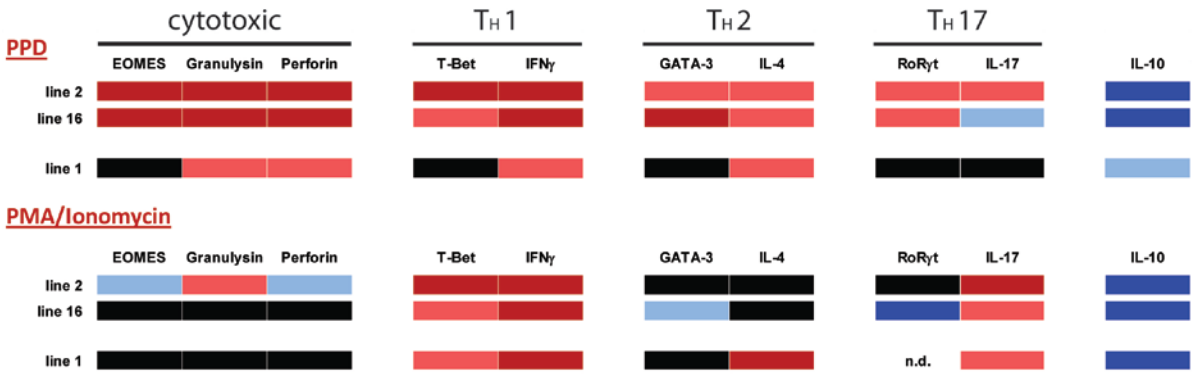


Figure 16: Heat map of quantitative real-time PCR (QPCR) results of T cell lines after stimulation with PPD or PMA + Ionomycin.

$1x10^5$ T cells were stimulated with $1x10^5$ irradiated PBMCs (30 gray) from the same donor for 5 days. Data are displayed as a heat map. Upper two lines representing T cell lines with high expression of granulysin (see Table 3) (line 2+16). The last row (line 1) is showing the fold changes for a T cell line that presented low expression of granulysin. Fold changes to unstimulated controls were individually calculated using ΔΔCt method [294] . As control gene GAPDH was used. Fold changes were color coded as followed: upregulation above 10 fold (dark red), upregulation between 10 fold and 2 fold (light red), genes not regulated (black), down regulation between 2 and 10 fold (light blue) and down regulation by more than 10 fold (dark blue). RoRγT could not be detected (n.d.) in T cell line 1 after stimulation with PMA and Ionomycin. Raw data are presented in Table 4.

The polyclonal stimulation with PMA and Ionomycin led to a complex picture in the context of genes associated with CTLs. Transcription of EOMES was down-regulated in T cell line 2 and otherwise not changed upon stimulation. T cell line 2 was also the only line showing increase in granulysin transcription by 2.4-fold, but an opposite effect could be seen for perforin which was down-regulated by 2.9-fold. In line 1 and 16 no change in expression of

any of the two genes could be detected. As expected, based on the FACS data, PMA and Ionomycin stimulation induced high IFNγ expression. Consequently the expression of T-Bet in all three T cell lines was increased. Genes associated with T_H2 polarization were partly influenced by the stimulation. While GATA-3 was downregulated by 3.3-fold in line 16, the transcription of IL-4 was up-regulated by 16-fold in line 1. The transcription factor RoRγt showed a high reduction in expression after polyclonal stimulation in T cell line 16, but remained unchanged in the other "granulysin high" T cell line. In T cell line 1, transcription of this transcription factor could not be detected. On the other hand, gene transcription of IL-17 was up-regulated in all three T cell lines. As it was shown after stimulation with PPD, the gene expression of IL-10 was strongly decreased after stimulation with PMA and Ionomycin.

In summary granulysin expressing T cell lines showed ability to induce transcription factor of different distinct T cell subsets. Consistent with the induction of transcription factors of T_H1, T_H2 and T_H17 subsets, transcription of associated cytokines was induced as well. On the contrary the gene expression of IL-10 was strongly downregulated. After stimulation with PMA and Ionomycin we could detect an activation of the transcription factor T-bet, which was not associated with induction of EOMES, another member of the T-Box family. Also, the nonexistent induction of the cytotoxic proteins granulysin and perforin after polyclonal stimulation indicates a direct association with the transcription factor EOMES, which is independent of T-Bet.

Table 4: Fold changes by real-time PCR (QPCR) of T cell lines after stimulation with PPD or PMA + Ionomycin compared to unstimulated samples.

PPD	cytotoxic			T_H1		T_H2		T_H17		
	EOMES	Granulysin	Perforin	T-Bet	IFNγ	GATA-3	IL-4	RoRγt	IL-17	IL-10
line 2	11.63	74.55	10.17	16.55	53.91	9.66	3.96	5.38	4.12	-42.19
line 16	14.63	42.25	19.53	5.90	26.39	12.10	2.14	2.38	-6.57	-10.93
line 1	-1.04	4.73	2.72	1.49	5.00	1.14	4.26	-1.58	-1.65	-4.33

PMA + Ionomycin										
	EOMES	Granulysin	Perforin	T-Bet	IFNγ	GATA-3	IL-4	RoRγt	IL-17	IL-10
line 2	-4.29	2.35	-2.87	10.67	34.99	-1.76	1.60	1.74	58.32	-24.91
line 16	-1.77	-1.64	-1.40	7.61	63.90	-3.30	1.99	-12.06	2.40	-29.57
line 1	-1.86	1.09	1.16	6.88	65.87	1.58	16.05		3.38	-53.94

3.2.6 Antigen-specific cytotoxic T cell lines expressing granulysin reduce viability of *M. tuberculosis* in infected target cells

To determine if the increased expression of granulysin also reflects in an improved inhibition of intracellular growth of *M. tuberculosis*, we infected autologous monocytes with *M. tuberculosis* H37Rv. After 24 hours incubation with the mycobacteria, cells were extensively washed to remove extracellular bacteria and T cells were added. For this experiment T cells from three lines that presented low expression of granulysin after PPD stimulation (lines 3, 5 and 21) and three lines with high frequencies of granulysin expressing cells (line 2, 16, 17) were chosen. T-cells were mixed with the target cells at a ratio of 10:1 and incubated together for additional 24 hours. As a next step cells were lysed and plated on 7H11 agar plates and the colony forming units (CFUs) were counted 4 weeks later (Fig. 17A).

T cell lines that had been demonstrated to express high amounts of granulysin after PPD stimulation ("granulysin high") decreased bacterial burden by around 43 percent when compared to infected monocytes without co-cultivation of T cells. This effect could not be seen in monocytes incubated with granulysin low expressing T cell lines.

These results could be confirmed in macrophages as well (Fig. 17B). For this experiment the [^{3}H]-uracil incorporation assay was used. This assay is a highly sensitive method to determine number of viable mycobacteria over time [338] . It is based on incorporation into mRNA during transcription of viable dividing cells. Measured counts per minute (cpm) in uninfected cells display the amount of [^{3}H-Uracil] by the target cells and T cell lines alone. Differences in cpm in comparison to infected cells directly correlate with the number of live bacteria actively growing during incubation. Since T cell lines were selected on the reactivity with PPD we co-cultivated infected cells with PPD to optimize cytotoxicity. *M. tuberculosis* showed a decrease in intracellular growth when co-cultivated with granulysin high expressing T cells (line 2, 16). Growth was reduced by an average of 37 percent. When co-cultivated with T cells from a granulysin low expressing T cell line (line 10) no difference in growth compared to macrophages alone could be detected.

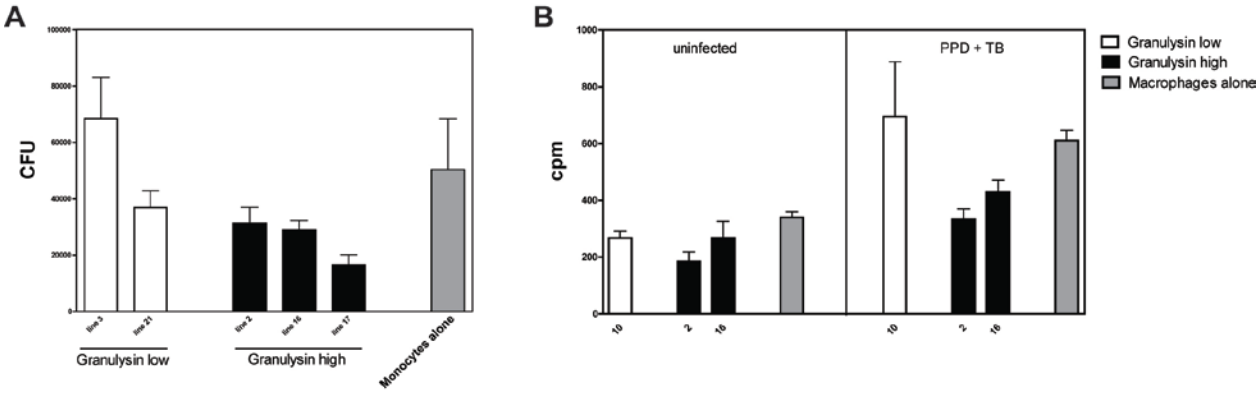


Figure 17: Effect of granulysin high- and low-expressing T cell lines on intracellular growth of *M. tuberculosis*.

A. $1x10^4$ autologous monocytes were infected with *M. tuberculosis* H37Rv (MOI 5) and incubated for 24h. Afterwards monocytes were incubated alone (grey bar), or with $1x10^5$ T cells from different T cell lines (Effector:Target ratio 10:1). Two different lines representing granulysin-low expressing (white bars) and three granulysin-high expressing (black bars) T cell lines were used. Colony forming units (CFUs) in cell lysates after additional 24 hours of incubation were measured 4 weeks later, as described in materials and methods. Mean of duplicates and serial dilutions were calculated with standard error of the mean (SEM) and plotted. **B.** $1x10^4$ autologous macrophages were infected with *M. tuberculosis* H37Rv (MOI 5), co-cultivated with PPD (10µg/ml), labelled with [^{3}H]-Uracil and incubated for 24h. Afterwards macrophages were incubated alone (grey bar), or with $1x10^5$ T cells from different T cell lines (Effector:Target ratio 10:1). One granulysin-low expressing (white bars) and two granulysin-high expressing (black bars) T cell lines were used. After additional 24h cells were fixed, harvested and scintillation counts were measured. Mean of biologic triplicates were calculated with standard error of the mean (SEM) and plotted.

3.2.7 Concluding remarks on granulysin expression in childhood tuberculosis

In sum, we analyzed the expression of granulysin of $CD4^+$ T cells from children with *M. tuberculosis* infection. The expression of this cytotoxic molecule was highly expressed in diseased children and absent in healthy controls. Cells showed a central- and effector-memory like phenotype and were long lived. Granulysin-positive $CD4^+$ T cells were party expressing IFNγ and were positive for several T_H1, activation and cytotoxic marker. Quantitative real-time PCR of granulysin expressing T cell lines revealed an upregulation of cytotoxic- and T_H1-trancription factors as well as T_H2 and T_H17 associated genes. In a final experiment we associated granulysin expression with the ability to reduce bacterial burden of infected target cells.

4 Discussion

Ever since Darrah et al. [76] proved that multi-functional T helper cells are crucial for vaccine-mediated immune protection against *Leishmania major*, the question has been whether this also holds true for the deadliest bacterial infection of all, TB. The need of new and better vaccines against TB is undeniable and progress in this field is urgently required [224] . Since *M. tuberculosis* is an intracellular pathogen, a vaccine will be successful only with an efficient induction of cellular immune response [339,340] . The first part of this study summarizes the results of a series of experiments conducted to answer an important question, whether the existence of multi-functional T cells in the immune response of LTBI protects them from developing active TB. Till today suitable markers for discriminating between latent and active TB and, the functional differences that lead to the outbreak remain unknown [341] . This thesis investigates for the first time the existence of polyfunctional T cells co-expressing T_H1 cytokines and GM-CSF in both TB patients and LTBI and demonstrates changes occurring in the T cell responses in the course of drug therapy. In the following sections the detailed experiments will be discussed in the context of current literature.

4.1.1 Differences in T_H1 cytokine expression between LTBI and TB patients

Before the potential of polyfunctional T cells as possible discrimination marker for protection could be evaluated, the general antigen-induced expression of all four cytokines after short-term stimulation had to be demonstrated. Both TB patients and LTBI showed induction of IFNγ, TNFα, IL-2 and GM-CSF at all time points after stimulation with PPD and ESAT6 (Fig. 1). Even though IFNγ has been shown to remain insufficient as a correlate of protection against *M. tuberculosis* [232] , it was demonstrated to be general expressed in TB patients and LTBI after stimulation with PPD and ESAT6 [342] . This induction of IFNγ after ESAT6 and also CFP10 stimulation led to an improved diagnostic test that has increasingly replaced the classical TST. The tests called IGRA can diagnose TB in adults and to lower degree in children, but they lack the ability to differentiate between latent and active TB [343,344,345,346,347,348] . Even though IFNγ has been shown to be crucial in immune protection against *M. tuberculosis*, its expression does not lead to protection against the pathogen [206,320,321] . The differential expression of IFNγ between LTBI and TB patients has remained a controversial issue. In our flow-cytometry based assay analysis of IFNγ at a single cytokine level did not reveal differences between latent and active tuberculosis after antigen-specific stimulation (Fig. 1B, 1C). Some studies have demonstrated a relative reduction of IFNγ responses in TB patients [270,309,349] , while others, in agreement to our study, showed no differences [350,351,352,353] . On the contrary, some studies even reported elevated IFNγ expression in patients [354,355,356,357,358,359,360,361] . One

explanation for the missing correlation between the existence of IFNγ induction and the inability to eradicate TB could be down-modulation of IFNγ-mediated signaling in macrophages by mycobacterial antigens [362] . Stimulation of lymphocytes in culture is subjected to a large number of variables in the methodology. Factors like serum, incubation time and type of culture plates can interfere with the results [363] .

No difference in the frequencies of IL-2 or TNFα expressing $CD4^+$ T cells could be detected after antigen-specific stimulation with PPD or ESAT6 (Fig. 1B, C). The production of IL-2 has previously described to be decreased in active TB patients [259,260,270,309,351,353,360] . However, the adjunctive treatment with recombinant IL-2 has been shown to be ineffective. Instead it leads to an induction of $CD4^+$ $CD25^+$ T cells, with regulatory functions and may prevent efficient clearance of the bacteria [261,364,365] . Similar to IFNγ, the role of IL-2 remains uncertain, since another study demonstrated an opposite effect with a higher induction in LTBI [366] . In a third set of studies the expression of IL-2 showed no difference between the two study groups. This could be observed by us as well [361,367] . To conclude, the equal expression of IL-2 after antigen-specific stimulation reflects its importance in the immune defense in both latent and active TB.

The induction of GM-CSF in $CD4^+$ T cells, after antigen-specific stimulation, has been described by our group for the first time [263] . In mouse experiments it could be shown that the lack of GM-CSF results in a reduced T cell response and the animals were unable to control *M. tuberculosis* infection [262] . However, an over-expression of this cytokine does not lead to protection, indicating the necessity of a fine regulation during the immune response [262] . In our initial study we focused on GM-CSF expression in the context of childhood TB. The results summarized in this study are the first to show expression of GM-CSF in adults with active or latent TB after antigen-specific stimulation. The only other study describing GM-CSF expressing T cells was conducted in the context of a vaccine trial showing GM-CSF induction after vaccination with modified vaccinia Ankara-expressing Ag85A (MVA85A) in children and adolescents [235] . Consistent with our data from childhood TB, equal expression of GM-CSF in both study groups could be determined. GM-CSF was also the only cytokine with increased expression frequency in TB patients compared to LTBI after antigen-specific stimulation with ESAT6 (Fig. 1C). This difference has not been detected in childhood TB and suggests an important role of this cytokine in the immune defense against active TB in adults.

In contrast to antigen-specific stimulation, co-incubation with staphylococcal enterotoxin B (SEB) revealed significant higher frequency of IFNγ expressing T cells in LTBI compared to TB patients (Fig. 1D). This increase was restricted to IFNγ and could not be detected for any of the other three measured cytokines. This observation goes along with the findings of other groups indicating a cellular hypo-responsiveness towards mitogens in a disease associated manner [357,360] . As additional proof of general decrease of IFNγ production in TB patients and to exclude a SEB-specific reaction, a QuantiFeron - TB Gold® test was performed in a group of TB patients and LTBI (Fig. 2). Consistent with the ICS data, measurement of IFNγ in

the supernatant revealed no difference after antigen stimulation, but confirmed lower induction of IFNγ after mitogen stimulation in TB patients. This suggests that lymphocytes are qualitatively or quantitative depressed in TB patients compared to LTBI. Since this difference could not be seen after antigen-specific stimulation and no differences in $CD4^+$ T cell percentages between study groups were detected. Therefore, it seems that in active TB patients *M. tuberculosis* is affecting naive $CD4^+$ T cells and leads to depression of IFNγ production in this T cell subset.

4.1.2 Effects of antimicrobial treatment on T_H1 cytokine expression

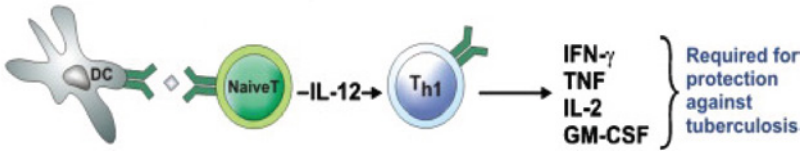
Next, we monitored changes during treatment of active TB patients on the single cytokine level (Fig. 1 B-D). After antigen-specific stimulation with PPD or ESAT6 an increase of cytokine expression of IFNγ, TNFα, IL-2 and GM-CSF over the first two months of treatment could be detected. This decreased again towards end of treatment (Fig. 1B, C). Interestingly, the time point of the highest response for IFNγ, IL-2 and TNFα differed between PPD and ESAT6 stimulation. While after PPD stimulation the frequency of $CD4^+$ T cells expressing three cytokines was highest after two months. The strongest expression of IFNγ, TNFα and IL-2 after ESAT6 stimulation could be measured already after one week of treatment. GM-CSF expressing T cells showed a similar pattern after both of the two antigen-stimulations with a peak of expression after two months of drug treatment. This induction can be explained by a better immune reaction and increased number of antigen-specific effector T cells in the first months of treatment and, a subsequent decrease to initial levels when the infection has been controlled. Memory T cells specific for ESAT6 seemed to reactivate in a faster manner and therefore express effector cytokines at earlier time points. Overall, these results emphasize the significance of all four chosen cytokines in host protection against *M. tuberculosis* infection. The importance of ESAT6-specific T cells for the immune response has been shown in several murine vaccine models [368,369,370,371] . For IFNγ such an increase under therapy has also been reported by Ulrichs et al. [321] , who described higher numbers of IFNγ expressing T cells after two months of therapy compared to untreated patients upon stimulation with ESAT6 but not with PPD. Since this study did not include further time points a conclusion about the kinetic of T cell response cannot be drawn. In the study conducted by Veenstra et al. [363] , the opposite effect was described. After start of therapy the numbers of IFNγ expressing $CD4^+$ T cells was initially higher in TB patients compared to LTBI, but normalized during treatment without an increase during the first months of treatment [363] . A different study also demonstrated an induction of both IFNγ and TNFα after two months of therapy in patients with active TB [372] . While the IFNγ expression further increased after end of treatment, the level of TNFα stayed significantly higher compared to healthy control, but did not increase further after the two months time point. This difference to our study could be explained by the usage of ELISA as the read-out system, which measures produced cytokines in an unspecific manner irrespectively of its origin. In a study performed by Ribeiro

et al. [373] , the amount of IFNγ expressing cells after stimulation with ESAT6 or CFP10 declined after end of therapy in comparison to untreated samples. Consistent with our data, the study conducted by Djoba Siawaya et al. [374] showed a significant increase of TNFα in the serum of TB patients after five weeks of treatment and normalized levels after end of treatment. This data suggest, that the short-term stimulation assay used in our study reflects the biologic processes *in vivo*.

4.1.3 Dynamic changes in CD4+ T cell cytokine profile under anti-tuberculosis drug treatment

As discussed previously, in-depth measurement of single cytokines between LTBI and TB patients under therapy could not associate any single marker with better protection against conversion to the active form of TB. Therefore, the notion is to move away from the search of a single marker towards a bio-signature, which is a combination of several markers [193] . In line with this, we used multi-color flow cytometry to integrate, for the first time, measurement of the four cytokines, IFNγ, TNFα, IL-2 and GM-CSF, in one single staining. To investigate our working hypothesis we grouped $CD4^+$ T cells according to their polyfunctionality in cytokine expression after stimulation (Fig. 3). Consistently, a proportion of $CD4^+$ T cells co-expressing all four cytokines could be detected in LTBI and TB patients after antigen-specific stimulation. This confirms the data obtained in childhood TB, where we could show that IFNγ and TNFα expressing T cells co-express GM-CSF [263] . In this previous study an association with IL-2 as a fourth cytokine expressed by GM-CSF positive multi-functional $CD4^+$ T cells in active and latent TB patients was missing. In the present work, it could be shown for the first time. The existence of IFNγ positive T cells that co-express either TNFα or IL-2 in TB patient after antigen-specific stimulation had been observed by Winkler et al. [360] previously. Due to technical limitations the simultaneous measurement of all three cytokines and the comparison to LTBI was missing [360] . The existence of polyfunctional T cells in humans in the context of TB has mostly been demonstrated in the context of vaccines [227,235,375] . The vaccine MVA85A, used in the study of Scriba et al., showed an induction of IFNγ, TNFα, IL-2 positive T cells that co-expressed GM-CSF and in addition IL-17 [235] . Hence, to date the conclusion that induction of multi-co-expressing T cells by vaccines correlates with a better protection against the infection with *M. tuberculosis* cannot be drawn. For the infection model this hypothesis is based on mouse data that correlated polyfunctional T cells expressing IFNγ, TNFα and IL-2 after vaccination with better protection against *M. tuberculosis* challenge [376,377] . In humans, all vaccine candidates are still in the first or second phase of clinical trials and years of further investigations will be needed to demonstrate this connection [224] . Nevertheless, the need of an efficient vaccine to induce a strong and long-lasting $CD4^+$ T cell response is indisputable (Scheme 12). It has to be pointed out that the data displayed in this thesis cannot answer the question, whether a high percentage of polyfunctional T cells after

vaccination might provide protection against an initial infection with *M. tuberculosis*. This has been just recently been negated by a study conducted by Kagina et al. [378] . This study demonstrates that the frequency of polyfunctional T cells after BCG vaccination does not differ between children that are protected against TB and children that are susceptible.



Scheme 12: Necessity of strong T_H1 induction for protection against *M. tuberculosis*. Modified after (Reece & Kaufmann, 2008)

Independent of their expression, the dissection of antigen-specific cytokine responses on the basis of functionality did not reveal any significant differences between active TB patients and LTBI (Fig. 3 A, B). Our data suggest therefore, that the existence of multi-cytokine-expressing T cells cannot be used as a correlate of protection against resuscitation of latent TB. Due to the lack of a reliable latency model in the mice, rabbit or guinea pig, this hypothesis could not been investigated [379] . The only animal model that resembles the different manifestations of TB quite similar to humans seems to be the non-human primates [380,381] . Unfortunately the polyfunctionality of T cells in these animals has not been further investigated at this point.

During the follow-up study of TB patients we could monitor the dynamic changes of the antigen-specific cytokine profile, as a consequence of therapy (Fig. 3 A, B). The PPD-specific quadruple positive $CD4^+$ T cells co-expressing IFNγ, TNFα, IL-2 and GM-CSF seemed to increase over the first two months of therapy before a reduced frequency could be detected after treatment. This correlates with previous data, where induction of the four cytokines could be detected when measured independently (Fig. 1 B). Also an induction of other polyfunctional subsets, co-expressing three or two measured cytokines simultaneously, within the first two months of treatment could be shown. The number of double positive T cells after PPD stimulation declined significantly at the end of therapy in comparison to the two months time point. Consistent with our working hypothesis the proportion of single positive T cells decreased significantly over time. This could also be seen after stimulation with ESAT6. This data suggest that under therapy an immune response constituting a shift from mono- to polyfunctional $CD4^+$ T cells occurs. This hypothesis is supported by the fact, that no significant loss of cytokine response can be detected in the single-cytokine analysis over time. After stimulation with ESAT6 a lowering of quadruple positive T cells could be found at the end of treatment. This can be explained by loss of TNFα expression in patients that completed therapy, which has been described in the previous analysis (Fig. 1C).

To determine the exact combination of cytokine co-expressions affected during treatment and in comparison to LTBI, we further subdivided the groups of polyfunctional T cells after PPD stimulation (Fig. 4). In line with the other data, most changes occurred during the first two months of treatment. Together with triple-positive T cells expressing IFNγ, TNFα and GM-CSF or IFNγ, TNFα and IL-2, also numbers of double positive T cells increased over this time period. At the time of the last measurements all subsets, except one population of triple-positive T cells co-expressing IFNγ, TNFα and IL-2, declined in their proportion after end of treatment. This suggests an important role of polyfunctional T cells during the time the immune system reduces bacterial burden in alliance with the anti-microbial drugs. Since the upregulated subsets almost exclusively expressed either TNFα or IFNγ or both, it is highly likely that these cells are mostly effector memory T cell (T_{em}). It has been shown in the context of TB, that the majority of cells corresponding to antigen-specific stimulation with the expression of effector cytokines like IFNγ and TNFα have T_{em} properties [263,382] . The importance of different T cell memory subsets will be discussed later. The population of multi-functional T cells expressing IFNγ, TNFα and IL-2 at the same time has been shown to be the only subset significantly upregulated during and after treatment. This suggests an increasing importance of these cells not only in the immune defense mechanism during therapy but also in the long life memory.

We next included the surface marker CD45RO into our analysis. It has been shown by us and others, that in short-term stimulation assays the vast majority of cytokines producing $CD4^+$ T cells after antigen-specific stimulation are found to be CD45RO positive [233,263,383,384] . Since naive T cells lack the expression of CD45RO the inclusion of this marker enabled us to focus only on memory induced cytokine responses [59] . As expected the frequencies of cytokine expressing T cells were higher in the memory compartment when compared to the complete $CD4^+$ T cell population (Fig. 5B). The same pattern of increased numbers of PPD-specific cytokine expressers under therapy could be confirmed. In contrast focussing on the memory T cells revealed not only higher expression of GM-CSF, but also IFNγ was upregulated in patients with active TB, after ESAT6 stimulation (Fig. 5C). Interestingly after stimulation with the superantigen SEB the lower IFNγ expression in TB patients we had seen in the total $CD4^+$ compartment could not be detected in memory $CD4^+$ T cells. This result indicates a suppression of IFNγ production in naive T cells that does not exist in memory T cells. Moreover higher induction of TNFα, IL-2 and GM-CSF was seen after SEB stimulation in memory T cells from active TB patients. This indicates a higher activation of memory T cells in patients that persist during time of treatment (Fig. 5D). When focussed on the polyfunctionality of memory T cells, the dynamic changes under therapy and in comparison to LTBI were quite similar to what was described in the former experiments (Fig. 6). This was expected since polyfunctional T cells were highly likely to be only found in the memory T cell compartment. Nevertheless, the inclusion of a memory T cell marker gave us additional information and revealed differences that would have been undetected without subdivision of the CD4 population. Our data suggest the usage of memory marker in all ICS of $CD4^+$ T cells.

The impact of further $CD4^+$ T cell subsets was investigated in an additional staining. We tested for IL-4 expressers which would indicate T_H2 involvement and also IL-17 positivity which is the characteristic cytokine of T_H17 T cells [308,385] . Both cytokines could not be detected after antigen-specific stimulation in our assays. The stimulation with SEB did not reveal different contribution of these subsets between LTBI and active TB patients or under therapy (Fig. 8). This could be explained by differences in assay conditions that would be needed for optimal induction of the expression of IL-4 and IL-17. Also the use of whole blood for stimulation instead of isolated PBMCs seems to increase proportion of IL-17 positive T cells upon stimulation. This suggests the necessity of certain co-factors that are getting lost in the process of PBMC isolation (personal communication from T. Scriba from University of Cape Town).

4.1.4 Interleukin-7 co-cultivation enhances antigen-specific T cell responses and polyfunctionality

It has been suggested by Feske et al. [386] that coincubation with low dose of Interleukin-7 (IL-7) enhances the antigen-specific memory T cell response. In their study, co-cultivation with low-dose recombinant IL-7 led to an increased IFNγ. IL-7 is a growth factor expressed by keratinocytes, bone marrow stem cells and thymic cells and also promotes B-cell survival [300] . IL-7 seems crucial for the transition from effector to memory T cells, since memory T cells fail to develop in the absence of this cytokine [53,54,387] . Also previous experiments have indicated that IL-7 is highly expressed by macrophages infected with *M. tuberculosis* [388] . This suggests that IL-7 administration during treatment or in the context of vaccine administration could lead to improved protection. A prerequisite for this would be an enhancement of cytokine expression and subsequently an increase in polyfunctionality of the $CD4^+$ T cells. Therefore, we compared cytokine responses after PPD stimulation with or without IL-7 co-cultivation of IFNγ, TNFα, IL-2 and GM-CSF. In both TB patients and LTBI this led to significant increase in all 4 cytokines (except TNFα in TB patients) (Fig. 7A). In the case of IFNγ expression these data go in line with the study by Feske et al., which was the only cytokine measured in their study [386] . TNFα levels were also shown in active TB patients [389] . In this study we could show an additional enhancement of two further cytokines that play an important role in the immune defense against *M. tuberculosis*. To verify, if this also led to an improvement in polyfunctionality and not only single cytokine expressing T cells we did co-staining for all four cytokines after PPD stimulation with or without IL-7. Exclusively quadruple-positive T cells and T cells expressing IFNγ, TNFα and GM-CSF simultaneously were upregulated under IL-7 co-cultivation (Fig. 7B).

This thesis presents for the first time the reactivity to IL-7 not only in comparison between LTBI and active TB patients, but also investigates its effect under anti-microbial drug treatment. The ability to induce higher numbers of cytokine producers by IL-7, like it was seen in newly diagnosed TB patients, was lost over time under treatment. This can be

explained by our previous data, which showed increased induction of effector cytokine producing T cells under treatment (Fig. 1-4). This argues for an upregulation of effector cells and would go in hand with the loss of IL-7 reactivity, since it has been shown that the IL-7 receptor is down-regulated on effector cells generated in response to an acute infection [55] . Taken these data into consideration, it strongly argues for the generation of a recombinant BCG vaccine co-expressing IL-7 to improve protection against *M. tuberculosis*. Moreover, since our data showed stronger induction of antigen-specific cytokine responses in TB patients, it could also be used to improve initial treatment. This idea is supported by a study performed in mice where the administration of recombinant IL-7 led to an enhanced survival of *M. tuberculosis* infected mice [390] .

4.1.5 Conclusion & Outlook

In summary, our findings show that T cell cytokine expression of $CD4^+$ T cells of TB patients compared to LTBI differs after antigen-specific stimulation. On a single cytokine level we could detect higher expression of GM-CSF in total $CD4^+$ T cells and to even higher extend when focussed on memory T cells. This emphasises the need for studies on GM-CSF expressing $CD4^+$ T cells in the context of active TB since this cytokine seems to be associated with the presence of viable bacteria. It would be interesting to investigate the role of GM-CSF expressed by T cells in the recruitment and stimulation of macrophages and granulocytes during TB. Additionally an adoptive transfer experiment in mice, wherein GM-CSF deficient T cells would be transferred into a T cell deficient recipient with subsequent challenge with *M. tuberculosis*, would be very useful. The differences in T cell responses and general clinical symptoms would be monitored and could give an insight in the importance of expression of this cytokine by T cells. In addition the possibility to negatively regulate gene expression by siRNA would be another way to investigate the role of GM-CSF produced by T cells during TB. T cells with reduced or lack of GM-CSF expression could be co-cultivated with *M. tuberculosis*-infected target cells and subsequently proliferation and cytokine expression could be monitored as well as changes in bacterial burden in the target cells.

By monitoring antigen-specific responses of patients during antimicrobial drug therapy a general increase in T_H1 cytokine expression during the first two months of treatment could be detected which decreased by the end of therapy. Detailed analysis of the cytokine profile identified an increase of polyfunctional T cells expressing a combination of at least two different T_H1 effector cytokines simultaneously as the main source of this differential expression. In a future study it would be extremely interesting to associate this effect with the sputum conversion and positive clinical outcome under drug therapy.

Stimulation with a superantigen revealed decreased IFNγ production by $CD4^+$ T cells of active TB patients. On the contrary memory T cells expressing TNFα, IL-2 or GM-CSF were detected

in higher frequencies in TB patients compared to LTBI indicating an IFNγ specific induction defect.

A very important finding of our study was that co-cultivation with recombinant IL-7 led not only to an antigen-specific increase of IFNγ, as it was described before, but also of IL-2, TNFα and GM-CSF. Till now only the administration of recombinant IL-7 in mice had been tested. In this model, survival after *M. tuberculosis* infection was significantly increased. Our data suggest the induction of T_H1 cytokines and the polarization towards polyfunctional T cells as the underlying mechanism. Based on our findings the design of a recombinant vaccine co-expressing IL-7 would be highly recommended. Since IL-7 receptor is highly expressed on memory T cells and gets downregulated on effector cells, administration of IL-7 in combination with a BCG vaccine would probably lead to an improved ratio of memory T cells. Since the induction of T cell cytokines by IL-7 was reduced in active TB patients and lost during therapy the possibility to administer IL-7 prophylacticly to immunocompromised latently infected subjects with increased risk of reactivation of TB, should be evaluated in latency animal models like in macaques.

4.2 Implication of cytolytic CD4+ T cells on childhood tuberculosis

CTLs are key players in the effector arm of the immune response that eliminates viral infections and transformed tumor cells [164,169] . Two publications from Stenger et al. [189,391] highlighted a role for CTLs also in the control of *M. tuberculosis* infection in human. CTLs and natural-killer (NK) cells can destroy their target cells via two different pathways. The first way is by engaging cell-surface death receptor, such as members of the tumor-necrosis factor receptor (TNFR), including FAS (CD95) [392] . The other way of cell-mediated cytotoxicity is the granule exocytosis pathway. The released granules contain cytolytic molecules such as perforin, granzymes and granulysin [155,189,393,394] . In the immune defense against *M. tuberculosis* the molecule granulysin plays a central role. It has been shown that granulysin directly kills extracellular *M. tuberculosis* and in combination with perforin also decreases the intracellular viability [189] . Most of the cytotoxic functions are driven by CD8 T cells. However, there are reports describing $CD4^+$ T cells with cytolytic activity in the response to mycobacterial infections [282,319,395] . Herein, we investigated the potential of granulysin expression by $CD4^+$ T cells as a marker for *M. tuberculosis* infection and its possible role for vaccination strategies. We also described for the first time, the phenotype of antigen-specific $CD4^+$ T cells positive for granulysin and emphasizing the important role of these cells during immune defense against *M. tuberculosis* in children.

In a first set of validation experiments the optimal assay to detect granulysin expressing CD4+ T cells was established. Since cytotoxic granules of activated CTLs are synthesized *de novo* after priming, the previously described short-term stimulation assay could not be used [164] . We found optimal results using a recently described assay based on two rounds of *in vitro* restimulation [290] . This assay combines a 7d long-term stimulation with restimulation 16h prior analysis. This assay revealed differences in cytokine expression, which could not be seen after shorter stimulation. Since this approach has not been used in the context of childhood TB, we used it to monitor the induction of memory $CD4^+$ T cells. After antigen stimulation with PPD and ESAT6-CFP10, all study groups of children infected with *M. tuberculosis* with or without treatment showed significant increase in the frequencies of $CD45RO^+$ memory T cells (Fig. 9B). As expected, children with an infection of NTM showed also heightened frequencies of memory T cells after PPD stimulation, due to cross-reactivity, but only background levels of CD45RO positive $CD4^+$ T cells after stimulation with ESAT6-CFP10. In healthy controls, no changes in frequencies could be measured after antigen-specific stimulation.

A possible reason for the observed induction of $CD45RO^+$ T cells over the period of seven days, could be the recruitment of naive cells to the memory compartment. The missing induction of $CD45RO^+$ memory T cells in healthy children and in children infected with a NTM after ESAT6-CFP10 stimulation argues against an unspecific effect induced by the long-term stimulation. To ensure that only cells out of the memory compartment where induced by the stimulation, we performed proliferation assays. This evidenced that only memory T cells

proliferated after stimulation with PPD (Fig. 9C) and ESAT6-CFP10 (data not shown). Summarizing, the 7d *in vitro* assay specifically induces antigen-experienced T cell memory which became granulysin producers.

ICS for granulysin expression revealed strong expression exclusively in $CD45RO^+$ memory T cells of children infected with *M. tuberculosis* (Fig. 10A+B) . Grouping all children that were infected with *M. tuberculosis* with or without treatment the induction of granulysin in memory T cells was highly significant ($p<0.0001$) compared to healthy controls (Fig. 10B). This increase remained significant when healthy controls were compared only to children that were freshly diagnosed with active or latent TB (Fig. 10C). Since the diagnosis of childhood TB remains a challenge (see Introduction), new ways for diagnosis are urgently needed [246] . Our data indicate a possible use of the 7d *in vitro* assay to confirm a potential TB in children. The idea of using flow-cytometry to diagnose TB has been investigated by different groups [396,397,398,399,400] . This revealed that identification of IFNγ and TNFα expressing cells after stimulation leads even to a higher sensitivity than the newly established IGRA in the diagnosis of TB. Therefore ICS-based assay could be soon the new standard for diagnosis TB in industrial countries. Based on our findings, the inclusion of granulysin in the testing for new diagnostic markers in an ICS-based assay in children is highly recommended. But the amount of equipment and knowledge needed for this kind of diagnosis would limit this technique to modern laboratories in industrial countries with low incidence rates of TB. Hence, we established an enzyme linked immunosorbent assay (ELISA) to measure granulysin in the supernatant (see methods section). We were able to detect granulysin in stimulated samples, but the protein concentration did not differ significantly to healthy controls (data not shown). It is possible, that cells of the innate immune system, like NK cells, also express granulysin [401] . This could mask the differences in $CD4^+$ T cell expression of granulysin that can be detected specifically in $CD4^+$ T cells by flow cytometry. At this moment the number of tested children is too low to exclude this assay as a possible diagnostic tool in labs without access to a flow cytometer but clearly requires further optimization.

As previously demonstrated, the 7d *in vitro* assay lead to strong induction of $CD45RO^+$ memory T cells (Fig. 9). Further we have shown, that granulysin expressing cells are almost exclusively CD45RO positive (Fig. 10). But till now, the proof that the proliferating memory T cells as the source of granulysin expressed by $CD4^+$ T cells has not been shown. A proliferation assay including ICS for granulysin revealed the expanded memory T cells as the major origin (Fig. 11). The existence of memory CTLs in TB has been demonstrated for CD8 T cells in mice in a previous study but not in man [402] . In this experiment, the antigen-specific CTLs showed a central memory (T_{cm}) phenotype. Staining for two discriminating markers revealed an equal contribution of central and effector memory-like T cells after PPD stimulation. But a majority of T_{em}-like $CD4^+$ T cells after ESAT6-CFP10 stimulation (Fig. 12). This difference could be explained by a better processing of the recombinant protein and therefore faster activation and transition from resting T_{cm}-like towards a T_{em}-like phenotype. These findings confirm data conducted by Bastian et al. [322] that used crude cell wall

extract of *M. tuberculosis* as stimulation. They could show that activated $CD4^+$ CTLs showed T_{em}-like phenotype.

We already discussed granulysin as a possible marker for diagnosis. In addition Di Liberto et al. [403] suggested serum levels of granulysin in children might be useful for monitoring improvement after chemotherapy. This hypothesis is based on a decrease of serum levels in children before treatment in comparison to healthy controls. This difference in serum levels of granulysin diminished after end of therapy. This effect could not be seen on the cellular level as it was done in our study (Fig. 10). This could be due to the fact, that children recruited for this study had finished treatment at various time points and a conclusion about elevated levels after a certain time point after treatment as performed by Di Liberto et al. is not possible. On the contrary, our data revealed a constant frequency of granulysin expressing memory T cells even years after end of treatment (Fig. 13A). In comparison the number of memory T cells expressing IFNγ after antigen-specific stimulation decreased over time. These data propose granulysin as an antimicrobial protein expressed by very long-lived memory T cells. This emphasises granulysin expression as a new potent tool to investigate the efficacy and long-term existence of memory T cells after vaccination or treatment. This has not yet been studied in human but two studies could be shown in cattle, that $CD4^+$ T cells highly express granulysin after vaccination with BCG [404,405] . Also the treatment with a recombinant *M. smegmatis*, co-expressing granulysin and IL-12, led to reduced CFU in the lung and increased levels of IFNγ and TNFα in *M. tuberculosis*-infected mice [406] . Based on these studies, we suggested this protein as a potent candidate for a protective biomarker. To verify this hypothesis, granulysin expressing memory T cells in stimulated PBMCs from children after vaccination or treatment would be needed to monitor and correlate these results with the protection against a primary or secondary TB at a later stage.

Interestingly the co-staining of granulysin and IFNγ revealed a proportion of memory T cells expressing IFNγ and granulysin simultaneously (Fig. 13B). Co-expression of these two key players in the immune defense against *M. tuberculosis* has been shown before in CFP10-derived clones [319] . The same could be demonstrated by Mutis et al. [407] , who detected IFNγ co-expression of T cell clones that were reactive with different mycobacterial antigens. In our study we could show for the first time the existence of such a subset in children after chemotherapy. We also noted a decrease of this subset over time towards single-granulysin-expressing memory T cells (Fig. 13B).

4.2.1 Phenotype of granulysin expressing $CD4^+$ T cells in childhood tuberculosis

The phenotype of human $CD4^+$ CTLs has not been investigated in detail. Therefore, we analyzed the expression of certain surface markers and also the co-expression of other cytotoxic proteins in granulysin-positive T cells (Fig. 14). Based on partial co-expression of IFNγ, a prominent cytokine of the T_H1 T cell subset, we investigated the expression of two

surface molecules associated with this polarization [408] . Interestingly while CXCR3 was significantly upregulated on granulysin positive T cells, CCR5 expression, mostly known for its role in the HIV entry in T cells, was not elevated [409] . This partially confirms the findings of Bastian et al. [322] , who found elevated of CXCR3 but also CCR5 after stimulation with crude wall extracts. Granulysin-positive $CD4^+$ T cells display mostly a T_{em}-like phenotype and therefore are highly activated. This could be confirmed by the increased expression of activation markers like CD40 ligand (CD40L), CTLA-4 and HLA-DR [325,410,411] . The important role of CD40-CD40L interaction between APCs and $CD4^+$ T cells in the activation of $CD8^+$ CTLs has been described previously [412] . The upregulation of this receptor on granulysin expressing $CD4^+$ CTLs has, to our knowledge, not been shown before. Next to the role of CTLA-4 as an activation marker, recent studies showed that CTLA-4 is also constitutively expressed on regulatory T cells [413] . CTLA-4 seems to have a dampening effect on T cell responses and CTLA-4 deficient mice represent a hyper-proliferative phenotype that is accompanied by massive tissue infiltration and organ destruction [35,414,415] . Interestingly, it has been shown that anti-CTLA-4 antibodies can inhibit IFNγ-production and the generation of an increased number of $CD8^+$ CTLs especially in secondary responses [416,417,418] . In the study by Hegel et al. [419] it was shown, that $CD8^+$ T cells from $CTLA^{-/-}$ mice had enhanced cytolytic function and a selective expression of Eomes, a transcription factor member of the T-box family, that will be discussed later [71,419] . Since we also detected an upregulation of this marker but also an upregulation of Eomes in granulysin expressing $CD4^+$ T cell lines (see 4.2.3), there seems to be an opposite effect in $CD4^+$ CTLs. This connection has not been described before and should be assessed in further investigations. The expression of CTLA-4 on *M. tuberculosis*-specific $CD4^+$ clones have been described previously [420] . After treatment with soluble anti-CTLA-4 antibody, which inhibits binding to CD80/CD86 molecules on APCs, a significant increase in cell-lysis could be detected. After cross-linking this molecule with antibody against another inhibitory receptor (CD85/LIR-1/ILT2) a significant reduction in specific lysis was observed. Therefore, it seems that CTLA-4 plays an important role in the control and containment of $CD4^+$ CTLs. The consequence of the blockage of CTLA-4 and other inhibitory molecules in our experimental setup has not been performed and will be investigated in further experiments.

Till now, we exclusively measured granulysin expression as marker of cytotoxicity. As previously described, further cytolytic marker are contained in the granule [169] . First, we detected higher expression of CD107a/b in granulysin positive $CD4^+$ T cells after PPD stimulation. This marker is expressed on the cell surface after activation-induced degranulation and is also necessary as a precursor of cytolysis [292] . This finding correlates with the data of Klucar et al. [319] that showed induced CD107a expression in T cell clones specific for CFP-10 from *M. tuberculosis*. Since neither Betts et al. nor Klucar et al. performed a co-staining of granulysin and CD107a/b it cannot be concluded, that these two proteins are always co-expressed. Our data for ESAT6-CFP10 stimulation would argue against it, since we could detect an equal upregulation of CD107a/b in granulysin-positive and –negative $CD4^+$ T cells upon stimulation with the recombinant protein ESAT6-CFP10. A CD107a-independent

delivery of granulysin to the outer cell space seems to be possible and will be addressed in further investigations. Two other cytolytic proteins associated with the cytotoxic granule and granulysin expression are perforin and granzyme B [169,189,421] . Canaday et al. [282] showed that $CD4^+$ T cells upregulate perforin and granzyme B after *in vitro* stimulation with *M. tuberculosis*. In this study they could demonstrate that the blockage of perforin did not affect T cell mediated restriction of mycobacterial growth. This goes in line with the work of Ochoa et al. [395] that could detect similar levels of expression of perforin across the clinical spectrum of leprosy. At the same time the frequency of granulysin expressing T cells in lesions correlated with the clinical form of disease. This suggests that even if perforin is upregulated in $CD4^+$ CTLs from children, it does not contribute to the cytolytic defense of these cells against *M. tuberculosis*. In summary, granulysin expressing $CD4^+$ T cells from children infected with *M. tuberculosis* show an activated T_H1 phenotype. We suggest that these cells are regulated in their activity via the CTLA-4 receptor, since this receptor is strongly upregulated on these cells. In addition, the increased expression of granzyme B and perforin supports the cytolytic role of these $CD4^+$ T cells in the immune defense against *M. tuberculosis*. As discussed earlier the alternative pathway for CTLs to act in a cytotoxic manner is via CD95/CD95L pathway [422] . This different route of killing can be excluded in our assay since no induction of CD95L on the cell surface of granulysin-positive T cells could be detected (data not shown). This confirms studies of clones and cell lines suggesting that $CD4^+$ CTLs use the cytotoxic granule mechanism rather than the CD95-dependent pathway [423,424,425,426,427] .

4.2.2 Granulysin-positive $CD4^+$ T cell lines show non-polarized properties and reduce bacterial burden

Till now, only cytokine and protein expression could be measured due to the need of fixation of the cells for ICS. This limited us to only investigate responses of bulk $CD4^+$ T cells. Thus we established a flow-cytometry based sorting panel that allowed us to establish single-cell derived T cell lines (see Methods section). Based on the granulysin expression after 5 day restimulation with PPD, the T cell lines were then grouped in “granulysin high”- and “granulysin low”-responders (Fig. 15). Interestingly, polyclonal stimulation with PMA and Ionomycin revealed the ability of granulysin-positive T cell lines to also express T_H1 cytokines, in this case IFNγ. The ability of CTLs from TB patients to express IFNγ has previously only be shown by Klucar et al., who measured IFNγ in the supernatants of T cell clones specific for a CFP-10 epitope [319] . The lack of protein induction above background after the stimulation with an unspecific antigen (TcF) evidenced the antigen-specificity of the established T cell lines.

Since we were now able to also investigate responses on transcriptional level, we isolated RNA of T cell lines of different groups and compared expression after different stimulations (Fig. 16 / Table 4). As suggested by previous experiments T-Bet, a transcription factor

characteristic for T_H1 polarization, as well as EOMES were upregulated [428] . Little is known about the role of the transcription factor EOMES in CTLs. It is, together with T-Bet a member of the T-Box family [429] . Lack of both molecules leads to an almost complete loss of memory $CD8^+$ T cells and a defect in the cytotoxic effector programming [327] . Furthermore, at least upon viral infection, the loss of both factors leads to an anomalous type 17 response of $CD8^+$ T cells [328] . Absence of only T-Bet can be compensated by EOMES as measured by IFNγ response in $CD4^+$ T cells as well as T_H1 differentiation. Nevertheless, these T cells were more susceptible to cytokine-driven T_H17 development [332] . Prior to this study, a direct link between granulysin expression and the induced expression of EOMES has not been shown. An upregulation of 12- and 15-fold in "granulysin high" T cell lines could be detected. The gene expression level of EOMES in "granulysin medium" and "granulysin low" showed no induction after antigen stimulation. This suggests a novel direct positive correlation between the induction of EOMES and granulysin in $CD4^+$ T cells. Interestingly, this induction appears to be T-Bet independent. This reasoning is based on the null induction of EOMES after polyclonal stimulation, while T-Bet expression showed up to 14-fold higher expression in comparison to unstimulated controls. As expected from the cytokine staining, gene expression of T_H2- and T_H17-associated transcription factors were upregulated after antigen stimulation. This finding has to be further investigated and suggests polyfunctional features of granulysin-positive T cells in the context of TB. Additionally, the expression of the regulatory cytokine IL-10 was highly suppressed in these granulysin expressing $CD4^+$ T cell lines. This finding stands in contrast to the work of Klucar et al. [319] who showed an induction of IL-10 in the supernatants of a CFP-10 specific cytolytic T cell clone. The loss of IL-10 expression can be explained by the activated state of the T cells as evidenced by the upregulation of characteristic markers (Fig. 14). This would consequently lead to loss of suppressive cytokine production such as IL-10. In the context of TB, IL-10 expression is associated with anergy in infected patients combined with reduced IFNγ response [430] . Loss of IL-10 in mice also results in improved antimicrobial immune response [431,432,433] . On the contrary decreased IL-10 secretion also leads to a hyper-immune response against pathogens [434,435] . In infants after BCG vaccination some children have either elevated levels of IL-10 or IFNγ production but not both [436] . In summary, IL-10 plays a crucial role in control of *M. tuberculosis* and the immune response. However, T cells producing granulysin upon stimulation lose the ability to produce this regulatory cytokine.

To demonstrate the correlation of granulysin expression and CTL activity, T cell lines of the "granulysin high"- and "granulysin low/medium"-group were incubated with *M. tuberculosis*-infected autologous monocytes (Fig. 17A). Bacterial burden could be directly related to granulysin expression. The reason for remaining bacteria could be explained by the specificity of the T cell lines. These were selected based on their specificity to the purified protein derivate (PPD) and not the processed pathogen itself. To mimic more physiologic circumstances, in a different set of experiment autologous macrophages instead of monocytes were used as target cells (Fig. 17B). Again, a reduction in bacterial growth could

be detected in presence of granulysin expressing T cells which was absent when co-cultivate with T cell lines lacking granulysin expression.

In summary, the generation of T cell lines allowed new insights into the T cell-differentiation and -expression pattern of granulysin expressing $CD4^+$ T cells in response to *M. tuberculosis*. We could demonstrate that these cells co-express T_H1, T_H2 and T_H17 transcription factor and also associated cytokines on the transcriptional level after PPD-stimulation. In addition we could show that the granulysin-positivity is associated with an improved antimicrobial cytotoxicity.

4.2.3 Conclusion & Outlook

The work presented in the second part of this thesis dealt with the identification and characterization of granulysin expressing $CD4^+$ CTLs in the context of childhood tuberculosis. By using a recently described 7d *in vitro* assay we could detect strong antigen-specific induction of $CD4^+$ $CD45RO^+$ memory T cells. We subsequently investigated the expression of the anti-microbial protein granulysin, which could be detected in high frequencies in PBMCs of TB experienced children. In this work we used only ESAT6-CFP10 and Antigen 85A and B as mycobacterial antigens, but due to the high induction of antigen-specific memory T cells in children we are convinced, that this novel restimulation assay could be used to identify various antigen responses. Especially against antigens with low frequencies of specific memory T cells as already demonstrated in adults. As a proof of principle we incubated PBMCs from healthy latently infected children with dormancy associated antigens and could detect frequencies of IFNγ expressing $CD4^+$ T cells up to three times higher than reported in adults.

The significant upregulation of granulysin by memory $CD4^+$ T cells in children with active TB as well as LTBI, emphasises on the value of this assay as a possible diagnostic tool to identify TB in children. This would require a larger clinical study with standardized operating protocols and should be realized in a high endemic area. Since a seven day stimulation period would be quite long for this purpose, we tested shorter stimulation periods and could detect significant increase in granulysin expression by memory T cells after five days already, even though to lesser extent.

Our data also revealed strong induction of granulysin in PBMCs from children that had received anti-microbial treatment more than 20 months ago. This makes granulysin a very interesting candidate to monitor vaccine efficiency and associated protection. It should also be considered to correlate the frequency of granulysin expressing $CD4^+$ memory T cells with the rate of re-infection or –activation of TB in children in high endemic areas.

In the framework of this thesis we also generated different T cell lines expressing and lacking granulysin expression. Experiments on the transcriptional level revealed gene induction of transcription factors which are characteristic for T_H1, T_H2 and T_H17 T cell subpopulations and which are described, to be induced exclusively. To pursue this finding the generation of single T cell clones out of the granulysin expressing T cell lines need to be performed. This would ensure that the detected induced expression of all three transcription factors can be traced back to a single clone.

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Appendix

Abbreviations

ADC	albumin, dextrose, catalase
AFB	acid-fast bacteria
Ag85a/b	Antigen85 a/b of *M. tuberculosis*
Alexa#	Alexa Fluor® #
APC	antigen presenting cell
APC	allophycoerythrin
APC-Cy7	allophycoerythrinCychrome7
ATP	adenosine tri-phosphate
BAK	BCG activated killer cells
BAL	bronchoalveolar lavage
BCG	*Mycobacterium bovis* bacillus Calmette Guérin
BCR	B-cell receptor
Bej	M. tuberculosis Beijing
BSA	bovine serum albumin
BSL3	biosafety level 3 (S3/L3 in Germany)
CCR5	chemokine (C-C motif) receptor 5
CD#	"cluster of differentiation" international nomenclature for cell surface molecules
CD40L	CD40 ligand
CFU	colony forming units
CFDA-SE	carboxyfluorescein diacetate, succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
cRPMI	RPMI-1640 supplemented with 10% AB Rh-positive heat-inactivated human serum, 100U/ml penicillin, 100µg/ml streptomycin, 1mM L-glutamine and 10mM HEPES
CTL	cytotoxic T-lymphocyte
CXCR-3	chemokine (C-X-C motif) receptor 3
DC	dendritic cell
d	days
DISC	death-induced signalling complex
DNA	deoxyribonucleic acid
DosR	Dormancy survival regulator
dp	double positive
EBV	Epstein-Barr virus
ER	endoplasmatic reticulum
E:T	effector:target ratio
eGFP	enhanced green-fluorescent-protein
ELISA	enzyme-linked immunosorbent assay
EOMES	Eomesodermin
ESAT6_CFP10	6 kDa early secretory antigenic target_culture filtrate protein 10 fusion protein of *M. tuberculosis*
FAAD	FAS-associated death domain

FACS	fluorescence activated cell sorting, flow cytometry
FCS	fetal calf serum
FITC	fluoreszeinisothiocyanat
FSC	forward scatter, in flow cytometry GAPDH glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony stimulating factor
Grz-A	Granzyme-A
Grz-B	Granzyme-B
h	hours
HCMV	human cytomegalovirus
HEV	high endothelial venules
HIV	human immunodeficiency virus
ICS	intracellular cytokine staining
IFNγ	interferon gamma
IGRA	interferon-gamma-release-assay
IL	interleukin
kDa	kilo-Dalton
LAK	lymphokine activated killer cell
LPS	lipopolysaccharide
LTBI	latent tuberculosis infection
M	mol/L
mAb	monoclonal antibody
MFI	median fluorescence intensity
MHC	major histocompatibility complex
min	minutes
MOI	multiplicity of infection
MTB-lysate	M. tuberculosis whole cell lysate
M. tuberculosis	Mycobacterium tuberculosis
MVA85A	modified vaccine Ankara-expressing *Mycobacterium tuberculosis* Antigen 85A
NK	natural killer cell
NTM	non-tuberculous mycobacteria
OD#	optical density, # indicates wavelength in nm
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pe	phycoerythrin
Pe-Cy5	phycoerythrin-Cychrome5
Pe-Cy7	phycoerythrinCychrome7
PerCP	peridinin-chlorophyll-protein complex
PerCP-Cy5.5	peridinin-chlorophyll-protein complex-Cychrome5.5
Pen	Penicillin
PHA	Phytohemagglutinin from *Phaseolus vulgaris*
PMA	phorbol 12-myristate 13-acetate
PPD	purified protein derivative of *M. tuberculosis*
PRR	pattern recognition receptor
QPRC	quantitative real-time polymerase chain reaction

Rif	rifampicine
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RORγT	RAR-related orphan receptor gamma
rpm	rounds per minute
RT	room temperature
SEB	Staphylococcus enterotoxin B
SSC	sideward scatter
sp	single positive
STAT	signal transducers and activators of transcription
Strep	Streptomycin
TAP	transporter associated with antigen processing
TB	tuberculosis
T_{cm}	central effector memory T cell
TCR	T-cell receptor
T. cruzi	Trypanosoma cruzi
T_{em}	effector memory T cell
TGF-β	Transforming growth factor beta
T_h1	T-helper cell type 1
T_h17	T-helper cell type 17
T_h2	T-helper cell type 2
TNFα	tumor necrosis factor alpha
TLR	Toll like receptor
T_{reg}	T-regulatory cell
TST	tuberculin skin test
v/v	volume per volume
w/v	weight per volume

Materials

Reagents

Reagent	Supplier
[5-6-^{3}H]-Uracil-1mCi/ml, 31.9Ci/mmol	Perkin Elmer, Waltham, USA
Antigen85a (Ag85a)	Colorado University, USA
Antigen85b (Ag85b)	Colorado University, USA
Anti-Granulysin (RB1) Monoclonal Antibody	MBL, Woburn, USA
Anti-Granulysin (RC8) Monoclonal Antibody	MBL, Woburn, USA
Biocoll Separation Solution	Biochrom AG, Berlin, Germany
Bovine Serum Albumin (BSA)	Serva, Heidelberg, USA
Brefeldin A from *Penicillium brefeldianum*	Sigma-Aldrich, Saint Louis, USA
$CD4^{+}$ T cell isolation Kit II	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CEF Peptide Pool	JPT Peptides, Berlin, Germany
CellTrace™ CFSE Cell Proliferation Kit	Invitrogen, San Diego, USA
Chimeric Chagas Multiantigen recombinant	JPT Peptides, Berlin, Germany
Cytomegalo Virus Pp65 protein recombinant	Prospec, Rehovot, Israel
Epstein-Barr Virus (HHV-4) EBNA1 recombinant	Prospec, Rehovot, Israel
Ethanol	Merck
HEPES Buffer	PAA, Pasching, Austria
human AB Serum	Sigma-Aldrich, Saint Louis, USA
Horseradish peroxidase (HRP)-streptavidin conjugate	Dianova, Hamburg, Germany
L-Glutamine	PAA, Pasching, Austria
Ionomycin calcium salt from *Streptomyces conglobatus*	Sigma-Aldrich, Saint Louis, USA

Macrophage colony-stimulating factor (M-CSF)	PeproTech, Rocky Hill, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, Saint Louis, USA
Phytohemaglutinin (PHA)	Invitrogen, San Diego, USA
Penicilin	PAA, Pasching, Austria
Purified protein derivate of *Mycobacterium tuberculosis* (PPD)	Statens Serum Institute, Copenhagen, Denmark
Rosewell Park Memorial Institute medium	Invitrogen, San Diego, USA
rhGranulysin	R&D Systems, Minneapolis, USA
rhInterleukin-1beta (IL-1B)	Biosource, Invitrogen, San Diego, USA
rhInterleukin-2 (IL-2)	Active Bioscience, Hamburg, USA
rhInterleukin-4 (IL-4)	Active Bioscience, Hamburg, USA
rhInterleukin-6 (IL-6)	Biosource, Invitrogen, San Diego, USA
rhInterleukin-7 (IL-7)	Active Bioscience, Hamburg, USA
Sodium-chromate	Hartmann Analytic, Braunschweig, Germany
Staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB)	Sigma-Aldrich, Saint Louis, USA
Streptomycin	PAA, Pasching, Austria
Tetanus toxin from *Clostridium tetani* (TT)	Sigma-Aldrich, Saint Louis, USA
Tween 20	Sigma-Aldrich, Saint Louis, USA
UltraPURE Water	Invitrogen, San Diego, USA

Other material

Material	Supplier
6-well / 24-well / 48-well / 96-well Flatt Bottom Plates	Nunc, Langenselbold, Germany
15ml Tubes	Sarstedt, Nümbrecht, Germany
50ml Tubes	Sarstedt, Nümbrecht, Germany
96-well Round Bottom Plates	Nunc, Langenselbold, Germany
BDVacutainer™ 8ml	BD, San Diego, USA
Cluster Tubes 1.8ml	Thermo Fischer, Dreieich, Germany
Combitips	Eppendorf, Hamburg, Germany
CryoTubes 1.8ml	Nunc, Langenselbold, Germany
Disposable Transfer Pipette	BD, San Diego, Germany
ELISA-plates Immuno MAXI-Sorp	Nunc, Langenselbold, Germany
FACS tubes 5.0ml	Sarstedt, Nümbrecht, Germany
Filter plates for Millex Syringe Driven Filter Units	Millipore, Billerica, USA
PARAFILM®	Brand, Wertheim, Germany
Plasticware	Corning, Sarsted or TPP
Steritops Sterile Filters	Millipore, Billerica, USA
Sterile filters and membranes	Schleicher & Schüll, Dassel, Germany
Stripette (5.0ml/10ml/25ml)	Corning, Amsterdam, Netherlands
Syringes	B.Braun, Melsungen, Germany

Instruments

Instrument	Supplier
7900HT Fast Real-Time PCR System	Applied Biosystems, Carlsbad, USA
Bio-Plex™ System	Bio-Rad, München, Germany
Cell counting chamber Neubauer improved	Brand, Wertheim, Germany
CO_2-Inkubator	Binder, Tuttlingen, Germany
ELISA-reader SpectrMAX190	Molecular Devices, Ismaning, Germany
Flow cytometer FACS Canto™	BD, San Diego, USA
Flow cytometer FACS Canto II™	BD, San Diego, USA
Flow cytometer BD LSR II™	BD, San Diego, USA
Lab-Shaker	Liebisch, Bielefeld, Germany
Microcentrifuge Biofuge fresco	Hereaus, Hanau, Germany
Microscopes	Leica, Solms, Germany
MuliScreen Filtration System	Millipore, Billerica, USA
pH-meter 761 calimatic	Knick, Berlin, Germany
Safety cabinet: Lamina HERAsafe	Hereaus, Hanau, Germany
TopCount NXT Microplate scintillation counter	Perkin Elmer, Waltham, USA
Vortex MS1 Minishaker	IKA Labortechnik, Staufen, Germany
Water purifier	Millipore, Billerica, USA

Buffers and solutions used for cell culture and flowcytometry

Reagent	
Cytofix/Cytoperm	BD, San Diego, USA
FACS Buffer	5% FCS in PBS
PBS Buffer	Invitrogen, San Diego, USA
Perm/Wash buffer (10x)	BD, San Diego, USA
RPMI complete medium	Rosewell Park Memorial Institute medium 1640 + 10%(v/v) heat inactivated human AB Serum 1.0mM L-glutamine 100U/ml penicillin 100µg/ml streptomycin 10mM HEPES Buffer

Buffers and solutions used for ELISA

Reagent	
3,3',5,5'-tetramethyl- benzidine peroxidase Substrate (TMB)	KPL, Gaithersburg, USA
Anti-Granulysin (RB1) Monoclonal Antibody	MBL, Woburn, USA
Anti-Granulysin (RC8) Monoclonal Antibody	MBL, Woburn, USA
Assay diluents (IFNγ ELISA)	PBS + 10% FBS (pH=7.0)
Blocking Buffer (Granulysin ELISA)	10% FBS in Washing Buffer
Coating Buffer (IFNγ ELISA)	1L: 0,1M Sodium carbonate (pH=9.5); 8,40g $NaHCO_3$; fill up to 1L with H_2O (pH=9.5)
Fetal bovine serum (FBS)	Sigma-Aldrich, Saint Louis, USA
Horseradish peroxidase (HRP)-streptavidin	Dianova, Hamburg, Germany

conjugate	
rhGranulysin	R&D Systems, Minneapolis, USA
Stop Solution	2N H_2SO_4
Tris Buffered Saline (TBS, pH 7.4)	Sigma-Aldrich, Saint Louis, USA
Washing Buffer (Granulysin ELISA)	PBS + 0.1% Tween-20
Washing Buffer (IFNγ ELISA)	PBS + 0.05% Tween-20

Antibodies or Kits used for ELISA

Antibody	**Clone**	**Isotype**	**Fluorochrome**	**Final concentration**	**Company**
α Granulysin (Capture)	RB1	Mouse IgG1	purified	5 µg/ml	MBL, Woburn, USA
α Granulysin (Detection)	RC8	Mouse IgG1	Biotinylated	0.1 µg/ml	MBL, Woburn, USA
BD OptEIA™ human IFN-γ ELISA Set					BD, San Diego, USA

Primer for qRT PCR

All primers were synthesised by Qiagen, Hilden, Germany and used at a final concentration of 10µM.

Name	**Targeted gene (human)**	**Detected transcript (NCBI reference sequence)**
Hs_GAPDH_2_SG	glyceraldehyde-3-phosphate dehydrogenase	NM_002046.3
Hs_IFNG_1_SG	Interferon gamma	NM_000619.2
Hs_EOMES_1_SG	Eomesodermin	NM_005442.2
Hs_GNLY_2_SG	Granulysin	NM_006433.3
Hs_PRF1_2_SG	Perforin	NM_001083116.1

Hs_TBX21_1_SG	T-Bet	NM_013351.1
Hs_TNF_3_SG	Tumor necrosis factor alpha	NM_000594.2
Hs_GATA3_1_SG	GATA binding protein 3	NM_001002295.1
Hs_IL4_1_SG	Interleukin 4	NM_000589.2
Hs_IL13_1_SG	Interleukin 13	NM_002188.2
Hs_RORC_1_SG	RAR-related orphan receptor C	NM_005060.3
Hs_IL17A_1_SG	Interleukin 17A	NM_002190.2
Hs_IL10_1_SG	Interleukin 10	NM_000572.2

Software

Software	**Supplier**
Adobe Illustrator CS4	Adobe, Delaware, USA
Adobe Photoshop CS3	Adobe, Delaware, USA
Bio-Plex Manager 4.1.1	Bio-Rad, München, Germany
Endnote X	Thomson, Carlsbad, USA
Excel 2007	Microsoft, Seattle, USA
FACS Diva v5	BD, San Diego, USA
FACS data Analyser (0.9.9)	courtesy of Christian Köberle
FCS Express 3.0	De Novo Software, Los Angeles, USA
FlowJo Software	Tree Star Inc., Stanford, USA
GraphPad Prism 5.0	GraphPad Software, La Jolla, USA
SoftMax Pro V5	MDS Analytical Technologies, Toronto, Canada
Word 2007	Microsoft, Seattle, USA

Web resources

Sequence information on *M. tuberculosis* genes:	http://genolist.pasteur.fr/TubercuList/
Information on cytokines and chemokines:	http://www.copewithcytokines.de/cope.cgi

Antibodies

Human antibody	Fluorochrome	Clone	Order #	Company
CCR4	PE	205410	FAB1567P	R & D
CCR5 (CD195)	A-647 (APC)	HEK/1/85a	MCA2175A647T	A&D Serotec
CCR7	APC	3D12	557734	BD Biosciences
	Pe-Cy7	3D12	557648	BD Biosciences
CD3	APC	UCHT1	555335	BD Biosciences
	APC-Cy7	SK7	557832	BD Biosciences
	FITC	UCHT1	555332	BD Biosciences
	PE	UCHT1	555333	BD Biosciences
CD4	Alexa-700	OKT4	56-0048	eBioscience
	APC	RPA-T4	555349	BD Biosciences
	APC-Cy7	SK3	341115	BD Biosciences
	FITC	RPA-T4	11-0049	eBioscience
	Pe-Cy7	SK3	557852	BD Biosciences
CD8	APC	SK1	345775	BD Biosciences
	APC-Cy7	SK1	557834	BD Biosciences
	PerCP-Cy5.5	SK1	341050	BD Biosciences
CD14	APC	TÜK4	130-091-243	Miltenyi
CD25	PE	M-A251	347647	BD Biosciences
	Pe-Cy7	M-A251	335824	BD Biosciences
CD40L	APC	TRAP1	555702	BD Biosciences
CD45RA	Pe-Cy7	HI100	337186	BD Biosciences
	FITC	HI100	555488	BD Biosciences
	Alexa700	HI100	304120	Biolegend
CD45RO	PE	UCHL1	347967	BD Biosciences
	Pe-Cy7	UCHL1	337168	BD Biosciences
CD56	PE	B159	555516	BD Biosciences
CD62L	APC	DREG-56	17-0629	eBioscience
CD95	APC	DX2	17-0959	eBioscience
CD95L	PE	NOK-1	12-9919	eBioscience
CD107a	FITC	H4A3	555800	BD Biosciences
CD107b	FITC	H4B4	555804	BD Biosciences

CD127	APC	40131	FAB306A	R & D
	PE	R34.34	IM1980	Beckman&Coulter
CD196	PE	11A9	559562	BD Biosciences
Human antibody	**Fluorochrome**	**Clone**	**Order #**	**Company**
CD279 (PD-1)	PE	eBioJ105	12-2799	eBioscience
CompBeads	-		552843	BD Biosciences
CTLA-4 (CD152)	APC	BNI3	555855	BD Biosciences
CXCR3 (CD183)	FITC	49801	FAB160A	R & D
CXCR5	A-488	RF8B2	558112	eBioscience
FoxP3	FITC	236A/E7	11-4777-73	eBioscience
GM-CSF	PE	BVD2-21C11	554507	BD Biosciences
	APC	BVD2-21C11	51-7337-73	eBioscience
Granulysin	FITC	RB1	558254	BD Biosciences
	PE	eBioDH2	12-8828-73	eBioscience
Granzyme B	Alexa 700	GB11	560213	BD Biosciences
HLA-DR	APC	L243 (G46-6)	559866	BD Biosciences
IFNg	APC	25723.11	341117	BD Biosciences
	FITC	B27	554700	BD Biosciences
	Pacific Blue	4S.B3	57-7319-73	eBioscience
	Pe-Cy7	B27	557643	BD Biosciences
	APC	MQ1-17H12	554567	BD Biosciences
IL-2	FITC	MP4-25D2	559361	BD Biosciences
	PE	MP4-25D2	559334	BD Biosciences
	APC	MP4-25D2	554486	BD Biosciences
IL-4	FITC	MP4-25D2	554484	BD Biosciences
IL-10	Pacific Blue	JES3-9D7	57-7108-73	eBioscience
	FITC	PVM13-1	11-7139-71	eBioscience
IL-17	A-647 (APC)	eBio64CAP17	51-7179-73	eBioscience
	FITC	eBio64DEC17	11-7179-73	eBioscience
Perforin	FITC	dG9	11-9994-73	eBioscience
TCR a/b	PE	T10B9.1A-31	555548	BD Biosciences
TCR g/d	FITC	11F2	347903	BD Biosciences
TNF-a	A-700	MAb11	557996	BD Biosciences
	FITC	6401.1111	340511	BD Biosciences
	Pacific Blue	Mab11	57-7349-73	eBioscience

Zusammenfassung

Charakterisierung des Zytokinprofils in Erwachsenen mit einer latenten oder aktiven Tuberkulose in einem hoch endemischen Gebiet

Die Tuberkulose (TB) stellt mit rund 2 Milliarden Infizierten weltweit ein globales gesundheitliches Problem dar. Während die große Mehrheit der infizierten Personen in der Lage sind die Krankheit zu kontrollieren, entwickelt sich bei ungefähr 10 % die aktive Form der TB aus. Der zugrunde liegende immunologische Prozess für diese Verteilung ist bis heute nicht bekannt und im Fokus dieser Arbeit. Das adaptive Immunsystem spielt eine entscheidende Rolle in der Immunabwehr gegen *Mycobacterium tuberculosis (M. tuberculosis)*, dem Erreger der TB. Hierbei sind besonders $CD4^+$ T-Zellen für die erfolgreiche Eingrenzung der Erkrankung verantwortlich. Im Vorfeld konnte bereits mehrmals eine Assoziation zwischen polyfunktionalen $CD4^+$ T-Zellen und einem Schutz gegen verschiedenste Krankheitserreger gezeigt werden. Im Rahmen dieser Doktorarbeit wird versucht die Frage zu beantworten, ob eine erhöhte Frequenz von polyfunktionalen $CD4^+$ T-Zellen auch gegen die Ausbildung einer aktiven TB schützen kann.

Zur Bearbeitung dieser Fragestellung wurde das T_H1 Zytokinprofil von Patienten mit aktiver TB untersucht und mit dem von gesunden latent infizierten Probanden (LTBI) verglichen. Desweiteren wurden die TB Patienten während der antimikrobiellen Therapie begleitet um Änderungen im Zytokinprofil von $CD4^+$ T-Zellen beobachten zu können. Im Rahmen dieser Arbeit wurde zum ersten Mal die simultane Expression der vier T_H1 Zytokine IFNγ, TNFα, IL-2 und GM-CSF mit Hilfe der multifarben Durchflusszytometrie untersucht. Nach antigenspezifischer Stimulation konnten sowohl in unbehandelten und behandelten Patienten mit aktiver TB ein großer Anteil an multifunktionale Gedächtnis-T-Zellen nachgewiesen werden, die alle vier Zytokine gleichzeitig exprimierten. Bemerkenswerterweise konnte diese Population ebenfalls in LTBI gezeigt werden. Nach den ersten zwei Monaten der Therapie war der Anteil an multifunktionalen T-Zellen signifikant erhöht welches auf einen positiven Einfluss dieser Zellen auf die Behandlung hinweist.

Um detaillierte Information über das Expressionspotential von $CD4^+$ T-Zellen zu gewinnen wurden PBMCs mit einem Superantigen inkubiert. Hierbei unterschied sich das Zytokinprofil zwischen den beiden Studiengruppen signifikant und veränderte sich ebenfalls unter Therapie. Während die Expression von IFNã in TB Patienten niedriger war als in LTBI, war die Frequenz von TNFá, IL-2 und GM-CSF-positiver $CD4^+$ T-Zellen signifikant höher in Patienten mit aktiver TB.

Zusammenfassend ist zu sagen, dass sowohl in TB Patienten vor und nach Therapie, als auch in LTBI, multifunktionale $CD4^+$ T-Zellen nachgewiesen werden können. Ein Unterschied in der Frequenz konnte dabei nicht festgestellt werden. Daher kann ein Zusammenhang zwischen

der Existenz von multifunktionellen $CD4^+$ T-Zellen und einem Schutz gegen eine mögliche Reaktivierung von der latenten zu der aktiven TB nicht beschrieben werden.

Die Rolle des zytotoxischen Proteins Granulysin in Kindertuberkulose

Mit einer Million Toten jedes Jahr, verbleibt Kindertuberkulose (TB) eine der Hauptursachen der Kindersterblichkeit weltweit. Es konnte gezeigt werden, dass Kinder ein erhöhtes Risiko für die Manifestation einer aktiven TB und für die Entwicklung eines extrapulmonären Verlaufes aufweisen. Auf Grund der geringen Teilungsrate von *M. tuberculosis* zeigen viele infizierte Kinder einen negativen Sputumtest auf. Daher gestaltet sich die Diagnose von TB in Kindern oft als sehr schwierig und neue Ansätze werden dringend benötigt. Auf Grund der zentralen Rolle von T-Zellen in der Immunabwehr gegen TB sollten neue diagnostische Ansätze auf diese Zellpopulation fokussieren. In dem zweiten Teil dieser Doktorarbeit werden $CD4^+$ zytotoxische T-Zellen (CTL) als möglichen neuen Kandidat für die Diagnose von TB beschrieben und untersucht. Der Fokus liegt hierbei auf der Expression des zytotoxischen Proteins Granulysin. Es konnte gezeigt werden, dass Granulysin extrazellulär und in Kombination mit Perforin auch innerhalb von Makrophagen hemmend auf *M. tuberculosis* wirkt.

Um die $CD4^+$ T-Zell Expression von Granulysin zu untersuchen, wurde ein neubeschriebener 7d *in vitro* Assay verwendet. Die Stimulation mit Antigenen von *M. tuberculosis* führte zu einem starken Anstieg von $CD4^+$ $CD45RO^+$ Gedächtnis-T-Zellen, welcher in gesunden Kontrollen fehlte. Gleichzeitig konnte eine signifikante Induktion von Granulysin in Kindern mit akuter oder behandelter TB beobachtet werden. Proliferierende Gedächtnis-T-Zellen konnten als die Hauptquelle der Granulysinexpression identifiziert werden. Weitere Experimente zeigten, dass diese Zellen über einen zentral- und effektor-Gedächtnisphänotyp verfügen. Desweiteren konnte Untersuchungen von Kindern, deren Therapie bereits abgeschlossen war, zeigen dass diese Zellen sehr langlebig sind und teilweise IFNγ koexprimieren. Phänotypische Untersuchungen dieser Zellen deuten auf die Hochregulierung von verschiedenen T_H1- und Aktivierungsmarkern hin. Granulysin exprimierende Zellen waren ebenfalls positiv für Granzym B und Perforin, zwei weitere zytotoxische Proteine. Durch die Generierung von Granulysin-positiven $CD4^+$ T-Zelllinien konnte die simultane Induktion von Transkriptionsfaktoren für T_H1, T_H2 und T_H17 Subpopulationen nach Antigenstimulation gezeigt werden. Desweiteren konnte eine Korrelation des Transkriptionsfaktor EOMES mit der Granulyinexpression beobachtet werden, welche unabhängig von dem T_H1 Transkriptionsfaktor T-Bet war. In einem abschließenden Experiment konnte dargestellt werden, dass granulysinexprimierende T-Zelllinien in der Lage sind das Wachstum von *M. tuberculosis* in infizierten Zielzellen zu hemmen.

Zusammenfassend konnte diese Arbeit zeigen, dass die Analyse von Granulysinexpression von $CD4^+$ T Zellen mit Hilfe des 7d *in vitro* Assay ein mögliches neues diagnostisches Mittel

für TB in Kindern darstellt. Die Untersuchung der Expression zu verschiedenen Zeitpunkten in Kindern nach der abgeschlossenen Therapie zeigt Granulysin als möglichen neuen Kandidaten für kommende Vakzine-Studien und als mögliches Korrelat für Therapieerfolge.

Publications

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Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als

die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in

dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde. Der Inhalt

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Henrik Müller