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The Immunoregulation of the Tuberculin Skin Test in HIV-seropositive and HIV-seronegative Persons

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Abbreviations

ABC Method	Avidin-Biotin-Complex Method
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen-Presenting Cells
ART	Antiretroviral Therapy
BCG	Bacille Calmette-Guérin
BSS	Buffered Saline Solution
CD 4/8/...	Cluster of Differentiation molecule 4/8/..., see table 2.1
CCR5	Chemokine (C-C motif) Receptor 5
CXCR4	Chemokine (C-X-C motif) Receptor 4
CFP-10	Culture Filtrate Protein-10
DAB	3,3´-Diaminobenzidine
DNA	Deoxy-Ribonucleic Acid
DTH	Delayed-Type Hypersensitivity Reaction = Type IV Reaction According to Gell and Coombs
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunospot
Env	HIV Glycoprotein Envelope
ESAT-6	Early Secreted Antigenic Target-6
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FoxP3	Forkhead Box P3
Gp120	Glycoprotein 120
HAART	Highly Active Antiretroviral Therapy
H_2O_2	Hydrogen Peroxide
HLA-DR	Human Leukocytes Antigen Subtype carrying “DR-Antigen”
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus, Species 1
HIV-2	Human Immunodeficiency Virus, Species 2
IFN-γ	Interferon-γ
IGRA	Interferon-γ Release Assay
IHC	Immunohistochemistry

IL-2	Interleukin 2
IL-10	Interleukin 10
IPT	Isoniazid Preventive Therapy
IQR	Interquartile Range
LTBI	Latent Tuberculosis Infection
mAb	Monoclonal Antibody
MDR-TB	Multidrug-Resistant Tuberculosis
MHC	Major Histocompatibility Complex
MHC class II	Protein of the Major Histocompatibility Complex Class II
MTB	*Mycobacterium tuberculosis*
NaCl	Sodium Chloride
NaN_3	Sodium Acid
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTI	Nucleoside Reverse Transcriptase Inhibitors
NTM	Non-Tuberculous Mycobacteria
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PI	Protease Inhibitors
PPD	Purified Protein Derivate
RD1	Recombinant *M. tuberculosis* region of difference 1
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Culture Medium
RT	Room Temperature
RT23 Tuberculin	Tuberculin Created by the Statens Serum Institute, Denmark
S.D.	Standard Deviation
SEM	Standard Error Mean
SFC	Spot-forming Cell
SPSS	Statistics program (=Statistical Package for the Social Sciences)
SSI	Statens Serum Institute, Denmark
TB	Tuberculosis

TGF-β	Transforming Growth Factor β
Th1	T Helper Cells Type 1
Th2	T Helper Cells Type 2
THT	Tuberkulin-Hauttest
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor-α
Tregs	Regulatory T Cells
TST	Tuberculin Skin Test
VCT	Voluntary Counselling and Testing
WHO	World Health Organisation
XDR	Extensively Drug-Resistant Tuberculosis

1 Introduction

1.1 Epidemiology of HIV infection and tuberculosis

Human Immunodeficiency Virus (HIV)-infection and tuberculosis (TB) are two of the most pressing public health problems in the world [1, 2], whilst the developing world, especially sub-Saharan Africa, is most severely affected by these two infections [3].

The World Health Organisation (WHO) estimates that over one third of the world's population is currently infected with *Mycobacterium tuberculosis* (*M. tuberculosis*, MTB), the etiologic agent of tuberculosis. The annual incidence of tuberculosis is approximately 9.2 million people per year according to recent publications [2]. Up to two million people die of tuberculosis every year. Approximately 29 percent of all tuberculosis cases worldwide and 34 percent of tuberculosis related deaths occur in Africa, which is home to 11 percent of the world's population. The challenges of controlling the disease have never been greater [4]. In contrast to this, Europe holds only five percent of all tuberculosis cases, and mortality rates are ten fold lower. Globally, around 5,000 people die of tuberculosis every day although tuberculosis is a curable disease with treatment success rates of approximately 80 percent reported for Europe [5]. Nevertheless, nowadays more people die of tuberculosis than ever before [2].

According to the WHO, in 2007 up to 40 million people were estimated to be living with HIV, approximately 2.5 million were newly infected that year and about 2.1 million people died of the acquired immunodeficiency syndrome, AIDS [1]. HIV is responsible for an estimated number of 6,000 deaths every day worldwide [1]. More than two thirds of all HIV infected patients live in sub-Saharan Africa, with approximately 1.7 million new cases in the year 2007 [1]. Eight countries in this region now account for almost one third of all new HIV infections and AIDS related deaths globally.

In South Africa, up to 1,000 people prematurely die per day because of HIV, many due to coexistent tuberculosis. It is estimated that up to 50 percent of all deaths among HIV infected people occur as a consequence of tuberculosis. About half of all tuberculosis patients are infected with HIV [2] (**figure 1.1**).

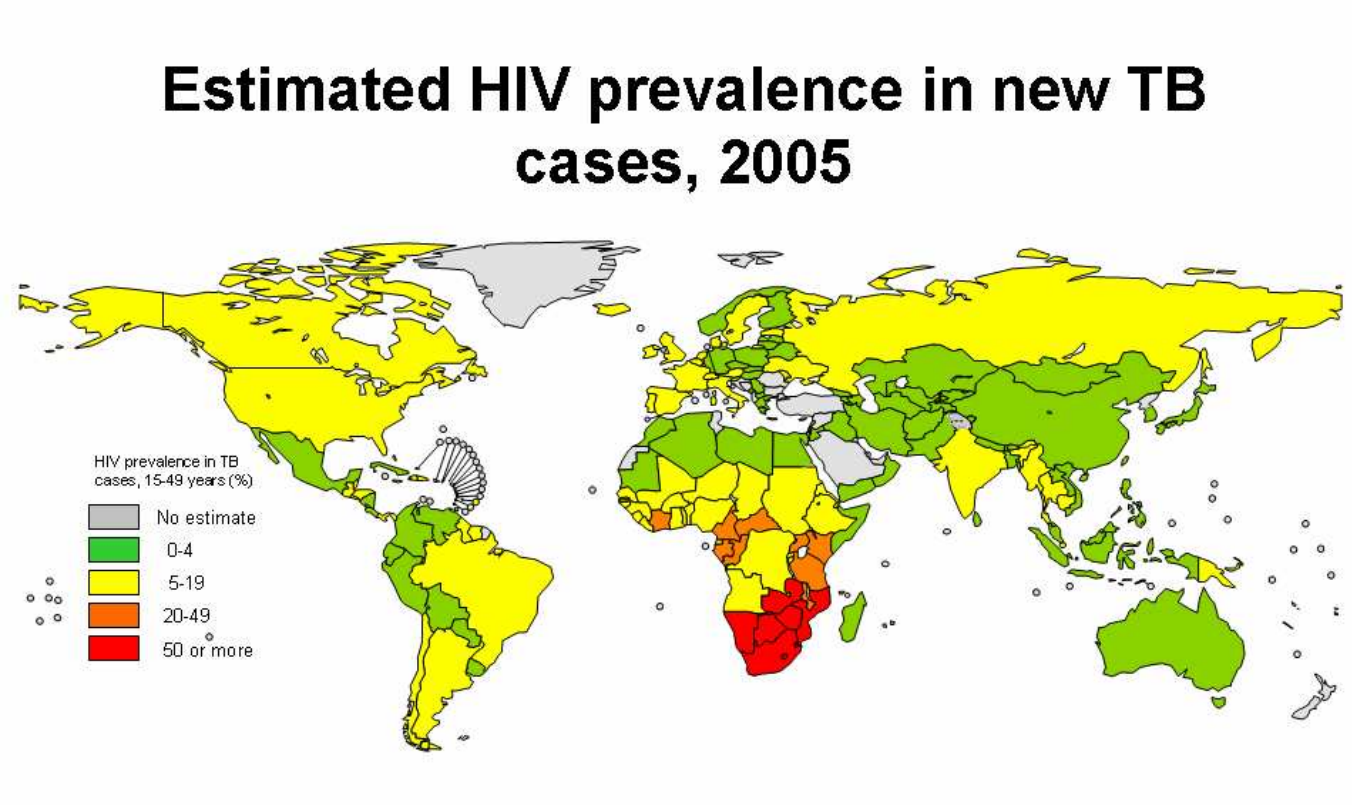


Figure 1.1 Estimated HIV prevalence in new tuberculosis cases in 2005 [6].

1.2 Pathogenesis of HIV infection, related opportunistic infections and coinfection with HIV/*M. tuberculosis*

The Human Immunodeficiency Virus is a member of the genus Lentivirus, part of the family of Retroviridae [7]. Lentiviruses are transmitted as single-stranded, positive-sense, enveloped RNA viruses. Entry of the target cell is mediated through interaction of the virion envelope glycoprotein (gp120) with the CD4 receptor on the target cells and also through chemokine coreceptors such as CCR5 (=chemokine (C-C motif) receptor 5) and CXCR4 (=chemokine (C-X-C motif) receptor 4) [8]. The viral RNA genome is then converted into double-stranded DNA by the virally encoded enzyme reverse transcriptase that is present in the virus particle. The viral DNA is subsequently integrated into the cellular DNA by the virally encoded integrase, along with different host cellular cofactors, and the genome is then transcribed [9]. After infection of the target cells, two pathways are possible: either the virus becomes latent and the infected cell continues to function,

or the virus becomes active and replicates, and a large number of virus particles are liberated that thus can infect other cells [10].

Two species of HIV have been described to infect humans: HIV-1 and HIV-2 [11]. HIV-1 was initially discovered and is more virulent, relatively easily transmitted, and the cause of the majority of HIV infections globally. HIV-2 is less transmittable than HIV-1 and is largely confined to Western parts of Africa.

Infections caused by HIV are characterised by a massive immune activation and a subsequent destruction of the acquired cellular immune system. HIV infects immune cells that carry the CD4 molecule, i.e. CD4+ T helper cells in the first place as well as dendritic cells and macrophages [10]. When a human organism has become newly infected, there is an initial massive increase of the viral load in the blood, and subsequently the number of CD4+ T lymphocytes in the majority of cases declines within the first few weeks of infection [10, 12]. The initiated immune response leads to a production of HIV specific antibodies (seroconversion) and to an induction of cytotoxic T cells [13, 14]. In the majority of chronically infected persons adaptive immune responses cannot achieve complete suppression of the viral replication. Yet in most cases immune responses can lead to a significant reduction of the viremic load and thus prevent progression of the disease by establishing a viral replication setpoint reflecting both HIV virulence and host immune responses [15, 16]. Within this latency period, which can take up to several years, patients usually do not develop HIV related symptoms deriving from opportunistic infections, although the incidence of some opportunistic infections, such as pneumococcal and candida infections, is already increased at this stage of HIV infection [17]. Nevertheless, without effective suppression of the viral replication, HIV infection is characterised by a continuous loss of CD4 T cells initiating a disease progression which finally leads to the stadium of AIDS [18].

Although HIV infection remains an uncurable disease, nowadays, several antiretroviral drug agents are available in order to prevent progression of HIV infection [19]. The prognosis of those living with HIV and AIDS has improved significantly due to highly active antiretroviral therapy (HAART), a combination therapy of at least three antiretroviral agents that was first initiated in 1996. The three initial classes of antiretroviral drugs used in HAART are the nucleoside reverse transcriptase inhibitors (NRTI, such as Lamivudine or Zidovudine), non-nucleoside reverse transcriptase inhibitors (NNRTI, such as Nevirapine) and

protease inhibitors (PI, such as Lopinavir or Ritonavir). New drugs are now available in these classes (second generation NNRTI and novel PI) as well as new classes of drugs, integrase inhibitors, CCR5 antagonists and fusion inhibitors [20].
Without sufficient antiretroviral treatment, most patients at one point progress to the stadium of AIDS. At the stage of AIDS patients may be affected by multiple complications deriving from the progressional immunodeficiency. These complications mainly include different opportunistic infections but also other illnesses such as malignancies.
The most common opportunistic infections belonging to the AIDS defining illnesses include infections caused by *M. tuberculosis, Salmonella spec., Pneumocystis jirovecii,* Candida spec., Herpes simplex virus, Cytomegalovirus, Epstein-Barr virus, Varicella zoster virus, *Toxoplasma gondii* or *Cryptococcus neoformans* [21].
As HIV infection progresses, the patients become more and more vulnerable to intracellular pathogens (*Mycobacterium avium* complex, *Toxoplasma gondii*, Epstein-Barr virus) and fungal infections (*Pneumocystis jirovecii* and Candida spec.), as overcoming those infections requires intact T cell responses of the host.
The point at which these conditions occur mostly depends on the status of immunodeficiency, which can be quantified by means of measuring the viral load and counting the number of CD4+ T lymphocytes in the blood (CD4 count) [22].
The normal range of the CD4 count is 500-1200 CD4+ cell per microliter (µl) blood.
In HIV infection most severe opportunistic infections occur when the CD4 count falls below 200 or even 100 cells per µl [23].

In this regard, *M. tuberculosis* (see **chapter 1.3**) is different from most other opportunistic infections, including all other mycobacterial infections. Although the risk of tuberculosis is increased with advanced immunosuppression, it is known to occur at all stages of HIV infection [24-27].
Coinfection with HIV and *M. tuberculosis* is among the greatest health problems in the world [3] with one condition worsening the other. Not only does HIV increase the risk of reactivating a latent tuberculosis infection (LTBI) [28], it also increases the risk of rapid tuberculosis progression soon after infection or reinfection [29, 30].
In immunocompetent persons with LTBI the lifetime risk of developing active tuberculosis is estimated to be 5-10 percent [2], whereas in HIV seropositive

persons with LTBI the risk of tuberculosis increases shortly after seroconversion, doubling within the first year [31]. Later on in HIV infection the annual risk exceeds 10 percent [32], with the risk increasing further with serious immunosuppression; an annual incidence of tuberculosis as high as 30 percent has been reported in South African patients with clinically advanced HIV [3, 30].

Tuberculosis in HIV seropositive individuals is almost certain to be rapidly fatal if undiagnosed or left untreated. Tuberculosis is the major cause of death among HIV infected persons in the developing world. However, infections with HIV and *M. tuberculosis* act synergistically in several ways: HIV replication takes place in *M. tuberculosis*-specific CD4+ T cells, while tuberculosis-induced immune activation may lead to a faster progression of HIV infection [33, 34].

1.3 Pathogenetic features of infections with *M. tuberculosis*

Mycobacterium tuberculosis is an obligate aerobe, slow-growing, acid- and alcohol-fast bacillus (AAFB, Ziehl-Neelsen or Kinyoun staining) lacking an outer cell membrane [35]. *M. tuberculosis* was first described in 1882 by Robert Koch [35], who subsequently received the Nobel Prize in Physiology or Medicine for this discovery in 1905.

Infections caused by *M. tuberculosis* spread through direct person-to-person contact and are usually conducted aerogenically by the bacilli being inhaled into the lung, with the lung being the most common and important primary site of tuberculosis infection. Following inhalation the bacilli reach the pulmonary alveoli. As Mycobacteria have a cell wall that contains high amounts of glycolipids and waxes, innate immune responses are often weaker than in other bacterial infections (such as bacterial pneumonia). In addition, *M. tuberculosis* has evolved mechanisms to escape the innate immune system [36].

Another important characteristic of *M. tuberculosis* is its ability to survive and replicate within macrophages, thus it is able to escape the host's immune response [37, 38]. Macrophages routinely engulf bacteria by phagocytosis (a specialised form of endocytosis for the uptake of large particles, including bacteria). Normally, the organelles formed around bacteria (phagosomes) merge together with lysosomes and mature into hydrolytic phagolysosomes. Here, the degradation

of bacterial proteins provides peptides for antigen presentation to lymphocytes. Phagosomes containing virulent *M. tuberculosis* are able to persist within the cell as organelles known as parasitophoruses or (mycobacterial) vacuoles, so that the fusion of phagosomes and lysosomes is inhibited. This prevents the induction of apoptosis of the macrophage so that *M. tuberculosis* does not get eliminated [38, 39].

Despite of *M. tuberculosis* having the ability to survive within macrophages, some bacteria do get eliminated or are at least stopped from replication by antibacterial mechanisms including reactive oxygen and nitrogen intermediates produced by activated macrophages [37].

M. tuberculosis-specific responses of the acquired immune system mainly consist of cellular responses, whereas humoral (B lymphocyte mediated) immune responses do not play an important role in the immune defense against *M. tuberculosis* [40]. The different T cell populations produce interferon-γ (IFN-γ) and hence are of the T helper 1 (Th1) type. This cytokine is the central mediator of macrophage activation and synergises with tumour necrosis factor-α (TNF-α) in activating macrophages [37].

Protective immunity to *M. tuberculosis* is characterised by the formation of granulomas at the site of infection [41]. These granulomas primarily consist of T lymphocytes and *M. tuberculosis*-infected macrophages [42] and do not only function to prevent dissemination of the mycobacteria, but also provide a local environment for the communication of different cells of the immune system. Within the granulomas, T lymphocytes (CD4+) secrete cytokines such as IFN-γ, which activate macrophages to destroy the bacteria they are infected with [37]. Cytotoxic T lymphocytes (CD8+) can also directly kill infected cells. Six to eight weeks after infection, and coincident with the development of a delayed-type hypersensitivity response manifested by Tuberculin Skin Test (TST) positivity (see **chapter 1.5**), these granulomas undergo caseous necrosis, resulting in the death of the majority of tubercle bacilli and destruction of the surrounding host tissue. The small proportion of surviving bacilli are thought to exist in a non-replicating hypometabolic state as an adaptation to the unfavourable milieu in the solid caseous material [41, 42].

1.4 Clinical aspects of tuberculosis, diagnostic tools, treatment standards and BCG-vaccination

After a newly acquired infection with *M. tuberculosis* a positive immune response against mycobacterial antigens can be observed after around 2-8 weeks [43]. Direct identification of individuals who are infected with viable *M. tuberculosis* but do not develop active tuberculosis is currently not possible. Diagnostic tests used to identify individuals latently infected with *M. tuberculosis*, i.e. the *in vivo* Tuberculin Skin Test (TST; see **chapter 1.5**) and the *ex vivo* interferon-γ release assays (IGRA; see **chapter 1.6**), are designed to identify a memory adaptive immune response to mycobacterial antigens. The proportion of individuals who truly remain infected with *M. tuberculosis* after TST or IGRA conversion is unknown. It is also uncertain how long adaptive immune responses to mycobacterial antigens persist in the absence of live mycobacteria. Based on the informative value presently derived by the TST and IGRA, a positive result at best implicates a lasting immune response to *M. tuberculosis*, but not necessarily a latent infection *[44, 45]*.

According to data derived from TST results, 5-10 percent of the persons with a positive reaction will develop active tuberculosis [2]. Recent studies on IGRA have suggested this number to be even higher [46]. Active tuberculosis can still manifest several years after initial contact to *M. tuberculosis.* Nevertheless, around 50 percent of active tuberculosis cases occur during the first two years after contact [47].

If tuberculosis manifests as active disease, clinical symptoms are cough, fever, weight loss, night sweats and loss of appetite. Risk factors for tuberculosis - regardless of social background - are: innate or acquired T cell dependent immune defects (such as HIV infection), immunosuppressive therapy, long-term steroid therapy, alcoholism, diabetes mellitus, chronic renal failure and gastrectomy [48].

Not only the lungs can be affected by tuberculosis: If *M. tuberculosis* bacilli gain entry into the bloodstream from an area of damaged tissue they may spread through the body and set up many foci of infection, all appearing as tiny white tubercles in the tissues, such as lung apices, peripheral lymph nodes, kidneys, brain and bones, in fact, all parts of the body can be affected. This severe form of tuberculosis is called miliary tuberculosis. It is more common in infants, the elderly

and immunosuppressed persons. In patients with this disseminated tuberculosis the fatality rate is approximately 20 percent, even with intensive treatment [49].
Tuberculosis can be very difficult to diagnose, mainly due to the difficulty in culturing this slow-growing organism in the laboratory. A complete medical evaluation for tuberculosis must include a medical history, a chest radiography, and a physical examination. Furthermore the diagnostics include a TST, sputum AAFB smears and cultures. Sputum smears and cultures for AAFB can be performed if the patient is producing sputum, otherwise specimens can be obtained by inducing sputum, or by fine needle aspiration or biopsy taking during bronchoscopy with bronchoalveolar lavage [50].
Approximately 50 percent of all culture confirmed tuberculosis cases are negative in three consecutive AAFB smears [51]. Therefore, even if the sputum smear is negative, tuberculosis must be considered and can only be excluded after negative cultures. If the smear is positive, PCR or gene probe tests can distinguish *M. tuberculosis* from other mycobacteria.

Bacille Calmette-Guérin (BCG) was developed in the early 20th century as a vaccine against tuberculosis. It is prepared from a strain of the attenuated (weakened) live bovine tuberculosis bacillus, *Mycobacterium bovis*, that has lost its virulence in humans by being specially cultured. The bacilli have retained enough strong antigenicity to become a somewhat effective vaccine for the prevention of human tuberculosis. At best, the BCG vaccine is 80 percent effective in preventing tuberculosis for a duration of 15 years [52], however, its protective effect appears to vary according to geography and is dramatically reduced in HIV-seropositive induviduals [53, 54].

The standard "short" course treatment for tuberculosis, in case of active disease, consists of isoniazid, rifampin, pyrazinamide, and ethambutol for two months (so called “first-line drugs”), then isoniazid and rifampin alone for a further four months. The patient is considered to be cured after six months when sputum tests are negative (although there is still a relapse rate of two to three percent). For latent tuberculosis, the standard treatment is six to nine months of isoniazid alone. Further treatment options are second-line drugs such as fluoroquinolones, capreomycin, amikacin, kanamycin.

Lately, the emergence of infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis* has created major problems concerning the treatment and outcome of tuberculosis [55]. MDR-TB is defined as resistant to rifampin and isoniazid, regardless of other drug resistances. Four percent of all TB-patients are estimated to be infected with an MDR-TB strain, the highest numbers of MDR-TB worldwide (10-40 percent) have been reported for Eastern Europe [55]. XDR-TB is by definition resistant to at least rifampin and isoniazid, in addition to any fluoroquinolone and at least one injectable second-line drug (capreomycin, amikacin, kanamycin). Extremely high death rates have been reported for XDR-TB cases among HIV-seropositive patients [56].

1.5 The Tuberculin Skin Test (TST)

The TST (synonym: Mendel-Mantoux-Test) was the gold standard for diagnosing (latent) tuberculosis infections in the last century [57]. Tuberculin, also known as purified protein derivate (PPD), was first described by Robert Koch in 1891 [58]. It consists of a precipitate of non-species-specific molecules obtained from filtrates of sterilised, concentrated cultures from tubercle bacilli. The test is named after Charles Mantoux, a French physician who developed the test together with Felix Mendel in 1907 based on the work of Koch and Clemens von Pirquet [59].

The internationally most widely used Tuberculin preparation consists of 2TU of Statens Serum Institute (SSI) Tuberculin RT23 in 0.1ml solution for injection. This standard dose of Tuberculin is injected intradermally preferably at the volar side of the forearm, the results are read 48 to 72 hours later. A reaction is considered positive if a visible and palpable induration greater than at least 5mm (not including erythema) appears in the skin at the sight of the injection. The induration is measured most reliably by the use of the ball-point pen and ruler method [60].

The immunological reaction to Tuberculin is classified as a delayed-type-hypersensitivity reaction (DTH) or type IV reaction according to Gell and Coombs [61]. It serves to test a person's immunological memory for tuberculous antigens by evoking a T cell dependent local inflammatory response. After inoculation of the antigen into the skin it is phagocytosed by antigen presenting cells (APC, i.e.

dendritic cells or Langerhans-cells), which subsequently present processed antigen peptides to CD4+ Th1 lymphocytes via the MHC-class II molecule. If the immune system has previously been exposed to *M. tuberculosis*, antigen specific memory T cells recognize the antigen and start secreting pro-inflammatory cytokines such as IFN-γ and TNF-α. These cytokines stimulate the expression of adhesion molecules at the endothelium and locally increase capillary permeability. Furthermore, monocytes and macrophages are activated and recruited to the site of antigen inoculation. Plasma and accessory cells invade the area.

The macroscopic result of the reaction is a visible and palpable inflammatory edema and induration in the skin. The maximum of the response can be expected 24-72 hours after the injection. Intense reactions may be associated with fever, more general swelling of the limb, general lymphadenopathy or rarely lymphangitis [62].

Microscopically the test area shows edema and a dense infiltration of the dermis by mononuclear cells, especially around small blood vessels [62]. It has been described that these cellular infiltrates predominantly consist of activated lymphocytes, especially T lymphocytes with a CD4+ phenotype, and monocytes [63-65]. Nevertheless, the exact composition of the cellular infiltrates and the interactions and immunological regulations of this DTH reaction to Tuberculin are still poorly understood.

Knowledge of TST sensitivity and specificity, as well as the positive predictive value, is required to interpret skin test reactions properly [66]. For persons with LTBI and normal immune responsiveness, test sensitivity approaches 100 percent [67]. However, false-positive TST results occur in persons who have been infected with non-tuberculous mycobacteria and in persons who have received BCG vaccination. These false-positive reactions result in a lower specificity and a low positive predictive value in persons who have a low probability of LTBI. Even if the test has a specificity approaching 99 percent, testing of persons in such low-prevalence groups would result in most positive tests being false-positive [68]. However, the specificity of the test is also dependent on the criterion used to define a “positive” test. The specificity can be improved by progressively increasing the reaction size that separates positive from negative reactors (at the

expense of decreasing test sensitivity) [67]. **Table 1.1** summarises criteria for appropriate interpretation of TST reactions among different risk groups.

Table 1.1 Criteria for the interpretation of a positive Tuberculin Skin Test reaction, stratified by risk groups, according to recent guidelines of the American Thoracic Society (ATS) [76].

Positive TST reaction if ≥ 5mm of induration	Positive TST reaction if ≥ 10mm of induration	Positive TST reaction if ≥ 15mm of induration
HIV infected persons	Recent immigrants (i.e. within the last 5 years) from high prevalence countries	Persons with no risk for TB
Recent contacts to active tuberculosis cases	Injecting drug users	
Patients on immunosuppression after organ transplantation	Residents and employees of high-risk congregate settings (prisons, hospitals, nursing homes, health-care facilities, homeless shelters)	
Patients on other immunosuppression (equivalent of ≥ 15 mg Prednisone/day for at least 1 month)	Mycobacteriology laboratory personnel	
	Persons with one of the following clinical conditions: silicosis, diabetes mellitus, chronic renal failure, lymphoma, leukemia, other specific malignancies, ≥ 10% weight loss of ideal body weight, gastrectomy, jejunoileal bypass	
	Infants and children younger than 4 years of age, children and adolescents exposed to adults at high-risk	

Furthermore, the TST can also resolve in false-negative results. These can generally occur if a person's immune system is not capable of mounting an adequate immune response to PPD. This can be the case during recently acquired and active infection with *M. tuberculosis* and other severe active illnesses, or can be due to different conditions of immunosuppression, most importantly HIV infection [69]. HIV-seropositive persons, particularly at advanced stages of immunecompromitation, often fail to mount a positive reaction in the TST despite a high likelihood of LTBI [70, 71]. As HIV infected individuals are at high risk to develop active tuberculosis, it is most crucial for this group that LTBI can be reliably detected so that the patients can subsequently receive preventive anti-tuberculous drug therapy [72-75].

1.6 Alternative diagnostic tests

Recently important advances have been made in establishing new tests for the immunodiagnosis of tuberculosis. Those new test assays are called (T cell) interferon (IFN)-γ release assays (IGRA). The principle of IGRA is the detection and quantification of IFN-γ production by peripheral blood mononuclear cells (PBMC) following *ex vivo* contact with the recombinant *M. tuberculosis* region of difference 1 (RD1)-restricted antigens early secreted antigenic target -6 (ESAT-6) and culture filtrate protein-10 (CFP-10) that are absent from BCG and most non-tuberculous mycobacteria (NTM).

Two IGRA test systems are commercially available: the ELISA test that measures IFN-γ production following antigen contact in whole blood (QuantiFERON TB-Gold In-Tube; Cellestis, Carnegie, Australia) and the ELISPOT test that allows the identification of single IFN-γ producing cells (spots) on the bottom of a microtiter plate following antigen encounter (T-Spot.TB; Oxford Immunotec, Abingdon, UK).

The main advantage of IGRA compared with the TST is that they are not influenced by prior BCG-vaccination or infections with most environmental (non-tuberculous) mycobacteria, which is reflected in a higher specificity [76, 77]. IGRA also have a higher sensitivity than the TST, especially in immunosuppressed persons [78-80].

IGRA have been designed to test for *M. tuberculosis* specific T lymphocytes in the circulating blood, but despite of showing a greater sensitivity and specificity than the TST, those tests are still not able to distinguish between active tuberculosis and latent infection. In order to overcome this problem, several recently conducted studies show promising results in using IGRA for testing of cells obtained directly from the actual sides of infection, i.e. from broncho-alveolar lavage [51, 81], from pleural fluid [82], pericardial or cerebro-spinal fluid [83].

1.7 Immunoregulatory mechanisms in HIV/*M. tuberculosis* coinfection: Regulatory T cells

In addition to the general depletion of CD4+ T cells during HIV infection, another factor possibly contributing to the high susceptibility of HIV infected persons to opportunistic infections could be the observed prevalence of remarkably high levels of regulatory T cells (Tregs) in advanced HIV infection [84-86].

Regulatory T cells are described as a subgroup of T lymphocytes with immunosuppressive properties, characterised by bearing CD25 as cell surface marker and expressing the transcription factor forkhead box p3 (FoxP3) [87]. Tregs play a crucial role in maintaining immunological self tolerance, i.e. by augmenting the suppression of immunological responses, for example by means of screting interleukin (IL)-10 and transforming growth factor (TGF)-β [88]. Furthermore, they are also generally upregulated at the site of an ongoing infection in order to prevent fulminant and thus self-destroying immune responses to the original pathogen. Tregs down-regulate alloreactive T cells that recognise antigens from tumors and allografts, and thereby reduce anti-tumor immunity, graft rejection, and graft-versus-host disease [89]. Tregs also control the balance between Th1 (cellular) and Th2 (humoral) immune responses [86].

Numbers of CD25+FoxP3+ Tregs have been shown to be remarkably increased in HIV infection [84]. Furthermore they have been associated with progressed stages of the infection [85, 86]. In HIV infected persons, Tregs contribute to the inhibition of effective immune reactions to HIV itself, but they can also promote susceptibility of the organism to opportunistic infections [84-86].

Regulatory T cells could furthermore play an important role during tuberculosis, as it has also been shown for patients with tuberculosis that high levels of CD25+FoxP3+ Tregs are detectable during the infection [90]. Also in this case Tregs seem to be contributing to ongoing infection by means of down-regulating the cellular immune responses to *M. tuberculosis* [91-93].
The role of Tregs during HIV/*M. tuberculosis* coinfection has not been clearly investigated so far, and it is thus not known whether they can be considered an important player in the interaction of HIV and tuberculosis.

1.8 Rationale for the study

Epidemiology and clinical features of HIV associated tuberculosis are well researched, but few studies have addressed the cellular mechanisms and immunological regulations in detail. Increased knowledge in this field could have important consequences when interventions that may improve the impaired immune ability of HIV infected people to resist *Mycobacterium tuberculosis* are considered.
As HIV infects CD4+ T lymphocytes, the currently most characteristic and useful index of immune depletion in HIV infection is the CD4+ lymphocyte count. Thus as HIV infection progresses and the CD4+ lymphocyte count falls, susceptibility to tuberculosis and the likelihood of exhibiting a false negative response in the TST increases [70, 94]. Furthermore, the likelihood of developing active tuberculosis or experience reactivation of LTBI is dramatically increased in HIV infection, while immunocompetent persons succeed in controlling and preserving the infection.
As the TST is operationally impaired by HIV infection, its administration is an opportunity to understand the response to mycobacterial antigens in HIV infected people better. It is safe and a relatively standardised test and its route of administration (intradermally) facilitates the characteristic DTH reaction. Tuberculin stimulates *M. tuberculosis* experienced T cells to expand in order to assist intracellular containment of *M. tuberculosis*. Currently it is not completely understood which parts of the immune system are involved in the generation of a positive reaction in the TST. Especially it is not known which mechanisms lead to an attenuation of this reaction in patients with advanced HIV infection. Additionally,

Tuberculin may also stimulate regulatory or anti-inflammatory T cells (FoxP3+) [87], that have been described to be increased in the peripheral blood in persons with HIV-associated tuberculosis [90, 92].

Detailed immunologic analysis of TST reactions in HIV infected compared to uninfected persons could therefore provide an invaluable opportunity to study, at a site of inflammation, the immune dysregulation that characterises the increased susceptibility of HIV infected persons to tuberculosis. Information from such sites may contribute to the design of interventions that could possibly improve the situations of HIV/*M. tuberculosis* coinfected persons.

In this study, reactions in the TST in HIV-seropositive and seronegative persons from a South African community with a very high incidence of tuberculosis and a high prevalence of HIV have been observed. In particular, different immunophenotypes of mononuclear cells from the peripheral blood and of immune cells prevalent in the skin at the site of Tuberculin injection have been investigated.

2 Material and Methods

2.1 Study design

This study was designed as a collaboration between the Institute of Infectious Diseases and Molecular Medicine (IIDMM) at the University of Cape Town, South Africa, the Center of Infectious Medicine (CIM) at the Karolinska Institute in Stockholm, Sweden, and the Research Center Borstel. The respective contributions of the different institutions are shown in **figure 2.1.**

The study was funded by the H.W.&J. Hector Foundation and the German Academic exchange program (DAAD).

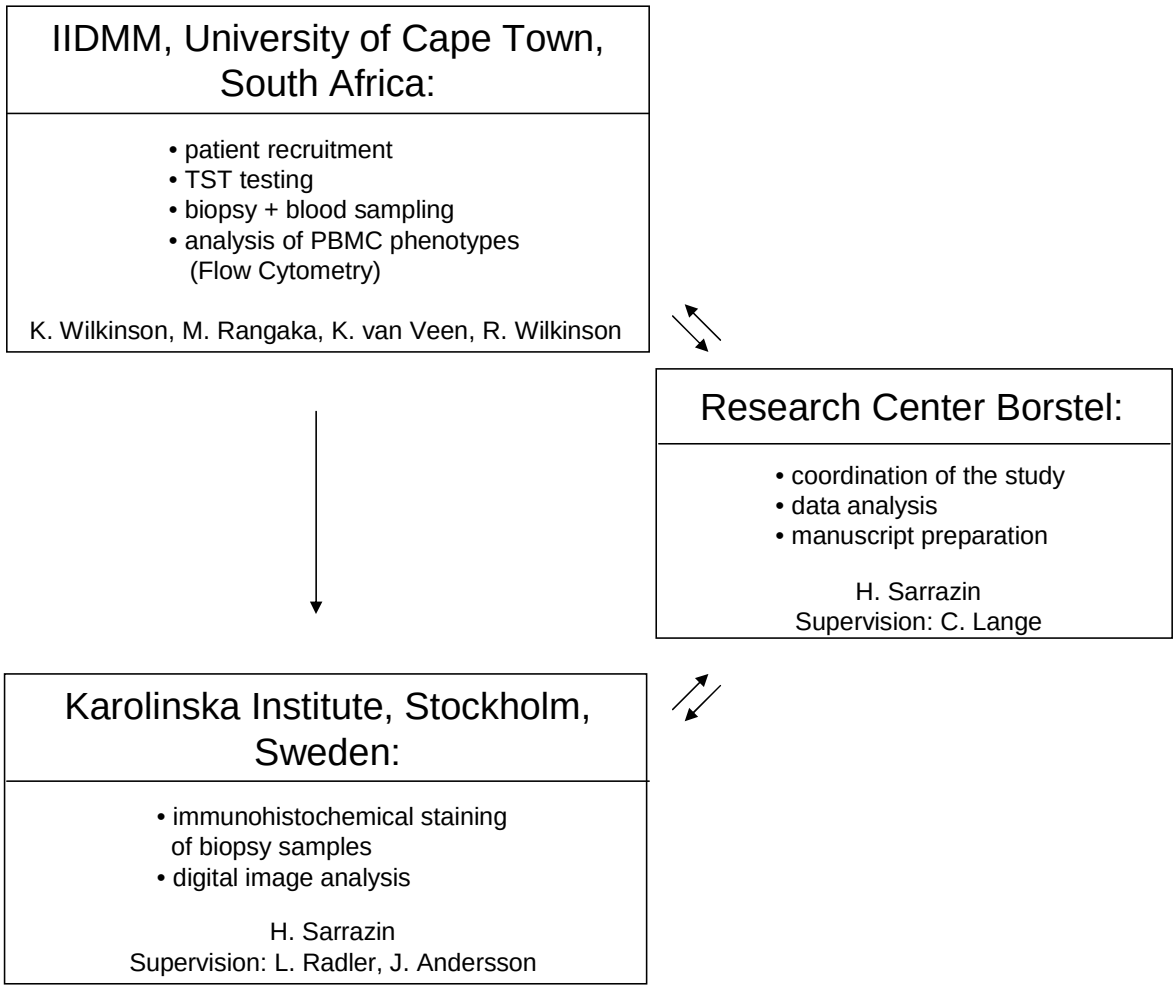


Figure 2.1 Study design

2.2 Patients included in the study

The patients that were recruited and included in this study were inhabitants of a South African township called Khayelitsha. Khayelitsha is situated in the north east of Cape Town, South Africa. It is the third biggest township in South Africa. According to recent estimates, approximately 500,000 – 1 million people live in Khayelitsha, most of them under very underprivileged conditions.

Figure 2.2 Photograph of Khayelitsha Township, South Africa.

According to latest calculations, tuberculosis incidence in Khayelitsha is 1,612 per 100,000 inhabitans per year [78, 95]. The prevalence of HIV-1 infection is also extremely high in Khayelitsha, it is believed that around 20 percent of the people are infected with HIV-1 [95].

Adults resident in Khayelitsha and attending Voluntary Counselling and Testing (VCT) for HIV infection were invited to participate. The study was approved by the research ethical committees of the University of Cape Town, South Africa (REC 175/2005) and the University of Luebeck, Germany (REC 04/176). All participants provided informed consent. Subjects enrolled in the study included both HIV-1 seropositive and HIV-1 seronegative persons. All HIV-1 infected persons were Antiretroviral Therapy (ART) naive. Persons testing seronegative for HIV-1 were

invited to participate after post-VCT counselling on the day of their negative test. Recruitment of persons found HIV-seropositive was deferred until their first visit to the HIV/AIDS clinic in order not to have them recruited before they had time to accept their HIV diagnosis. Prior tuberculosis, isoniazid preventive therapy (IPT), steroid therapy, pregnancy, and in HIV infected people current opportunistic infections or a Karnofsky score ≤ 60%, indicating a progressed stadium of the disease (the Karnofsky score system provides information on the status of disease referring to symptoms and reaches from 100%=no current symptoms or signs of disease to 0%=death), were exclusion criteria. The assessment of eligibility included a symptom screening questionnaire and physical examination for active tuberculosis based upon validated approaches [96, 97]. The presence of any symptoms such as cough, chest pain, recent weight loss, night sweats, fever, loss of appetite, swelling of lymph nodes or generalised tiredness formed an exclusion criterion and triggered referral.

2.3 Performance of the Tuberculin Skin Test and biopsy taking

The patients included in the study underwent Tuberculin skin testing. 0.1ml 2TU Tuberculin (RT23, Statens Serum Institute) were injected intracutaneously on the volar side of one of their forearms.

Additionally to the TST on the one forearm, the same site at the other forearm was used for injecting 0.9% NaCl as a negative control.

TST reactions (diameter of the visible and palpable induration in the skin in millimeters) were read 48-72 hours after the injection of Tuberculin by the use of the ball-point pen and ruler method (**figure 2.3**) [60].

Positivity was defined as an induration diameter of >5mm according to ATS guidelines [98].

Figure 2.3 Photograph of a positive reaction in the Tuberculin Skin Test measured by the ball-point pen and ruler method. The diameter of the shown reaction measures 13mm.

Biopsy samples were obtained from the site of the Tuberculin injection: A 4mm sample was extracted via punch biopsy after anaesthesia of the surrounding skin by infiltration of 1% lidocaine. Steristrips were used for closing up the wounds.
The same procedure was applied for taking samples of normal skin from the control sites infiltrated with NaCl, in this case the samples measured only 2mm.

2.3 Blood samples

In order to analyse phenotypes and distributions of the peripheral blood mononuclear cells (PBMC), each patient had a 20ml blood sample drawn from a peripheral vein.

2.4 Immunophenotypisation of mononuclear cells in the blood

After the venous blood draw PBMC were separated by the use of standard protocols. Cells were left resting overnight in 96U plates at 10^6/ml cell culture

medium (Roswell Park Memorial Institute (RPMI) / 10% fetal calf serum (FCS)), plated at 200µl/well.

In the morning cells were stained for 20 minutes on ice; this was done by using the following fluorescent antibodies (all from BD Biosciences, San Diego, California, USA): CD4-PerCP, CD62L-FITC, CD45RA-PE, CD45RO-FITC, HLA-DR-PE, CD28-FITC, CD8-PerCP. After being washed, stained cells were fixed in phosphate buffered saline (PBS) / 2% FCS containing 1.6% paraformaldehyde (PFA) and analysed on a BD- Fluorescence Activated Cell Sorting (FACS) Calibur Flow Cytometer. Intracellular staining for Foxp3 was performed with the PE anti-human FoxP3 staining set from eBioscience (San Diego, California).

Cells were first surface stained as described above, followed by incubation for 45 minutes on ice in eBioscience Fix/Perm buffer. Cells were washed in eBioscience Permeabilisation Buffer and incubated for 15 minutes on ice in 2% normal rat serum, followed by incubation for 30 minutes on ice in 10µl anti-human FoxP3 (PCH101) antibody. Cells were washed again, fixed in PBS / 2% FCS containing 1.6% PFA, and analysed on a BD-FACS Calibur Flow Cytometer.

2.5 Interferon-γ RD 1 based ELISPOT analysis

20ml of blood were drawn for overnight IFN-γ ELISPOT analysis employing the recombinant RD1 encoded antigens CFP-10 and ESAT-6 as it has been described in previous studies [95, 99, 100]. The ELISPOT assay, based on the principle of a sandwich capture ELISA, detects IFN-γ molecules in the immediate vicinity of the T cell from which they are secreted, while they are still at a high concentration. After its development each resulting spot thus represents the “footprint” of an individual antigen-specific IFN-γ secreting T cell, or spot-forming cell (SFC). The *ex vivo* ELISPOT assay for IFN-γ is sufficiently sensitive in order to detect antigenspecific T cells directly from peripheral blood, without the need for a prior *in vitro* stimulation step. Moreover, since the *ex vivo* ELISPOT assay enumerates antigen-specific T cells with rapid effector function, only short incubation periods are required. Separated and cultured PBMC were put onto 96-well polyvinylidene difluoride–backed plates (MAIPS45; Millipore, Billerica, MA, USA) precoated with

15 mg/ml of anti-IFN-γ monoclonal antibody (MAb) 1–D1K (Mabtech, Sweden). PBMC were then blocked with RPMI+ 10%FCS (R10) for two hours and were afterwards added in 100ml R10/well, and each of the ESAT-6- and CFP10-derived peptides was individually added to single wells at 10 mg/ml. Positive control wells containing phytohaemmagluttinin (5 µg/ml) were included. After a 14-hour incubation at 37.7℃, 5% CO_2, plates were washed with PBS 0.05% Tween-20 (Sigma Aldrich, USA). Next, 100 ml of 1 mg/ml biotinylated anti–IFN-γ MAb, 7-B6-1-biotin (Mabtech), was added. After two hours, the plates were washed, and streptavidin-alkaline phosphatase conjugate (Mabtech) was added to a dilution of 1:1000. After one hour and further washing, 50ml of chromogenic alkaline phosphatase substrate (Biorad, CA, USA), diluted 1:25 with deionized water, were added. After 20 minutes the plates were washed and allowed to dry [99].
A positive response was defined as ≥ 20 spot forming cells/million PBMC. Absence of reactivity to this stimulus in the absence of reactivity to any antigen was scored as indeterminate.

2.6 Storage and transport of the biopsy samples

All biopsy samples were immediately placed into a 1ml screw-topped sterile eppendorf, labelled and snap-frozen at the clinic in steel thermos containing liquid nitrogen and a small block of dry ice. They were then transported to the laboratory and racked in the -80℃ freezer.

After the sample collection was completed the samples were transported on dry ice overseas to the Karolinska Institute in Stockholm, Sweden, where further analyses were conducted in the laboratory of Prof. Jan Andersson at the Center of Infectious Medicine.
There the biopsies were also stored in the -80℃ fr eezer.

2.7 Preparation and fixation of the biopsy samples

The biopsy samples were carefully removed out of the eppendorfs using cooled tweezers. They were then cryoconserved in OCT mounting medium (Tissue-Tek Sakura Finetek Europe B.V Woeterwoude, NL). The samples were cut at a Cryostat into 8µm thin sections (at a temperature of -16 to -19℃). The freshly cut sections were put onto SuperFrost+ glass slides (Menzel-Gläser, Menzel GmbH & Co, Braunschweig, Germany), each slide containing six sections of one biopsy sample, and immediately fixed for 20-30 minutes with 2% formaldehyde (Sigma Aldrich, St. Louis, USA) diluted in 0.01M PBS (phosphate buffered saline). After the fixation the slides were stored in a freezer at -4℃.

2.8 Immunohistochemistry of the biopsy samples

2.8.1 General aspects of immunohistochemistry

Immunohistochemistry (IHC) is the localisation of antigens in tissue sections by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualised by a marker such as fluorescent dye, enzymes, radioactive elements or colloidal gold [101].

Albert H. Coons and his colleagues were the first to label antibodies with a fluorescent dye, and used it to identify antigens in tissue sections [102]. With the expansion and development of the immunohistochemistry technique enzyme labels have been introduced such as peroxidase and alkaline phosphatase. Colloidal gold label has also been discovered and used to identify immunohistochemical reactions at both light and electron microscopy level. Other labels include radioactive elements, and the immunoreaction can be visualised by autoradiography.

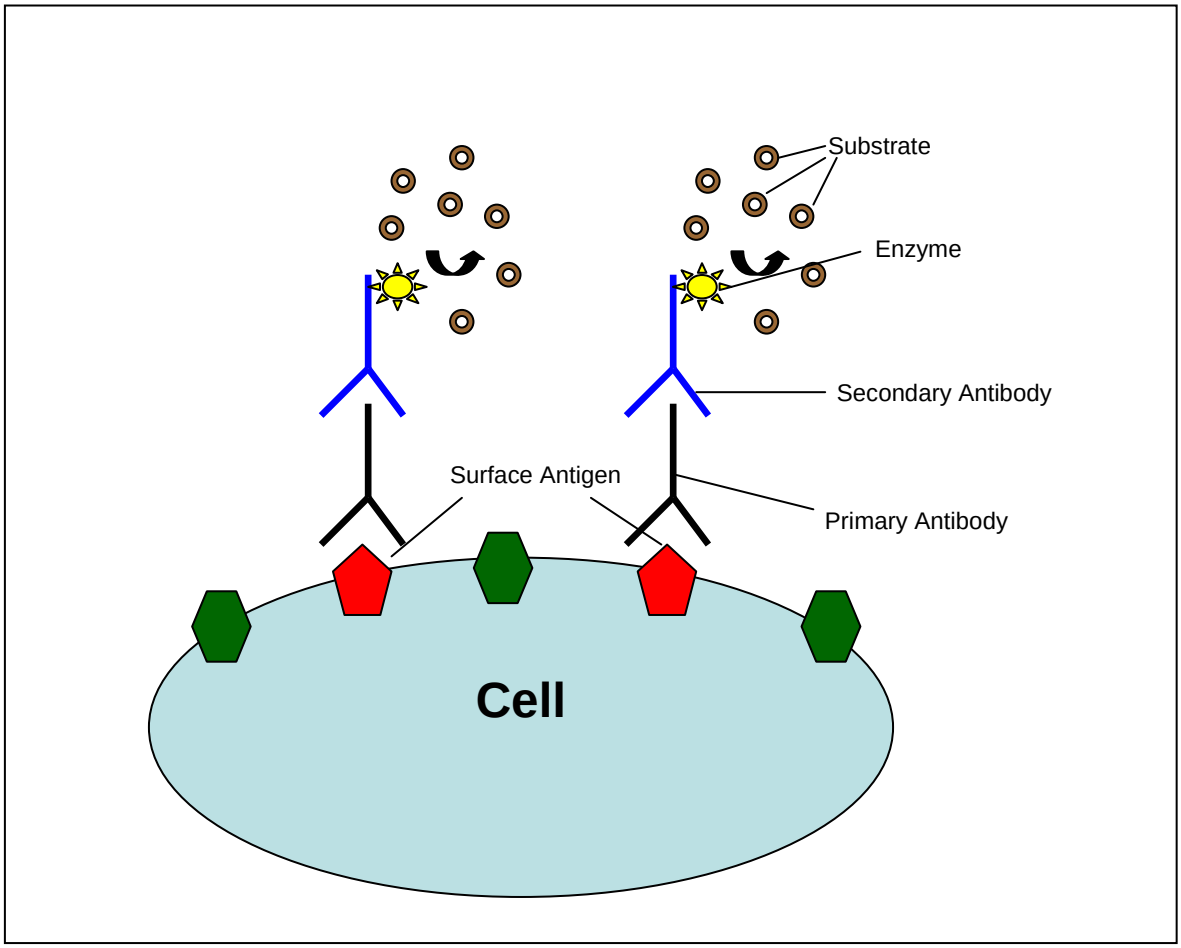


Figure 2.4 Illustration of the general theory of immunohistochemistry.

After binding of the primary antibody to a specific surface antigen on the cell the secondary antibody binds to the primary antibody. After addition of an enzyme complex and a respective substrate (for example containing colour reagents), the enzyme catalyses a reaction of the substrate which then creates a visible colour reaction in order to label the cell carrying the specific antigen.

2.8.2 Performance of the immunohistochemical stainings of the skin samples, ABC-method

After letting the slides defrost at room temperature, they were put onto trays containing wet sponges to prevent the slides from drying out (**figure 2.5**):

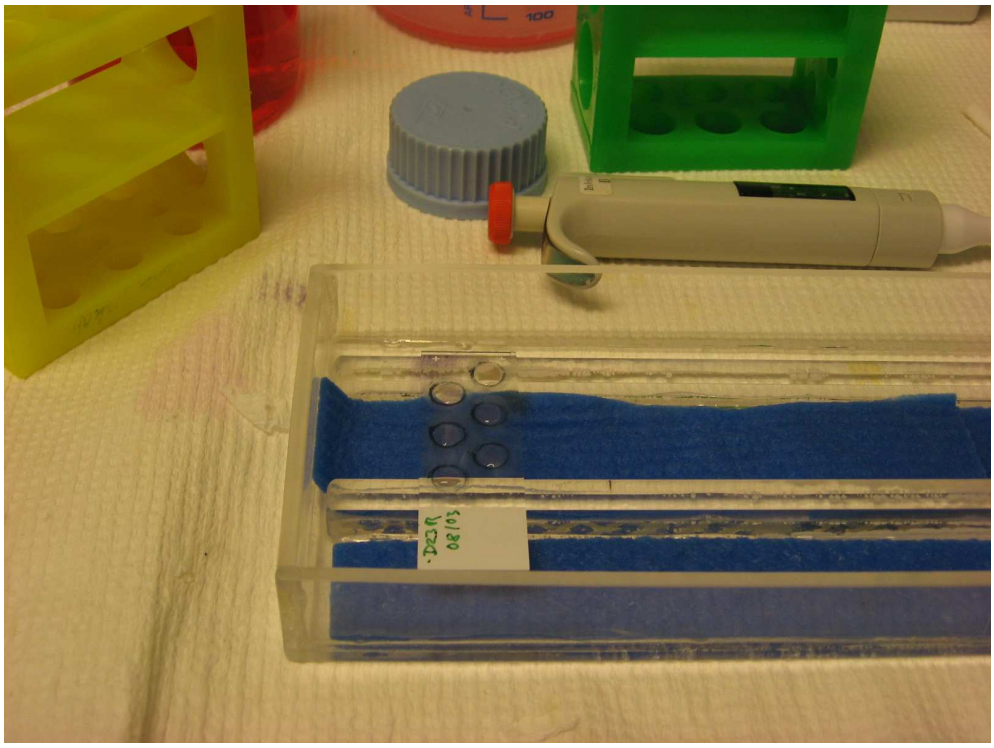


Figure 2.5 Photograph of the experimental setting for the immunohistochemical staining procedure.

Six sections of one biopsy sample are fixed within the six circles (holes) on a glass slide. Staining reagents are put onto the holes on the glass. Trays contain wet sponges, in between the steps they are covered with lids.

The immunohistochemical staining was performed by the use of the **Avidin-Biotin-complex method (ABC method)** [103]:

The ABC method is a standard IHC method and one of the most widely used techniques for immunhistochemical staining. Avidin, a large glycoprotein, can be labelled with peroxidase or fluorescein and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules such as antibodies.

The technique involves three layers. The first layer is an unlabeled primary antibody. The second layer is a biotinylated secondary antibody. The third layer is a complex of avidin-biotin peroxidase. The peroxidase is then developed by substrates such as the DAB (3,3´-diaminobenzidine) to produce different colorimetric end products.

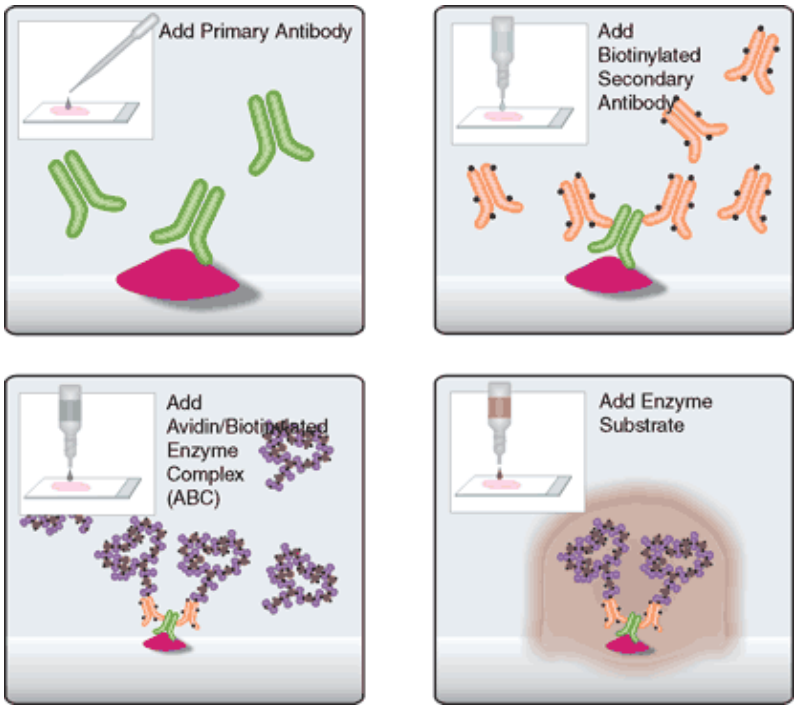


Figure 2.6 Illustration of the major work steps of the Avidin-Biotin-complex (ABC)-method [104].

The first step in our protocol was performed in order to block the endogenous peroxidase contained in the sample tissue: 20-30µl of 2% H_2O_2 (Sigma Aldrich, St. Louis, USA) diluted in 0.02M buffered saline solution (BSS) containing 1% Hepes (buffer, Sigma Aldrich) and 0.1% Saponine (Sigma Aldrich), a detergent which increases cell membrane permeability, and 0.2% NaN_3 (Sigma Aldrich) as an antimicrobial toxin, were added onto the samples. This blocking step is essential for the following staining procedure as the enzyme complex used for labelling the secondary antibodies bound to the tissue consists of horseraddish peroxidase, and endogenous peroxidase could therefore create a disturbing colour reaction.

Furthermore, for using the ABC method endogenous biotin has to be blocked in the tissue by the use of the Avidin/Biotin Blocking Kit (Vector Laboratories INC Burlingame, CA, USA):

At first, 20-30µl of Avidin were added to the samples to incubate for 15 minutes at room temperature (RT).

Secondly, 20-30µl of Biotin were added to the samples to also incubate for 15 minutes at RT.

After each step the tissue samples were washed with BSS + 1% Hepes + 0,1% Saponin. This was performed to make sure that the samples were covered with liquid. The gaps in between the holes had to be dried, for this filter paper was used in order to avoid connecting “water bridges” between the different holes.
After the blocking steps the primary antibodies against the cellular antigens were applied.

For the staining of the samples from the TST reaction sites the following antibodies were used (see also **supplement table 1**):

Table 2.1 Antibodies used for immunophenotypisation of the immune cells in the skin samples

Cellular Antigen	Distribution	Function	Staining Antibody
CD 4	Effector T cells (TH_1/TH_2), Monocytes, Macrophages	MHC-class-II co-receptor; T cell activation and differentiation	Anti-CD4 (BD Bioschiences, San Jose, CA, monoclonal mouse IgG1 anti-human)
CD 8	Cytotoxic T cells (CTL); NK cells	MHC-class-I co-receptor; T cell activation and differentiation	Anti-CD8 (BD Biosciences, San Jose, CA, monoclonal mouse IgG1 anti-human)
CD 28	T cells; Plasma Cells	T cell costimulation	Dako Cytomation, DK, monoclonal mouse IgG1 anti-human
CD 45RA	Naive T cells	Enhances T and B cell receptor signalling	Dako Cytomation, DK, monoclonal mouse IgG2a anti-human
CD 45RO	Memory T cells	Lymphocyte activation	Dako Cytomation, DK, monoclonal mouse IgG2a anti-human
CD 62L	Naive and Memory T cells	Homing receptor	DakoCytomation, DK, monoclonal mouse IgG2b anti-human

Cellular Antigen	Distribution	Function	Staining Antibody
HLA-DR	MHC-class-II cell surface receptor; activated T cells	Ligand for T cell-receptor	BD Biosciences, San Jose, CA, monoclonal mouse IgG2a anti-human
IFN-γ	Cytokine produced in activated T cells	Activates Macrophages (e.g. Tuberculosis)	Mabtech, S, monoclonal mouse IgG1 ani-human
Granulysin	Cytolytic protein, produced in CTL	Induces cytolysis	Santa Cruz Biotechnology, Inc., ploy clonal goat anti-human
FoxP3	Transcription factor in CD4+CD25+ Regulatory T-Cells	Differentiation of Regulatory T cells	Abcam Ltd., GB, polyclonal goat anti-human
CD1a	Dendritic Cells; Langerhans Cells	Antigen presentation	Dako Cytomation, DK, monoclonal mouse IgG2a anti-human

We further used the following solutions as negative controls:

- Negative control mouse IgG1 (Dako Cytomation, DK)
- Negative control mouse IgG2a+2b (Dako Cytomation, DK)
- Negative control goat (normal goat IgG, R&D Systems) .

The control samples from normal skin were only stained for CD4, CD8 and CD1a by using the same antibodies as given above.

All antibodies were diluted in BSS+Hepes+Saponin+0,02% NaN_3 in specific concentrations (see **supplement table 1**).

Prior to application of the FoxP3 antibody the tissue sample had to be incubated with Trypsin (Sigma Aldrich, diluted 1:1000 in BSS), a cytolytic enzyme that supports the penetration of the antibody into the nucleus of the cell where it binds to the transcription factor FoxP3.

The antibody solutions were then added to the samples to incubate over night at RT in a tray covered with a lid to avoid exposure to light.

After overnight incubation the samples were thoroughly washed with BSS+Saponin.
In the next step the samples were blocked with 20-30µl of 1% rabbit serum (Dako Cytomation, diluted in BSS+Saponin) for 30 minutes at RT. This was performed in order to block non-specific binding sites in the tissue as the secondary antibodies were polyclonal antibodies from rabbits.

After flipping the liquid (no wash), the secondary antibodies were added and incubated for 30 minutes at RT:

- 20-30µl of biotinylated polyclonal rabbit anti-mouse antibodies (DakoCytomation) were used to label cells marked with antibodies against CD4, CD8, CD28, HLA-DR, CD45RA, CD45RO, CD62L, CD1a, IFN-γ.
- 20-30µl of biotinylated polyclonal rabbit anti-goat antibodies (Dako Cytomation) were used to label cells marked with antibodies against Granulyzin and FoxP3.

After the incubation of the secondary antibodies the samples were washed with BSS+Saponin.
Then 20-30µl of the VECTASTAIN® Elite ABC (Vector Laboratories INC Burlingame, CA) diluted in BSS+Saponin were added to the samples to incubate for 30 minutes at RT in the dark. VECTASTAIN® Elite ABC contains the avidin and biotinylated peroxidase enzyme complex.
After this step the samples were washed three times each with BSS and with BSS+Saponin.
In the last step the DAB (3,3´-diaminobenzidine) Substrate (Vector laboratories) was added and incubated for approximately five minutes in the dark. The substrate creates a colour reaction with the peroxidase on cells that have been labelled with the respective antibodies. The reaction was stopped by the sample being washed thoroughly with BSS.

Counterstaining, i.e. marking the cell cytoplasm and not the cell surface, was performed by dipping the slides into hematoxyline (Histolab produkt AB Göteborg, Sweden) diluted 1:1 in tab water.

The slides were then mounted with Glycerol (Merck Eurolab Alberlund, DK) diluted 1:10 in 0.01M PBS and covered with cover glasses.

2.9 Image analysis and data evaluation

Analysis of the immunohistochemically marked biopsy samples was performed at the Microscope Leica DMRX (Leica Wetzlar,Germany) in connection with the computerised image analysis system “Leica Qwin 550IW” (Leica Imaging System Cambridge, UK, **figure 2.6**).

In order to quantify the cell areas within the sample and to show the distribution of labelled cells, the sections were digitally organised with the help of different colour thresholds, so that the frequency of stained cells could be measured, and the distribution was calculated *in situ* at the single cell level. The overall area measured depended on the size of the sample and varied between 2.0×10^5 µm^2 and 2.0×10^6 µm^2. Epidermal layers were excluded from evaluation. For each sample section it was intended to analyse an average of 10-15 different areas, between which calculated cell distributions were averaged to representative values until average values were stable and a standard deviation of <0.5% was reached.

Exact calculations of the different subsets of cells staining positive for the different phenotypic markers were achieved. The results were transferred into an Excel Chart containing data from all 38 persons with information on their race, sex, date of birth, HIV status, CD4 count in the blood and the TST induration diameter.

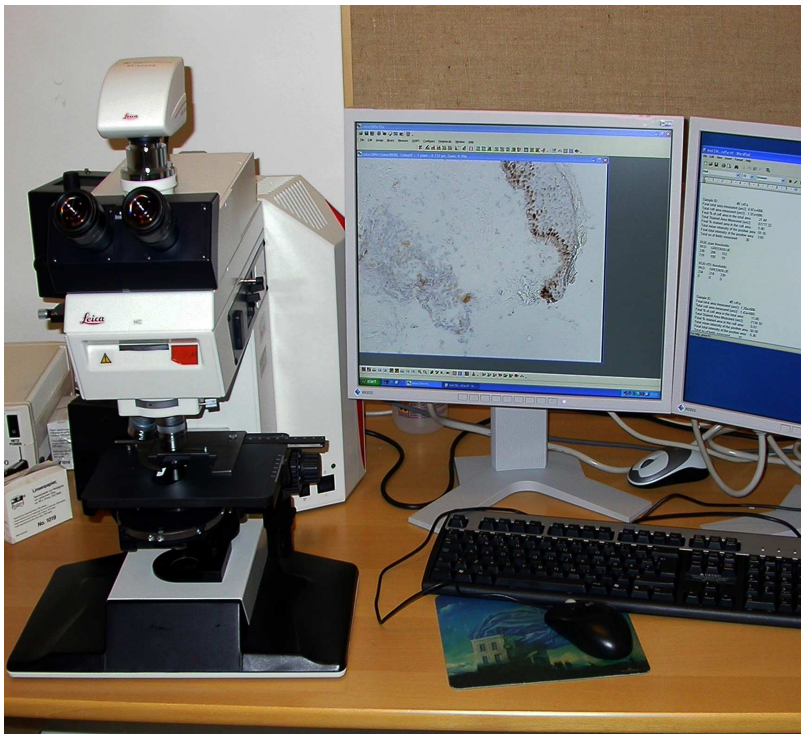


Figure 2.7 Photograph of the setting of the image analysis system.

Glass slides with immunohistochemically stained biopsy samples are observed through the Leica DMRX microscope. The images are transferred to the computer (middle screen). Analysis and calculations of the staining signals are conducted with the imaging programme Leica Qwin 550IW (right screen).

2.10 Statistical analysis

Statistical analysis was performed using the statistics programme SPSS (Version 8.0, 1998, Chicago, IL, USA).

The normality of the data was assessed by the Shapiro-Wilk test. Normally distributed variables are shown as Mean ± standard deviation (S.D.) and between group comparisons the student's unpaired t-test was employed. Non-parametric variables are shown as Median + interquartile range (IQR) and between group comparisons the Mann Whitney U test was used. Where normality was incongruent between clinical groups non-parametric distributions were assumed and analysed appropriately. Where numbers were too small, no statistical comparison was attempted.

Graphs were created by the use of the programme GraphPad Prism (Version 4.03, 2005, San Diego, CA. California).

3 Results

3.1 Baseline characteristics

Thirty-eight persons were recruited, 15 persons were HIV-1 seropositive and 23 were HIV-1 seronegative. The baseline characteristics are shown in **table 3.1**.
The groups did not differ regarding sex, age and tuberculosis exposure history.
One biopsy sample from one person was excluded from the analysis as the sample had very poor quality concerning size, tissue structure and staining signal and was therefore considered inappropriate for analysis.

Table 3.1 Baseline characteristics of subjects enrolled in the study

	HIV infected n=15	HIV uninfected n=22	p value
Gender			
Female	10 (67%)	13 (59%)	1.0
Age			
mean (s.d)	31.6 (5.2)	31.4 (8.6)	0.94
CD4			
Median (Range)	325 (125-972)	NR	NA
TST-Reactions			
Mean induration (s.d.)	10.6 (9.9)	16.9 (6.7)	0.02
Greater than 15 mm	6 (40%)	17 (77%)	0.037
Greater than 10 mm	9 (60%)	19 (86%)	0.12
Greater than 5 mm	9 (60%)	21 (95%)	0.01
0 – 5 mm	6 (40%)	1 (4%)	<0.001

3.2 Interferon-γ RD1 based ELISPOT results

24 patients provided sufficient blood cells (11 HIV infected and 13 HIV uninfected, **table 3.2**) for an RD1 based IFN-γ ELISPOT analysis to be conducted. One assay in each group was indeterminate. 11 out of 13 scored positive in the HIV uninfected group, and eight out of eleven scored positive in the HIV infected group, including positives in three of the six patients who were TST unreactive.

Table 3.2 Results of RD1 based ELISPOT assay

RD1 based ELISPOT	HIV infected n=15	HIV uninfected n=22	p value
Positive	8/11	11/13	0.63
Negative	2/11	1/13	0.58
Indeterminate	1/11	1/13	1.0

3.3 Reactions in the Tuberculin Skin Test in HIV infected vs. HIV uninfected persons

Comparing TST reactions of HIV infected versus HIV uninfected persons, it was observed that the mean induration diameter of the TST reaction was greater (16.9 ± 6.7 mm) in the HIV uninfected group than in the HIV infected (10.6 ± 9.9 mm, p=0.02).

This difference was also reflected in the greater proportion of HIV uninfected persons scoring positive in the TST. A positive reaction in the TST was defined as an induration diameter of greater than 5mm, thus nine out of 15 (60%) HIV infected persons and 21 out of 22 (95%) HIV uninfected persons were regarded as TST positive (p=0.01). Six HIV infected persons (40%) were unreactive in the TST while only one of the 22 HIV uninfected persons was TST negative (**figure 3.1**).

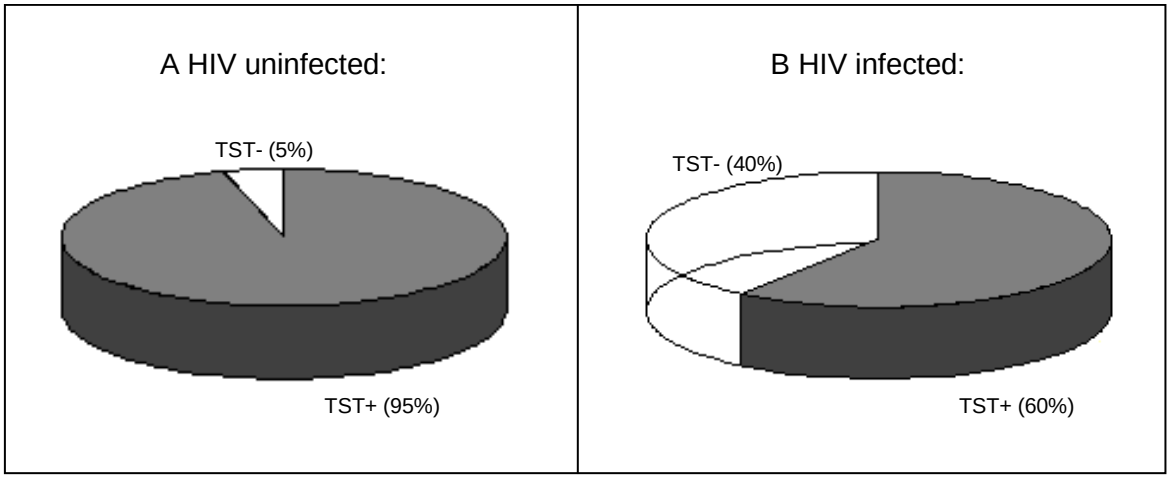


Figure 3.1 TST reactions in HIV uninfected and HIV infected persons.

A HIV uninfected subjects. 21 of the 22 HIV uninfected persons (95%) showed a positive reaction in the Tuberculin Skin Test, one person (5%) did not react.

B HIV infected subjects. Nine out of 15 HIV infected persons (60%) showed a positive reaction in the Tuberculin Skin Test, six persons (40%) did not react.

3.4 Phenotypes of peripheral blood mononuclear cells

3.4.1 Distribution of cells

For analysis of the distribution of PBMC subsets obtained by FACS both HIV infected and HIV uninfected persons were compared as well as TST reactors and TST non-reactors.

The total percentage of CD4+ cells in the blood of HIV infected persons was lower than in HIV uninfected persons (14.1, IQR 10.1-21.6 versus 24.4, IQR 20.4-37.0, p=0.0005, **supplement table 2**). There was no difference between reactors and non-reactors according to the total percentage of CD4+ cells. Frequencies of CD4+ T cells bearing the costimulatory marker CD28 were also significantly reduced in HIV infected persons (20.8, IQR 13.2-23.5 versus 34.7, IQR 30.1-42.9, p=0.0001, **supplement table 2**), but again reactors and non-reactors did not differ according to this marker. Regarding CD8+CD28+ T cell distribution, no significant differences could be observed between the two groups.

In TST unreactive HIV infected persons there was a decrease in the percentage of CD4+CD45RA-RO+ cells when compared to HIV infected reactive persons (19.2, IQR 16.6-26.1 versus 31.6, IQR 26.1-54.7, p=0.005), which suggests an important role for T cells with a memory phenotype.

Conversely the percentage of CD4+FoxP3+ regulatory T cells was 1.78 times higher in TST unreactive HIV infected persons than in reactive HIV infected persons (13.7, IQR 7.8-17.3 versus 7.7, IQR 6.4-10.2, p=0.036, **figure 3.2**).

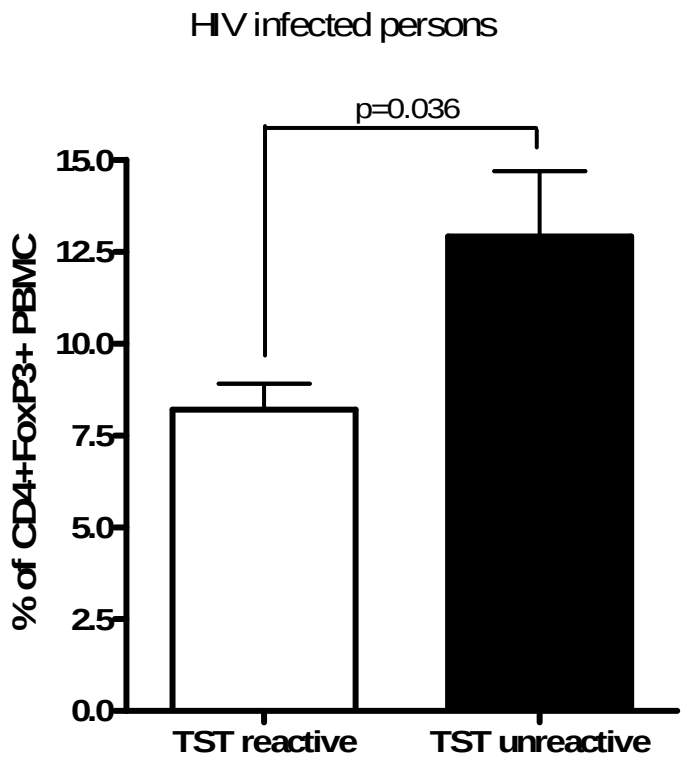


Figure 3.2 Frequency of CD4+FoxP3+ mononuclear cells in peripheral blood from HIV infected persons reactive in the Tuberculin Skin Test and HIV infected unreactive subjects. Unreactive persons show significantly higher levels of CD4+FoxP3+ regulatory T cells in the blood than reactive persons. The graph shows mean + standard error mean (SEM).

There was a greater percentage of activated (HLA-DR+) and antigen experienced (CD45RA-RO+) CD8+ cells in the PBMC of HIV infected people (CD8+HLA-DR+ 18.9, IQR 16.5-34.2 versus 10.8, IQR 6.1-13.4, p=0.0007 and CD8+CD45RA$^-$

CD45RO+ 11.3, IQR 7.1-16.6 versus 8.2, IQR 3.3-11.9, p=0.006, **supplement table 2**) than in HIV uninfected persons. Despite of this, distributions of activated HLA-DR+ CD4+ cells were not different between the groups.

There was a trend towards a lower percentage of CD8+CD45RA-CD45RO+ cells in the PBMC of TST unreactive HIV infected persons, but this was not significant (p=0.088).

The proportions of CD45RA+CD62L+ cells did not reveal any significant differences between the groups.

3.4.2 Correlations

Next it was explored whether correlations between the TST diameter and any peripheral blood T cell subset existed. A significant negative correlation between the percentage of CD4+FoxP3+ regulatory T cells and the TST diameter was observed. This could be observed in the entire patient group (Spearman r = -0.39, 95% CL -0.07 to -0.64, p=0.015) and more strongly in the HIV infected group (Spearman r = -0.57, 95% CL -0.06 to -0.84, p=0.026, **figure 3.3**).

Absolute CD4 count numbers were available for the HIV infected persons, which also allowed an alternative exploration of correlation with absolute numbers. When analysed this way a weak but significant positive relationship was noticed between the total number of CD4 cells HLA-DR+ and the TST diameter (Spearman r = 0.53, 95% CL 0.01-0.82, p=0.04).

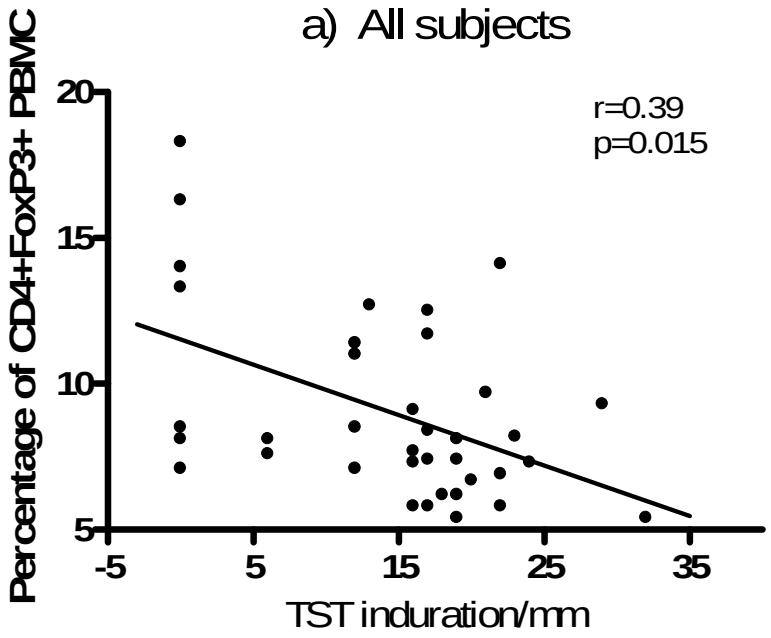


Figure 3.3 a) Correlation between the frequency of CD4+FoxP3+ peripheral blood mononuclear cells and the Tuberculin Skin Test induration diameter in all persons. The frequency of peripheral CD4+FoxP3+ regulatory T cells inversely correlates with the induration diameter of the reaction (r=-0.39, p=0.015).

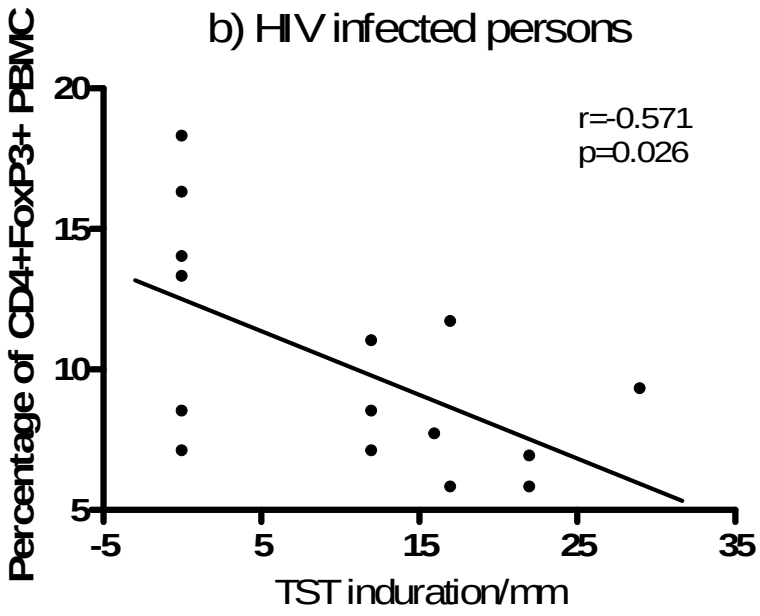


Figure 3.3 b) Correlation between the frequency of CD4+FoxP3+ peripheral blood mononuclear cells and the Tuberculin Skin Test induration diameter in HIV infected persons. The frequency of peripheral CD4+FoxP3+ regulatory T cells inversely correlates with the induration diameter of the reaction (Spearman r=-0.571, p=0.026).

Data was then explored for correlations between the peripheral CD4 count of the HIV infected persons and the TST induration diameter.

The CD4 count of the peripheral blood and the TST induration diameter were not significantly correlated (**figure 3.4**), although the mean CD4 in those unreactive in the TST was lower (243 ±100 versus 442 ± 260 /mm^3 p=0.75, **table 3.1**).

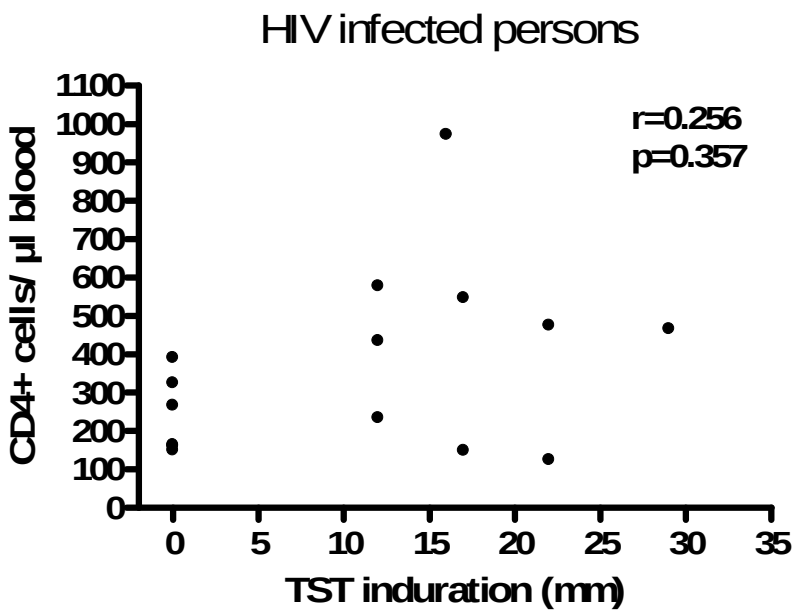


Figure 3.4 Correlation between the CD4 count (number of CD4+ T cells per µl blood) and the Tuberculin Skin Test induration diameter in HIV infected persons. The number of circulating CD4+ T cells does not correlate with the size of the induration (Spearman r=0.256, p=0.357).

In contrast to this, the absolute CD4 count was observed to be strongly correlated with the number of peripheral CD45RA+CD62L+ naïve cells (Spearman r = 0.91, p<0.0001, **figure 3.5**).

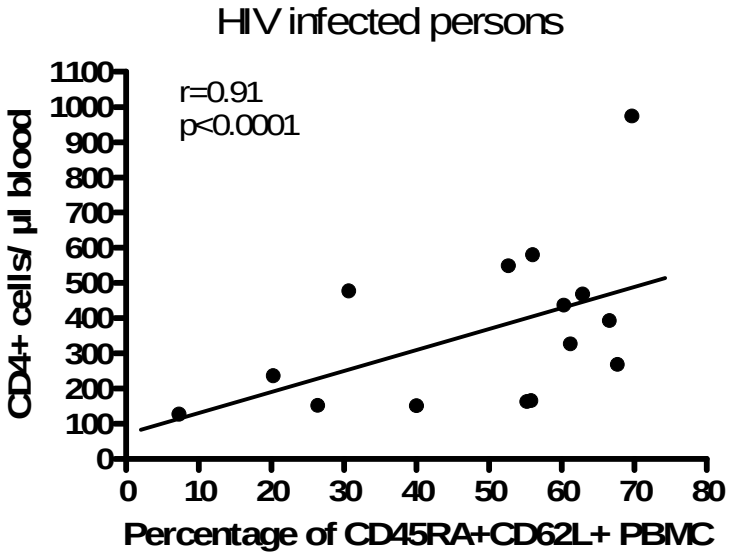


Figure 3.5 Correlation between the CD4 count (number of CD4+ T cells per µl blood) and the percentage of CD45RA+CD62L+ PBMC in HIV infected persons. The number of circulating CD4+ T cells significantly correlates with the percentage of CD45RA+CD62L PBMC (Spearman r=0.91, p<0.0001).

3.5 Skin biopsies

In order to evaluate the different cell populations prevalent in the skin biopsies, an overall number of approximately 4,000 digital images from both normal skin and TST reaction biopsies have been evaluated.

3.5.1. Distribution of cells in normal skin controls

The median values of the total percentage of the cell area within the whole section area (total % of cells) did not significantly differ between the HIV infected and the HIV uninfected group (3.6% vs. 4.0% of the total area measured, p=0.734, **table 3.2.1**) in normal skin control biopsies. There was also no difference in total cellular infiltrates between TST reactive vs. TST unreactive persons (3.9 vs. 3.6, p=0.880, **table 3.2.2**).

When the HIV infected group was compared to the HIV uninfected group according to the distribution of CD4+, CD8+ and CD1a+ cells in normal skin (**table**

3.2.1), there could not be found any significant differences, either. The same was revealed when TST reactive persons were compared to unreactive persons (**table 3.2.2**). It can thus be concluded that any differences that might be observed in reactional tissue would not relate to initial cellular composition but reflect recruitment or local expansion.

Table 3.2.1 Distribution of cell populations in normal skin controls in HIV infected versus uninfected persons (medians + interquartile range IQR).

	Total % of cells*	**CD4+ (%)#**	**CD8+ (%)#**	**CD1a+ (%)#**
HIV-	4.0 (IQR 3.0-4.0) n=19	3.3 (IQR 0.5-9.1) n=18	2.4 (IQR 0.8-3.9) n=18	0.7 (IQR 0.5-0.8) n=7
HIV+	3.0 (IQR 3.0-4.5) n=13	4.4 (IQR 1.2-5.8) n=13	0.9 (IQR 0.6-3.8) n=13	0.4 (IQR 0.3-0.8) n=10
p-value	0.734	0.692	0.417	0.417

Table 3.2.2 Distribution of cell populations in normal skin controls in Tuberculin Skin Test reactors versus non-reactors (medians + interquartile range IQR).

	Total % of cells*	**CD4+ (%)#**	**CD8+ (%)#**	**CD1a+ (%)#**
TST+	3.0 (IQR 3.0-4.0) n=19	3.7 (IQR 0.7-7.0) n=18	2.1 (IQR 0.7-4.1) n=18	0.6 (IQR 0.4-0.9) n=7
TST-	3.0 (IQR 3.0-4.5) n=13	4.0 (IQR 1.9-10.6) n=13	1.2 (IQR 0.4-2.6) n=13	0.3 (IQR 0.2-0.8) n=10
p-value	0.880	0.589	0.358	0.300

3.5.2. Distribution of cells at the reaction site of the Tuberculin Skin Test

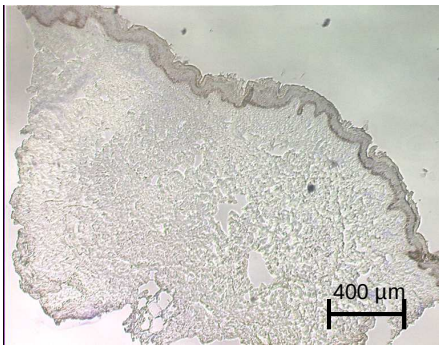
The analysis was focused on two major aspects:

1. Comparison of cellular compositions in samples from HIV infected vs. HIV uninfected persons.
2. Comparison of cellular compositions in samples from TST reactive vs. TST unreactive persons.

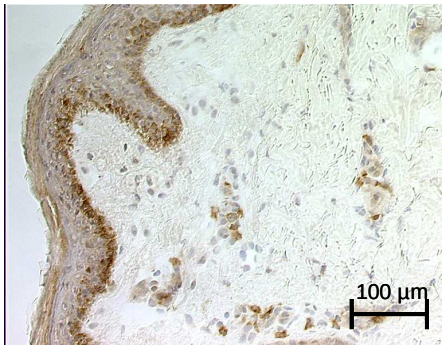
* Median values of the percentages of cell area measured within the total section
# Median values of the percentages of positive cells within the cell area of the section

Typical staining results of different skin samples are shown in **figure 3.6**.

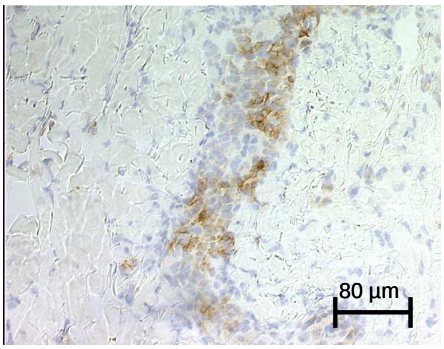
Overview of total sample section (no positive staining):



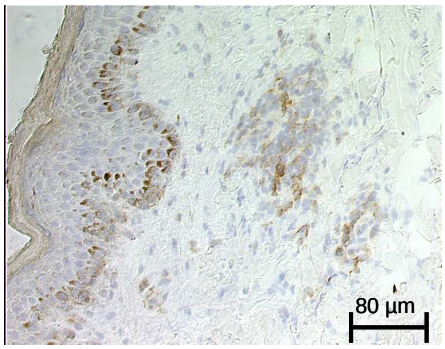
Control sample (normal skin), stained for CD4:



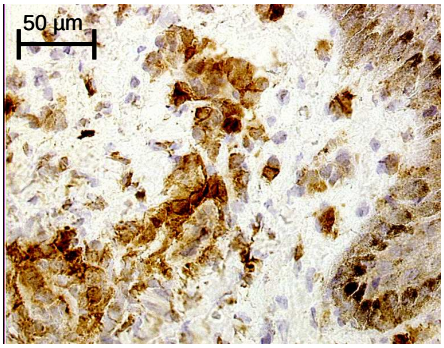
TST reactive sample, stained for CD4:



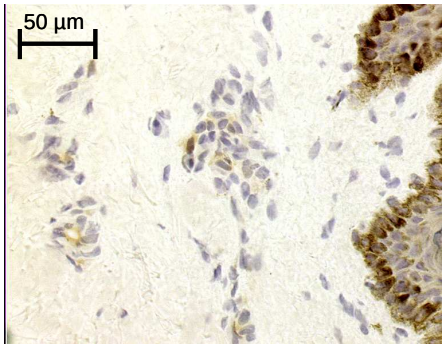
TST reactive sample, stained for CD8:



TST reactive sample, stained for CD45RO: *



TST unreactive sample, stained for CD45RO:



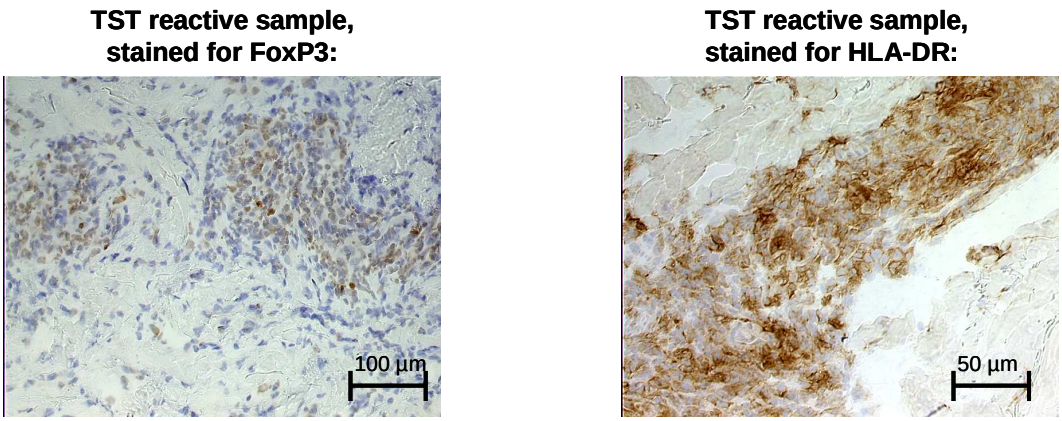


Figure 3.6 Microscopic view of immunohistochemically stained skin samples.
The brown colour indicates positive staining signal of the respectively investigated marker. All pictures show samples with positive staining at the cell surface, except for the picture showing a sample stained for FoxP3 (nuclear staining signal).
* The photograph of a TST reactive sample stained for CD45RO will appear as front cover picture of the April 2009 issue of the Journal of Infectious Diseases where this work will be published.
The TST reactive sample shows more infiltrating cells than the unreactive sample (at the right, also stained for CD45RO).

The total cellular infiltrate, expressed as percentage of the cell area measured within the total area of the biopsy sample, was at 6.0 (median value, IQR 5.0-13.0) in HIV infected persons, and thus lower than in HIV uninfected persons (12.0, median value, IQR 9.0-14.0), p=0.045, **supplement table 3, figure 3.7**). In HIV infected persons this difference was entirely due to a reduction of cells in unreactive HIV infected persons (4.5, median value, IQR 3-6) in contrast to 11.0 (median value, IQR 6.5-15.5) in reactive HIV infected persons (p=0.008, **supplement table 3, figure 3.9**). These findings indicate that a positive reaction in the TST is in fact due to an influx of cells to site of the antigen inoculation and that it is not just interstitial inflammatory edema that creates the visible and palpable induration in the skin.

When the different cellular subgroups were analysed it became clear that the percentage of skin cells staining positive for CD4 was predictably lower in HIV infected persons (12.3, median value, IQR 8.0-20.9 versus 7.7, median value, IQR 3.0-11, p=0.005, **figure 3.7**). This difference reduced but remained significant when only reactive biopsies were considered (12.6, median value, IQR 9.4-23.3 in

HIV uninfected versus 9.2, median value, IQR 4.4-11.0 in the HIV-1 infected, p=0.022, **figure 3.8**).

A similar trend was seen for the percentage of cells staining positive for CD62L, which was lower in HIV infected than in uninfected persons (0.7, median value, IQR 0.6-3.5 versus 3.5, median value, IQR 1.2-6.1, p=0.038, **figure 3.7**) with this difference again remaining significant when only reactive biopsies (0.7, median value, IQR 0.5-3.4 in HIV uninfected versus 3.5, median value, IQR 1.2-6.1 in HIV infected persons, p=0.037, **figure 3.8**) were considered.

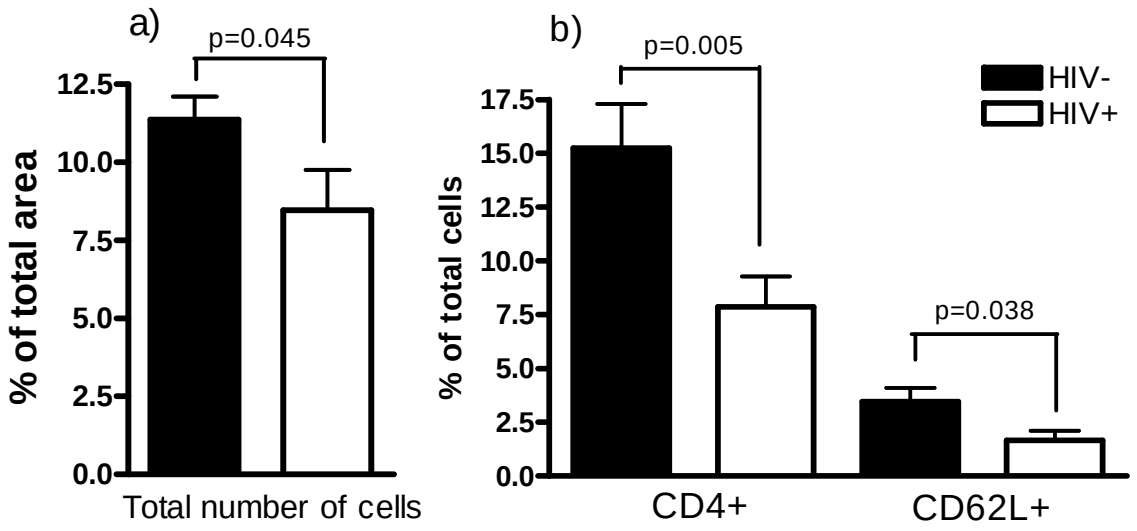


Figure 3.7

a) Cellular infiltrates at the Tuberculin Skin Test (TST) reaction site from HIV uninfected vs. HIV infected persons. Numbers are expressed as the percentage of the cell area within the total area of the skin sample being obtained 48h after tuberculin inoculation. Samples from HIV infected persons show lower numbers of cells infiltrating the TST reaction site than samples from HIV uninfected persons (p=0.045). The graph shows means + standard error mean.

b) Distribution of cellular infiltrates at the Tuberculin Skin Test reaction site from HIV uninfected vs. HIV infected persons. The numbers of positive cells are displayed as percentages relative to the total number of infiltrating cells. Samples from HIV infected persons show significantly lower frequencies of cells staining positive for CD4 (p=0.005) and CD62L (p=0.038) than samples from HIV uninfected persons. The graph shows means + standard error mean.

Taken together these results suggest that cells other than CD4+ and CD62L+ may contribute proportionately more to the infiltration seen in the reactions of HIV infected persons.

Indeed HIV infected persons showed a higher proportion of cells positive for HLA-DR+ (activated cells) than uninfected persons in TST reactive skin samples: 37.8 (median value, IQR 31.0-43.1) vs. 26.2 (median value, IQR 16.4-34.7, p=0.046, **figure 3.8**). Unreactive HIV infected persons had lower values (22.9, median value, IQR 13.7-41.2) but this difference did not attain significance.

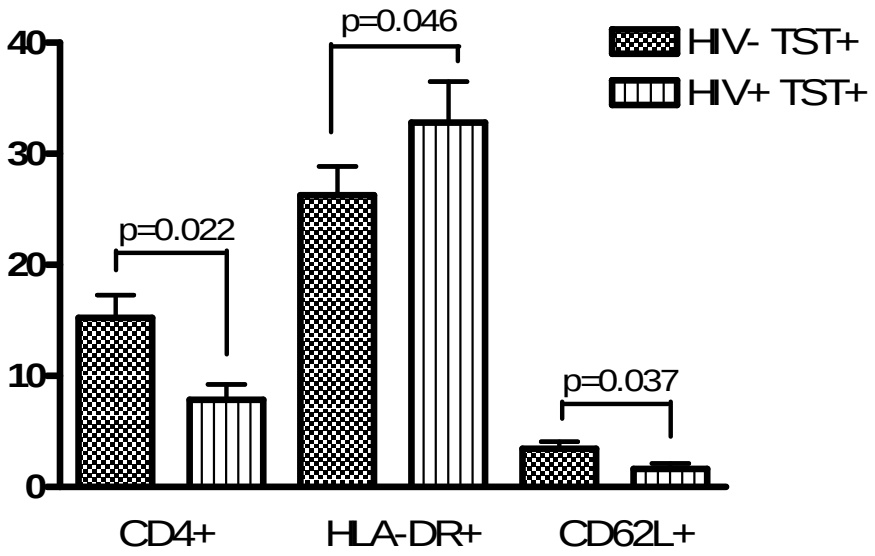


Figure 3.8 Cellular infiltrates at the Tuberculin Skin Test (TST) reaction site in HIV infected and HIV uninfected TST reactive persons. The numbers of positive cells are displayed as percentages relative to the total number of infiltrating cells. Samples from HIV infected persons show lower numbers of CD4+ (p=0.022) and CD62L+ cells (p=0.037) than samples from uninfected persons, whereas there are more HLA-DR+ (activated) T cells (p=0.046) in samples from HIV infected persons. The graph shows means + standard error mean .

When unreactive and reactive HIV infected persons were compared there were also significantly decreased numbers of CD45RA+ cells (1.7, median value, IQR 0.1-3.5 versus 4.4, median value, IQR 2.9-7.8, p=0.036) and CD45RO+ cells (5.7, median value, IQR 1.7-10.0 versus 16.0, median value, IQR 13.8-23.8, p=0.003) in samples from TST unreactive persons (**figure 3.9**).

This indicates that decreased cellular influx to the site of an unreactive TST is due to a reduction in the recruitment of naive T cells (CD45RA+), and furthermore a lack of memory T cells (CD45RO+) can be associated with a non-reactive TST response.

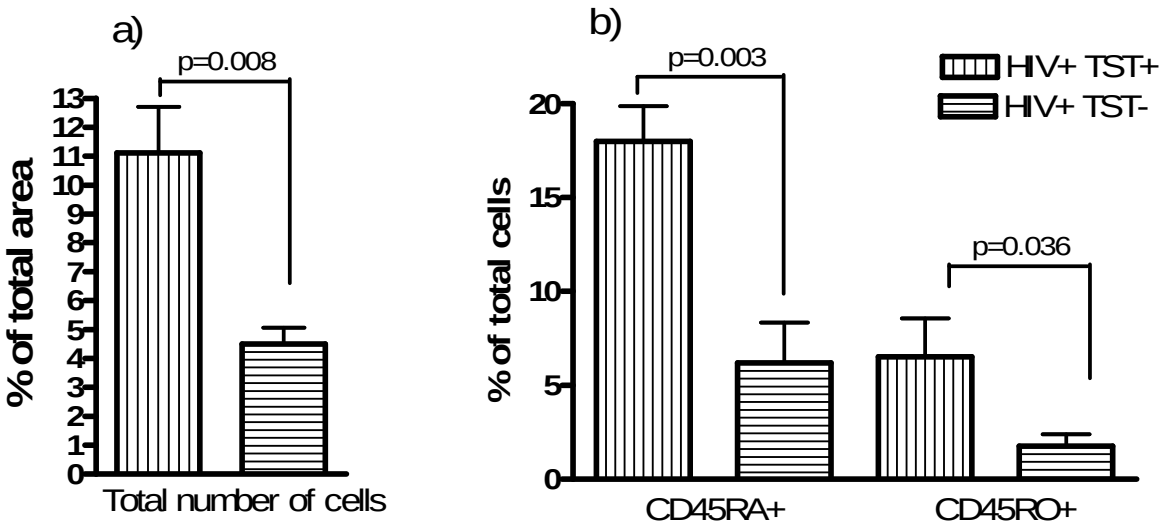


Figure 3.9

a) Cellular infiltrates at the Tuberculin Skin Test (TST) reaction site from HIV infected persons with postive reactions vs. HIV infected persons with negative reactions. Numbers are expressed as the percentage of the cell area within the total area of the skin sample obtained 48h after tuberculin inoculation (cut-off >5mm). Samples from HIV infected TST unreactive persons show lower concentrations of cellular infiltrates at the reaction site than samples from HIV infected TST reactive persons (p=0.008). The graph shows means + standard error mean.

b) Distribution of cellular infiltrates at the Tuberculin Skin Test (TST) reaction site from HIV infected TST reactive vs. HIV infected TST unreactive persons. The numbers of positive cells are displayed as percentages relative to the total number of infiltrating cells. Samples from HIV infected TST unreactive persons show significantly lower frequencies of cells staining positive for CD45RO (p=0.003) and CD45RA (p=0.036). The graph shows means + standard error mean.

The analysis of the other cellular markers (CD8, CD28, CD1a, IFN-γ, Granulysin and FoxP3) in the skin biopsies did not reveal any significant differences between either HIV infected and uninfected persons or reactive and unreactive persons.

3.5.3 Correlations

It was examined whether any correlations existed between the different cellular subgroups and the induration diameter of the TST reaction.

Overall, in the entire patient group the total number of infiltrating cells (Spearman r=0.559, p<0.001, **figure 3.10**), the number of infiltrating CD4+ cells (Spearman r=0.406, p=0.013), the number of CD1a+ cells (Spearman r= 0.362, r=0.030, data not shown) and the number of infiltrating CD45RO+ cells (Spearman r=0.365, p=0.029, data not shown) were positively correlated with the size of the TST induration.

When only the HIV-seropositive group was analysed, the total number of infiltrating cells (Spearman r=0.757, p=0.001, **figure 3.10 a)**) and the number of CD45RO+ cells (Spearman r= 0.670, p=0.006, **figure 3.10 b)**) were positively correlated with the size of the TST induration.

No correlations were observed between the distribution of CD4+, CD8+ and CD1a+ at the TST site compared with normal skin controls.

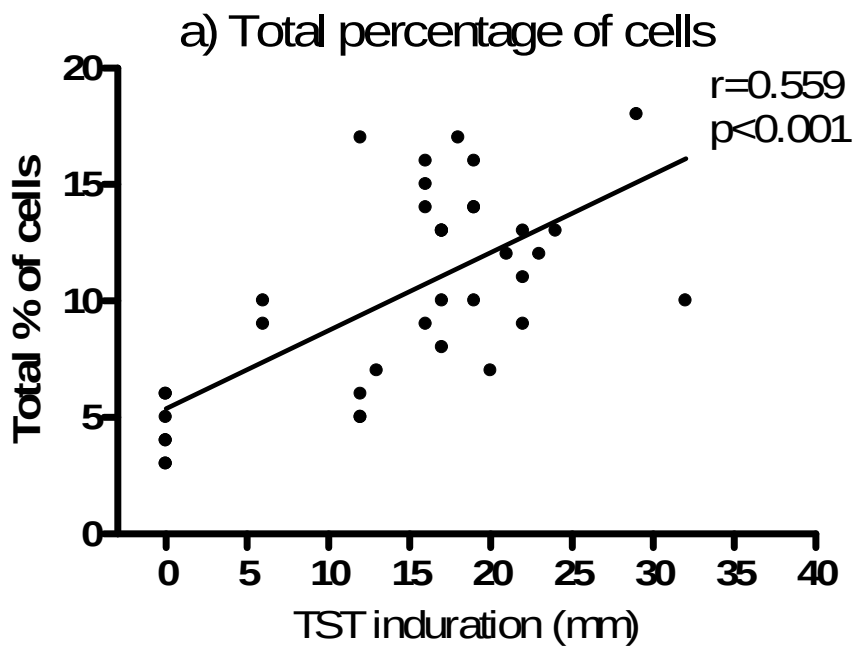


Figure 3.10 a) Correlation between the total number of infiltrating cells at the Tuberculin Skin Test (TST) reaction site and the TST induration diameter. The total number of infiltrating cells (Spearman r=0.559, p<0.001) correlates significantly with the size of the TST induration.

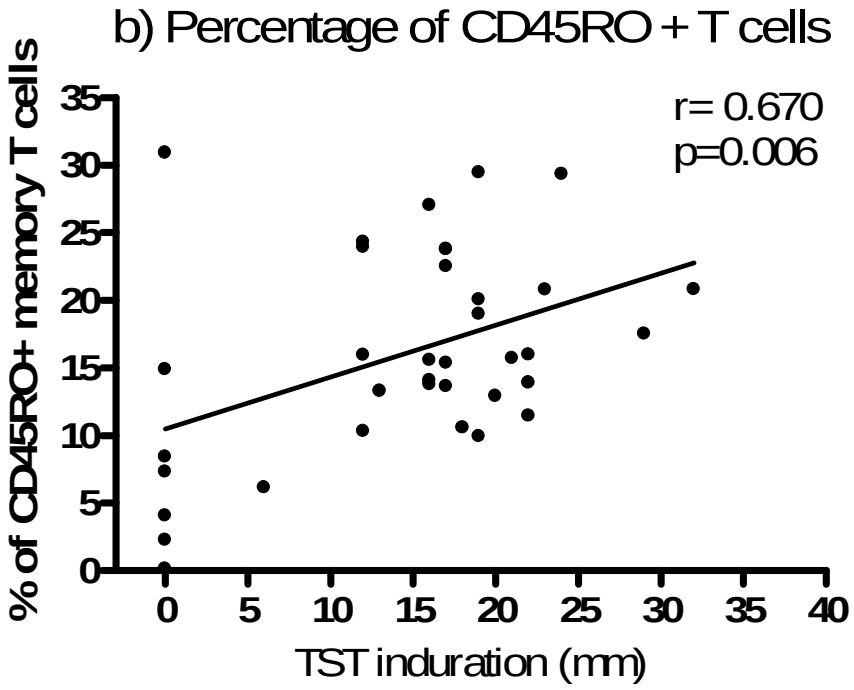


Figure 3.10 b) Correlation between the percentage of CD45RO+ memory T cells at the Tuberculin Skin Test (TST) reaction site and the TST induration diameter. The percentage of CD45RO+ memory Tcells (Spearman r= 0.670, p=0.006) correlates significantly with the size of the TST induration.

4 Discussion

Coinfection with HIV and *M. tuberculosis* is currently one of the most pressing health problems in the world, especially in sub-Saharan Africa [3]. In the last few years many research studies have investigated the clinical and epidemiological features of the two diseases as well as their coexistence and coinfection [25, 29, 33, 105-107].

It is long since known that the Tuberculin Skin Test is unreliable to detect infections with *M. tuberculosis* among HIV infected persons [71], and in the last few years new and better diagnostic tests for tuberculosis, most importantly interferon-γ release assays, have been developed [45].

Despite of its clinical limitations the TST offers an opportunity to study *M. tuberculosis*-specific immune regulations. We therefore used the TST to study, at the site of its application, the immunological features and regulations being involved in tuberculosis related immune responses in immunocompromised HIV infected persons.

This study investigated the immunoregulation of the DTH reaction in the TST in HIV infected and uninfected persons.

Phenotypes of PBMC and cells from skin biospy samples out of the TST reaction area from 22 HIV-seronegative and 15 HIV-seropositive individuals from Khayelitsha Township, South Africa, where tuberculosis and HIV are highly endemic, were explored. All persons underwent TST testing and had biopsy samples taken from the test area in the skin and from skin infiltrated with normal saline for controls. The skin samples were deep-frozen, cut into thin slides and then immunohistochemically stained for several phenotypic markers for different mononuclear cell subtypes. Analysis was performed by using a computerised image analysis system. Furthermore, blood samples were obtained and PBMC subsets were analysed by FACS.

4.1 Reactions in the Tuberculin Skin Test

In our study cohort, 21 out of the 22 HIV uninfected persons (95 percent) had a positive reaction in the TST at a cut-off of 5mm induration (**table 3.1**), indicating that *M. tuberculosis* infections are highly endemic in Khayelitsha [95]. In the HIV infected group, nine participants scored positive (60 percent) and six did not mount a positive reaction (40 percent). This is in concordance with previous studies that reported TST anergy rates between 30-50 percent among HIV infected persons [70, 71, 78].

It was not possible to confirm suspected LTBI in all enrolled persons by ELISPOT assay and thus it could also not be proven with absolute certainty that TST unreactive persons were in fact anergic to Tuberculin. The uncomplete ELISPOT results were mainly due to a lack of sufficient amounts of PBMC obtained from the blood samples. Nevertheless, overall rates of TST positivity and RD1 based ELISPOT positivity (**table 3.2**) support the assumption that the vast majority of the enrolled individuals had previously been exposed to *M. tuberculosis*. This is very highly consistent with previous observations in this community, as Rangaka et al. demonstrated the extremely high prevalence of LTBI among HIV seropositive inhabitants from Khayelitsha Township who are frequently exposed to tuberculosis [78].

4.2 Influence of the CD4+ T cell count on reactivity in the Tuberculin Skin Test

The status of immunodeficiency and the stage of disease during HIV infection are generally described and classified by using the CD4+ T cell count in peripheral blood (expressed as CD4+ cells per microliter blood). Severe conditions and life-threatening infections usually occur at low CD4 count levels below 200 cells per microliter blood [22, 23]. Infections caused by *M. tuberculosis* are known to occur even at earlier stages of HIV infection [24, 25, 108], although extrapulmonary tuberculosis and infections with non-tuberculous mycobacteria usually occur at

further progressed stages of HIV infection and often lead to very severe conditions and outcomes of the patients [109, 110].

While susceptibility to opportunistic diseases increases when the CD4 count falls, the ability to mount sufficient immunologic responses towards antigenic challenges decreases dramatically. As the Tuberculin Skin Test relies on efficient functioning of the immune system in order to recall immunologic memory to *M. tuberculosis*, the likelihood of HIV infected persons with advanced immunodeficiency to fail in mounting sufficient immune responses is generally considered to increase with decreasing CD4 count numbers [70, 71].

Surprisingly, in this study the CD4 count did not correlate with the TST reactivity of HIV infected persons (**figure 3.4**). This supports earlier findings from Lange et al. [111] who showed that T cell responsiveness to various antigens, including Tuberculin, was not related to the CD4 count of HIV infected individuals.

It can therefore be argued that generally efficient immune responses towards *M. tuberculosis* are at miss even at early stages of HIV infection. Clinically, the risk of developing active tuberculosis is already increased at those early stages [31].

4.3 Relationship between CD4+ and CD8+ T cell subsets in peripheral blood and HIV status

As HIV mainly infects immune cells carrying the CD4 molecule, the CD4 T cell count drops remarkably during early infection. In contrast to this, numbers of circulating CD8+ cytotoxic cells are known to rise at the same time and remain at a relatively steady state until very late in the disease. This increase of CD8+ cytotoxic T cells is associated with decreasing levels of the plasma viral load soon after the initial peak of viremia [112].

With regards to further specification of the cellular subgroups of CD4+ and CD8+ T lymphocytes during the course of HIV infection, a profound loss of naïve cells (both CD4+ and CD8+) has been described, whereas cells with a memory phenotype seem to be increased respectively [113-115].

It has been shown that the proportion of naïve CD8+ cells is decreased in HIV-1 infection [113], a finding that was reproduced in this study (**supplement table 2**). The same study by Roederer et al. also documented a reduction in the proportion

of naïve CD4+ cells as HIV-1 infection progressed. Such a decrease was not documented among the subjects of this study. This difference between studies may be related to undoubted differences in pathogen exposure between California and South Africa, gender (most of the patients in this study were female), and prior antiretroviral therapy (all patients in this study were antiretroviral naïve). The finding of Roederer et al. that the absolute number of naïve cells is strongly related to the absolute CD4 count was corroborated in this study (Spearman $r=0.91$, $p<0.0001$).

It was furthermore shown in this study that HIV infected persons had more activated HLA-DR+ CD8+ T cells in their peripheral blood than uninfected persons. This was to be expected as chronic viral infections including HIV usually go along with increased numbers of activated T cells [116-118]. Activated T cells are known to increase further with disease progression. This generalised immune activation may play a role in maintaining an asymptomatic stage of the infection for a certain period of time, or it may on the other hand contribute to the onset of symptoms in HIV disease.

4.4 The role of regulatory T cells during the immune response to tuberculin

Regulatory T cells (Tregs), determined by a CD4+CD25+ phenotype and by expression of the transcription factor FoxP3, have been shown to have immunosuppressive properties [87]. Their natural function is to prevent the immune system to react or even overreact towards its own body, i.e. producing autoimmune reactions [88]. Tregs are known to be generally upregulated during active infections in order to avoid collateral tissue damage caused by vigorous immune responses towards the pathogen [119].

Regulatory T cells are known to be expanded in chronic HIV infection [84, 120, 121] and high FoxP3 expression levels are linked to severity of HIV disease [85, 86, 122] as HIV-specific T cell responses are suppressed by Tregs [121]. High levels of FoxP3+ Tregs have been shown to be directly correlated with the plasma viral load [85].

Numbers of circulating Tregs have also been shown to be upregulated during tuberculosis [90] and have been related to the suppression of *M. tuberculosis*-specific immune responses in active tuberculosis [92, 93]. It has been demonstrated by Scott-Browne et al. that Tregs proliferate and expand at the site of tuberculosis [123]. High frequencies of CD4+CD25+ T cells and high FoxP3 expression levels are known to suppress otherwise efficient T cell responses to *M. tuberculosis* and the production of IFN-γ in active tuberculosis [91, 124].

In addition, Tregs could also play a role in suppressing immune responses needed for an adequate DTH response to Tuberculin in the Tuberculin Skin Test.

In this study it was demonstrated that the reaction in the TST, measured in millimeters of induration, is negatively correlated to the frequency of CD4+FoxP3+ Tregs in the peripheral blood. This relationship can be observed in all persons (HIV infected and HIV uninfected) and presents itself even stronger in the HIV infected subgroup (**section 3.4.2** and **figure 3.3**). High numbers of Tregs are thus related to weak reactions in the TST or even to unreactivity. It was a statistically significant finding in this analysis that TST unreactive subjects had higher numbers of Tregs in their blood than TST reactive persons (**section 3.4.1, figure 3.2**).

Li et al. have demonstrated in their study about the role of Tregs in the control of cellular immune responses in tuberculosis that Tregs are able to inhibit the production of IFN-γ by CD4 T cells in response to stimulation with BCG [125].

Our data extend these observations suggesting that Tregs also contribute to the suppression of antigen specific responses to Tuberculin in the TST. Although we did not directly investigate the immunosuppressive properties of the cells staining positive for FoxP3, we suggest that they may play a role in the regulation of the response to the antigen challenge, as it has been shown before that the function and activity of Tregs depends upon FoxP3 expression [87].

It can furthermore be speculated that Tregs that are usually upregulated during HIV infection contribute to both the increased susceptibility to tuberculosis in HIV infected patients and to the lack of appropriate immune responses to *M. tuberculosis*.

There also seem to be other factors during HIV infection that could be contributing to the suppression of HIV-specific T cell responses rather than just Tregs. Hu et al. have recently published a study on the role of HIV-envelope glycoprotein (Env)-dependent suppression of T cells during HIV infection [126]. Env seems to induce

suppression of HIV-specific T cell responses independent of FoxP3 expression and Tregs.
However, the background of immunoregulation in HIV infection is still poorly understood and will remain a great target for research in the future.

4.5 The composition of cellular infiltrates at the reaction site of the Tuberculin Skin Test

In this study the phenotypes of the cellular infiltrates at the TST reaction site from skin biopsies of HIV infected and HIV uninfected persons were investigated by immunohistochemical stainings and computerised image analysis.
Observations from this study have confirmed that the visible and palpable induration of the TST reaction in the skin is caused by a localised lymphocytic cell infiltrate rather than just by an inflammatory edema of the skin. The study furthermore provides new information on the cellular subgroups being involved in reactions in the TST and on differences between cell populations from HIV infected and HIV uninfected persons. Furthermore, differences of cell compositions between positive and negative reactions in the TST have been described.

A few studies and investigations into the cellular background of DTH responses have so far been conducted in the past. The studies that are discussed in the following mainly used light microscopy and early immunological techniques.
In 1974 Dvorak et al. [65] conducted a quantitative analysis of the inflammatory response during DTH-reactions in the skin by using light microscopy and immunofluorescence. They showed that at early stages during DTH reactions (4-8 hours) there is an infiltration of lymphocytes mainly around small blood vessels which over the course of the next hours and days increases progressively in intensity, reaching a maximum after three days. Infiltrates were noticed to subsequently extend into the intervascular dermis and epidermis.
Platt et al. further specified in their study from 1983 the different immune cell populations being involved in DTH reactions [63]. Confirming the quantitative evaluations made by Dvorak et al. [65], they extended these observations by

describing that the majority (75-90 percent) of the mononuclear cells consists of T lymphocytes and monocytes, with CD4+ T cells being more numerous than CD8+ T cells. Activation markers were found to be progressively increased during the course of the response.

It has furthermore been reported that the lymphocytic cell infiltrate is composed of CD4+ and CD8+ T cells at a ratio of 2:1 throughout a 72-hour time course [64]. This study by Poulter et al. also documented a rapid increase of Langerhans cells in the epidermis and suggested an important role for interdigitating cells in expressing the local immune response. Another discovery of this study was the absence of B lymphocytes and plasma cells in the DTH reaction.

Similar results concerning cellular infiltrates during DTH reactions were presented by another study on reactions to Tuberculin with regard to infections by *Mycobacterium leprae* [127, 128].

Fullmer et al. [129] investigated Tuberculin reactions of patients with active pulmonary tuberculosis. They particularly described a predominant mixture of lymphocytes and macrophages with lymphocytes presenting activated after 24 hours of the reaction course. They also reported that lymphocytes were producing IL-2 and IFN-γ which they interpreted as reflecting the occurence of a DTH reaction.

Starting at this point of knowledge from times when digital analysis was not yet available, this study is the first to describe in detail the lymphocytic subgroups of the cellular infiltrates in DTH reactions to Tuberculin by means of applying digital image analysis which allows for quantification of the positive immunoreactivity at the single-cell level *in situ*.

The finding by Poulter et al. [64] that CD4+ and CD8+ T cells are present at a ratio of approximately 2:1 was reproduced in this study, but this was only the case in HIV uninfected persons (**supplement table 2**). In HIV infected persons numbers of CD4+ T lymphocytes were significantly reduced at the skin site of the TST which is in concordance with the general depletion CD4+ T helper cells during HIV infection [18].

On the other hand, numbers of CD8+ T cells in HIV infected persons did not differ significantly from those in uninfected persons, although there was a slight trend towards higher numbers of CD8+ T cells in HIV infected persons. This agrees with

the known shift of predominating T cell numbers towards a CD8+ cytotoxic phenotype during HIV infection [112].
With regard to further differences of the cellular infiltrates between HIV infected and HIV uninfected persons it was observed, in addition to the first finding that HIV infected persons had less CD4+ T cells in their skin reactions, that they also had significantly less CD45RA+ and CD62L+ T cells in their skin reactions (**figure 3.7**) than uninfected persons. This emphasizes that TST reactions depend upon a clonal expansion of T lymphocytes which seems to be impaired in HIV infected persons.
It was furthermore discovered that memory CD45RO+ was the dominating phenotype of CD4+ T lymphocytes at the site of the TST reaction. The induration diameter of the TST reaction was strongly correlated to the total number of infiltrating cells and also to the number of infiltrating CD45RO+ memory T cells (**figure 3.10**).

Skin samples from TST reactive HIV infected and TST reactive HIV uninfected persons were compared in order to make out differences in the cellular infiltrates of HIV infected and those of uninfected persons that are not due to differing reactivity. It was again observed that HIV infected people had less CD4+ T cells in their skin than uninfected persons, and again numbers of CD62L+ T cells were reduced in infected compared to uninfected persons (**figure 3.8**). On the other hand, HIV infected TST reactive persons had significantly more HLA-DR+ activated T cells in their skin than uninfected persons.
This is in concordance with the observation that HLA-DR+ activated T cells are more dominant in the blood of HIV infected persons compared to the blood of uninfected persons. This also agrees with previous descriptions in the literature [116-118].

It was also investigated whether differences in cellular infiltrates could be observed between samples from persons that showed positive reactions in the TST and those from people who were unreactive. For this the cellular infiltrates in skin samples from TST reactive and TST unreactive HIV infected persons were compared. It was observed that unreactive persons had significantly lower total

numbers of infiltrating cells at the TST site, and especially of CD45RO+ memory T cells.

This observation suggests that unreactivity in HIV infected persons is due to a lack of influx of memory T cells to the site of the TST in the skin. It highlights that immunologic memory is dramatically impaired in HIV infected persons. Reactions in the TST are based on immunologic memory to *M. tuberculosis*. Persons that have been exposed to *M. tuberculosis* and thus are thought to have developed an immunologic memory towards *M. tuberculosis* should theoretically exhibit positive DTH reactions to Tuberculin. This reaction is believed to be generated by memory T cells that recognise the antigen and subsequently initiate recruitment and clonal expansion of lymphocytes and thus lead to an inflammatory response at the site of the TST administration. In HIV infection, such immunologic responses are reduced due to decreased numbers of T helper cells and thus immunologic memory as well as effector functions are impaired. HIV infected persons are therefore often not capable of mounting positive reactions to antigen challenges, including immunologically based diagnostic tests such as the TST.

4.6 Are cell accumulations in the skin test area due to cell recruitment or clonal expansion of existing cells?

Two possible models could be used to explain the differences observed between different skin samples regarding cellular infiltrates and cell distribution in the TST area: differences could either be due to differences in preexisting cell populations in the skin which then clonally expand upon PPD stimulation, or they could result from differences in cell recruitment.

To approach this question, numbers of CD1a+ (antigen presenting cells), CD4+ (T helper cells) and CD8+ (cytotoxic T cells) cells from normal skin (PPD uninfiltrated control samples) and from the TST reaction site were compared. No correlation was found between the distribution of these T cell subsets prevalent in normal skin and the TST induration diameter. Furthermore there were no correlations observed between numbers of those cell populations in normal skin compared to TST reactive tissue.

It can therefore be concluded that active recruitment of immune cells to the TST site is necessary in order to evoke the typical reaction in the skin and that this happens irrespectively of the cells that are prevalent under normal conditions. Clonal expansion of preexisting cell clones does not seem to play a major role in establishing the cellular infiltrates during TST reactions.
The study was nevertheless not designed to investigate the mechanisms of cell recruitment or cell expansion and their contributions to the TST reaction in detail. For instance, no specific chemokine analyses were conducted which could have provided further information on that matter. Future studies will be needed in order to approach this further.

4.7 Conclusions, limitations of the study and future prospects

This is the largest study to date to compare immunophenotypes of cells in peripheral blood and at the site of Tuberculin injection in HIV infected and uninfected persons. It is also the first study to investigate the role of different T cell populations in the peripheral blood and at the site of the TST reaction and the influence of human HIV infection on reactivity in the TST.
It was demonstrated that a positive TST-reaction is characterised by a local accumulation of T cells, predominantly of CD45RO+ memory T cells. HIV infected persons show a reduction of infiltrating cells at the TST reaction site, especially of CD4+ T cells. Reduced TST reactivity seems to be related to the number of circulating CD4+Foxp3+ regulatory T cells rather than to the number of circulating CD4+ T cells in the blood.

A limitation of the study is the lack of ultimate proof of the general prevalence of LTBI among all of the enrolled persons, although overall rates of TST positivity and ELISPOT results support the assumption that the vast majority of the enrolled individuals had previously been exposed to *M. tuberculosis*.
Yet, even if LTBI is confirmed by additional tests it still is not clear whether this represents an ongoing chronic infection with *M. tuberculosis* or whether it simply reflects a positive immune response and immunologic memory to *M. tuberculosis.*

Another limitation was the varying quality of the skin biopsies that led to exclusion of one biopsy from the analysis. Nevertheless, biopsies from all other participants provided enough and sufficient material for appropriate analysis.
For logistic reasons the immunohistochemical analysis was performed on frozen material. It could be argued that the results might have slightly differed if the analysis had been conducted on fresh samples.
With regards to the immunohistochemical stainings, it could have added further interesting information to the existing data if double or triple stainings had been conducted, which means combining two or three different antibodies against phenotypic markers in one staining in order to further specify the cellular subgroups at the TST reaction site.
Furthermore, only the marker FoxP3 was used to identify regulatory T cells, although it would have been more exact to also analyse the Treg surface marker CD25.
In contrast to measuring TST reactions in persons with suspected LTBI it could be interesting to also observe TST reactions in persons with active tuberculosis. Observations in this study were obtained from individuals from a community with high tuberculosis incidence. Performance of the same analyses on persons from low incidence settings could possibly provide additional information on TST responsiveness and anergy rates among those populations.

This study has gained some interesting information on the immunoregulations that are involved in TST reactions in HIV infected persons. Yet there are still many open questions that could be further investigated in the future. It would for example be interesting to further look into functional aspects of the immunoregulations, such as cell recruitment or local cell expansion at the TST reaction site. In order to approach this issue, cytokine and chemokine profiles could be analysed in addition to the cell phenotypes. It would furthermore be interesting to gain more information on the role of regulatory T cells in suppressing immune responses to Tuberculin. The actual immunosuppressive properties of Tregs during TST reactions could be further investigated in *in vitro* studies on cell interactions and possible cell depletions induced by Tregs. It could also be investigated whether Tregs play a role in false-negative TST results during active tuberculosis.

5 Summary

Human immunodeficiency virus (HIV)-infection and tuberculosis are the most pressing public health problems in Africa. The epidemiology and clinical features of HIV associated tuberculosis are well researched, but few studies have analysed the immunophenotypes of T cell populations during HIV/*M. tuberculosis* coinfection in detail. The Tuberculin Skin Test (TST) was the diagnostic gold standard for tuberculosis in the last century. Investigations of the immune responses to the TST provide an opportunity to better understand the responses to *M. tuberculosis* antigens in HIV infected people, which may also be important for future anti-tuberculosis vaccine developments. The TST is safe and a relatively standardised test and its route of administration (intradermally) facilitates antigen presentation via dendritic cells to *M. tuberculosis* reactive T cells. These cells expand upon restimulation in the skin and give rise to the characteristic type IV delayed type hypersensitivity (DTH) reaction. A detailed immunologic analysis of TST reactions in HIV infected and uninfected people could therefore provide an invaluable window to study, at a site of inflammation, the immune dysregulation that characterises the increased susceptibility of HIV infected people to tuberculosis better.

Twenty-two HIV-1 seropositive and 15 HIV-1 seronegative persons from Khayelitsha, South Africa, a community with a high incidence of tuberculosis and a high prevalence of HIV infection, were enrolled in this study. Skin biopsies were taken from the TST site at the volar aspect of a forearm 48 to 72 hours after the intradermal injection of 0.1 ml (2 TU) Tuberculin RT23. Control biopsies were taken from the other forearm 48 to 72 hours after the intradermal application of 0.1 ml normal saline. Biopsies were immediately snap frozen in liquid nitrogen for later analysis. Eight micrometer thin histological sections were prepared from the biopsies and were then immunohistochemically stained with monoclonal antibodies against CD4, CD8, CD28, CD45RA, CD45RO, CD62L, CD1a, HLA-DR, granulyzin, interferon-γ (IFN-γ) and FoxP3 in order to observe the distribution of different mononuclear cell subgroups. The stained sections were analysed by digital imaging at the single-cell level *in situ*. Peripheral blood mononuclear cells (PBMC) were obtained from blood samples of all individuals and analysed by

Fluorescence Activated Cell Sorting (FACS) analysis using monoclonal antibodies against CD4, CD8, CD28, CD45RA, CD45RO, CD62L, HLA-DR and FoxP3 for determination of immunophenotypes. In addition, Region of difference (RD)-1 based IFN-γ ELISPOT analyses were conducted on PBMC for determination of LTBI.

Out of 15 HIV infected persons, 9 showed a positive reaction in the TST (60 percent) whereas 6 persons were unreactive (40 percent). In contrast, only one out of 22 HIV uninfected persons was TST unreactive ($p=0.01$). In biopsies from the TST reaction site, HIV infected persons had lower absolute numbers of cells ($p=0.045$). Numbers of CD4+ T cells ($p=0.005$) were especially reduced in skin biopsies from the TST reaction site of HIV-infected persons compared to the biopsies of HIV uninfected persons. In contrast, HIV infected persons had more HLA-DR+ activated T cells ($p=0.037$) in samples from positive TST reactions compared to uninfected persons. In HIV infected persons, the total number of cells ($p=0.008$) and especially numbers of CD45RO+ memory T cells ($p=0.003$) were significantly higher in TST reactive than in unreactive skin samples. No correlation was found between immunophenotypes in normal skin and in TST reactive skin.

In HIV infected persons, the TST induration diameter inversely correlated with the frequency of circulating CD4+FoxP3+ T cells in the blood ($p<0.026$), but was unrelated to the number of circulating CD4+ T cells.

In conclusion, the TST reaction in HIV infected persons depends upon an influx of memory T cells to the TST reaction site in the skin and relates better to numbers of circulating FoxP3+ regulatory T cells in the blood than to the circulating CD4 T cell count.

6 Ausführliche deutsche Zusammenfassung

HIV und Tuberkulose gehören zu den bedeutendsten Infektionskrankheiten weltweit. Die WHO schätzt, dass ca. ein Drittel der Weltbevölkerung mit *Mycobacterium tuberculosis*, dem Erreger der Tuberkulose infiziert ist. Etwa 33 Millionen Menschen weltweit sind mit dem Humanen Immundefizienzvirus (HIV) infiziert. Die Tuberkulose stellt die wichtigste opportunistische Infektion von HIV-Infizierten dar.

Nach Kontakt mit *M. tuberculosis* erkranken schätzungsweise 5-10 Prozent immunkompetenter Personen im Laufe ihres Lebens an einer aktiven Tuberkulose. Die übrigen Personen, die nach Kontakt zu *M. tuberculosis* eine spezifische Immunreaktion ausbilden, gelten als latent infiziert. Personen mit einer latenten *M. tuberculosis*-Infektion sind nicht an einer Tuberkulose erkrankt und sie können den Erreger nicht auf andere Personen übertragen. Bei einer Änderung der Immunkonstitution kann im späteren Leben eine aktive Tuberkulose entstehen. Etwa 50 Prozent der Fälle einer aktiven Tuberkulose treten in den ersten zwei Jahren nach der Primärinfektion auf. Anders als immunkompetente Personen haben HIV-seropositive Personen ein vielfach höheres Risiko, an einer aktiven Tuberkulose zu erkranken. Im Gegensatz zu den meisten übrigen opportunistischen Infektionen tritt die Tuberkulose häufig schon in frühen Stadien der HIV-Infektion auf.

Der Tuberkulin-Hauttest (THT) gilt weltweit als Goldstandard zur Immundiagnose einer Infektion mit *M. tuberculosis*. Allerdings kann mit dem THT, ähnlich wie mit neueren immunologischen Verfahren an mononukleären Zellen im peripheren Blut, den Interferon-γ Release assays (IGRA), eine aktive Tuberkulose nicht von einer latenten Infektion mit *M. tuberculosis* unterschieden werden. Bei der Durchführung des THT wird Tuberkulin, ein gereinigtes Antigengemisch von *M. tuberculosis* (purified protein derivate, PPD), intradermal gespritzt. Bei bestehendem T-Zell-Gedächtnis gegenüber den Antigenen bildet sich eine sicht- und tastbare Hautreaktion aus, die auf einer Hypersensitivitätsreaktion des verzögerten Typs (nach Coombs und Gell) beruht. Eine Induration von mehr als 5mm Durchmesser gilt bei HIV-seropositiven Personen als positive Testreaktion. Positive THT-Reaktionen können auch bei Infektionen mit anderen, nicht-

tuberkulösen Mykobakterien, oder nach erfolgter Tuberkulose-Impfung mit dem Bacille Calmette-Guèrin (BCG)-Impfstoff auftreten. Mit Fortschreiten der Immundefizienz im Verlauf der HIV-Infektion werden zunehmend falsch negative Testergebnisse beim THT beobachtet.

Die Pathogenese der Koinfektion mit HIV und *M. tuberculosis* wird bislang nur unzureichend verstanden. Unter anderem ist unklar, welche Faktoren die Ausbildung einer THT-Reaktion beeinflussen und welche Mechanismen dazu führen, dass bei der HIV-Infektion gehäuft falsch negative THT-Ergebnisse auftreten. Studien zur Pathogenese der THT-Reaktion bei HIV-seropositiven Personen könnten dazu beitragen, ein besseres Verständnis für die Mechanismen der Immunregulation bei der HIV/*M. tuberculosis* Koinfektion zu entwickeln.

Ziel dieser Studie war es, die THT-Reaktionen von HIV-seropositiven und HIV-seronegativen Personen und die daran beteiligten Immunzell-Subtypen in der Haut und im peripheren Blut detailliert zu untersuchen.

HIV-seronegative und HIV-seropositive Personen aus dem Township Khayelitsha bei Kapstadt (Südafrika), einer Population mit sehr hoher HIV-Prävalenz und Tuberkulose-Inzidenz wurden für diese Studie rekrutiert. Für den THT wurde den Probanden 0,1ml (2 TU) Tuberkulin RT23 an der Innenseite eines Unterarms intradermal injiziert. Zur Kontrolle diente die intradermale Injektion von 0,1ml NaCl (0.9%) an der Innenseite des anderen Unterarms. Im Intervall von 48-72 Stunden nach der Injektion wurde der Durchmesser der Induration im Testareal mit der Kugelschreibermethode vermessen. Anschließend wurden aus den Testarealen an beiden Unterarmen Hautbiopsien (THT-Gebiete: 4mm Durchmesser, Kontrollen: 2mm Durchmesser) entnommen. Die Hautbiopsien wurden sofort vor Ort tiefgefroren. Später wurden die Biopsien nach dem Transport ins Labor in 8µm dünne Schnitte geschnitten und dann mittels Immunhistochemie mit monoklonalen Antikörpern gegen verschiedene Zellmarker und Zytokine (CD4, CD8, CD28, CD45RA, CD45RO, CD62L, CD1a, HLA-DR, Granulyzin, Interferon-γ und FoxP3) angefärbt. Die Analyse der gefärbten Schnitte erfolgte mit Hilfe eines digitalen Bildanalyseverfahrens zur flächenbezogenen Auswertung der positiven Farbsignale. Außerdem wurden Blutproben der Studienteilnehmer zur Immunphänotypisierung der mononukleären Zellen im peripheren Blut gewonnen. Die Blutproben wurden mittels FACS-Analyse auf die Verteilung der verschiedenen Immunzell-Subtypen hin mit monoklonalen Antikörpern gegen CD4,

CD8, CD28, CD45RA, CD45RO, CD62L, HLA-DR und FoxP3 untersucht. Außerdem wurden RD-1 spezifische Interferon-γ ELISPOT-Analysen zur Feststellung einer latenten Infektion mit *M. tuberculosis* durchgeführt.

Neun von 15 HIV-seropositiven Personen (60 Prozent) und 21 von 22 HIV-seronegativen Personen (95 Prozent) zeigten positive Reaktionen im THT (p=0,01).

Die Anzahl der Zellen und die Verteilung der Zellpopulationen aus den Kontrollbiopsien unterschieden sich nicht zwischen HIV-seropositiven und HIV-seronegativen Studienteilnehmern. Die Anzahl der Zellen und die Verteilung der Zellpopulationen aus den Kontrollbiopsien korrelierten nicht mit dem Ausmaß der Reaktion im THT oder mit den Zellverteilungen in den Tuberkulin-Testbiopsien.

In den Hautbiopsien aus reaktiven THT-Arealen wiesen HIV-seronegative Personen mehr Zellen insgesamt (p=0,045) und insbesondere mehr CD4+ T Zellen (p=0,005) im Vergleich zu den HIV-seropositiven Personen auf. Im Gegensatz dazu fanden sich bei HIV-infizierten Personen mehr HLA-DR+ aktivierte T-Zellen in den Biopsien aus den THT-reaktiven Testarealen als bei HIV-nicht-infizierten Personen (p=0,037). In den Hautbiopsien von HIV-seropositiven Personen wiesen diejenigen mit einer positiven THT-Reaktion mehr Zellen (p=0,008), insbesondere mehr T-Gedächtniszellen (CD45RO+, p=0,036) und naive T-Zellen (CD45RA+, p=0,003), auf als diejenigen mit einer negativen THT-Reaktion.

Das Ausmaß der THT-Reaktion (in Millimetern Induration) war negativ mit der Anzahl an zirkulierenden CD4+FoxP3+ regulatorischen T-Zellen im Blut korreliert (r=-0,39, p=0,015 bei allen Personen, r=-0,571, p=0,026 bei HIV-Infizierten). Das Ausmaß der THT-Reaktion korrelierte hingegen nicht mit der Gesamtzahl an CD4+ T-Helferzellen im Blut, welche klinisch sehr häufig als Parameter für das Stadium einer HIV-Infektion herangezogen wird.

Zusammenfassend lässt sich daher feststellen, dass eine positive Reaktion im Tuberkulin-Hauttest durch das Einwandern von Zellen, insbesondere von T-Gedächtniszellen, in das Reaktionsgebiet in der Haut bestimmt wird. Bei HIV-seropositiven Personen ist die Anzahl an T-Helferzellen im THT-Reaktionsgebiet gegenüber HIV-seronegativen Personen erniedrigt. Das Ausmaß der THT-Reaktion hängt von der Anzahl an regulatorischen T-Zellen im Blut, hingegen nicht von der Gesamtzahl der CD4+ T-Zellen im Blut, ab.

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8 Supplements

Table 1. Staining antibodies

Antibody	Company	Isotype	Concentration	Conjugate	Dilution
CD1a	Dako Cytomation, DK	mouse IgG2a	0,145mg/ml	0	1:10
CD4	BD Biosciences, USA	mouse IgG1	0,0125mg/ml	0	1:10
CD8	BD Biosciences, USA	mouse IgG1	0,0125mg/ml	0	1:10
CD28	Dako Cytomation, DK	mouse IgG1	0,145mg/dl	0	1:20
CD45RA	Dako Cytomation, DK	mouse IgG1	0,145mg/dl	0	1:50
CD45RO	Dako Cytomation, DK	mouse IgG2a	0,145mg/dl	0	1:50
CD62L	Dako Cytomation, DK	mouse IgG1	0,145mg/dl	0	01:10
Foxp3	Abcam, GB	goat polycl.	0,5mg/ml	0	5µg/ml
Granulysin	Santa Cruz Biotech, USA	goat polycl.	0,2mg/ml	0	1:200
HLA-DR	BD Biosciences, USA	mouse IgG2a	0,025mg/ml	0	1:50
IFN-γ	Mabtech, Sweden	mouse IgG1	1mg/ml	0	2µg/ml
Neg.contr. IgG1	Dako Cytomation, DK	mouse IgG1		0	
Neg.contr. IgG2a	Dako Cytomation, DK	mouse IgG2a		0	
Mouse Ig	Dako Cytomation, DK	rabbit polycl.	0,79mg/ml	biotin	1:500
Goat Ig	Dako Cytomation, DK	rabbit polycl.	0,76mg/ml	biotin	1:500

Table 2. Analysis of the phenotype of cells in peripheral blood

	All HIV uninfected	Reactive HIV uninfected	All HIV infected	Reactive HIV infected	Unreactive HIV infected
	n=23	n=20	n=15	n=9	n=6
Marker	% of cells positive (median values)				
Total $CD4^+$	24.4	24.8	14.1[1]	14.0[2]	15.4
IQR	20.4-37.0	20.1-37.2	10.1-21.6	8.7-24.3	9.7-20.0
$CD4^+$ $CD28^+$	34.7	37.1	20.8[3]	22.0[4]	18.0
IQR	30.1-42.9	30.7-43.5	13.2-23.5	10.0-24.2	13.4-34.0
$CD4^+$ HLA-DR^+	2.7	2.7	2.4	3.4	2.2
IQR	2.3-3.9	2.2-4.2	2.0-4.5	2.2-5.6	1.7-3.6
$CD4^+$ $CD45RA^-$ RO^+	39.2	38.2	26.2	31.6	19.2[5]
IQR	30.0-45.1	29.2-43.9	19.6-40.4	26.1-54.7	16.6-26.1
$CD4^+$ $CD45RA^+CD62L^+$	43.9	44.1	55.9	52.8	58.6
IQR	33.9-50.1	34.6-49.2	30.8-63.0	25.6-61.7	40.9-67.3
$CD4^+$ $FoxP3^+$	8.1	7.8	8.5	7.7	13.7[6]
IQR	6.7-9.1	6.5-9.4	7.1-13.3	6.4-10.2	7.8-17.3
$CD8^+$ $CD28^+$	14.0	13.6	14.3	14.3	15.1
IQR	13.2-18.8	13.1-17.2	9.9-18.7	6.9-19.9	10.2-18.3
$CD8^+$ HLA-DR^+	10.8	10.5	18.9[7]	21.7[8]	18.0
IQR	6.1-13.4	6.0-12.6	16.5-34.2	13.0-39.7	12.0-28.7
$CD8^+$ $CD45RA^-$ RO^+	8.2	8.3	11.3[9]	14.7[10]	8
IQR	3.3-11.9	3.4-10.9	7.1-16.6	8.9-21.8	4.9-14.4
$CD8^+$ $CD45RA^+CD62L^+$	46.1	45.9	33.4	35.9	32.6
IQR	38.6-51.6	39.1-49.0	26.8-41.3	25.2-44.1	28.9-38.6

[1] p = 0.0005 by comparison with HIV uninfected.

[2] p = 0.010 by comparison with PPD reactive HIV uninfected.

[3] p = 0.0001 by comparision with HIV uninfected.

[4] p = 0.0004 by comparison with PPD reactive HIV uninfected

[5] p = 0.005 by comparison with PPD reactive HIV infected.

[6] p = 0.036 by comparison with PPD reactive HIV infected.

[7] p = 0.0007 by comparison with HIV uninfected.

[8] p = 0.009 by comparison with PPD reactive HIV uninfected.

[9] p = 0.006 by comparison with HIV uninfected.

[10] p = 0.040 by comparison with PPD reactive HIV uninfected.

Table 3. Analysis of the phenotype of cells in reactional skin

	All HIV uninfected	Reactive HIV uninfected	All HIV infected	Reactive HIV infected	Unreactive HIV infected
	n=22	n=21	n=15	n=9	n=6
Marker					
Total % of cells *	12.0[11]	13.0	6.0	11.0[12]	4.5
IQR	9.0-14.0	10.0-14.0	5.0-13.0	6.5-15.5	3.0-6.0
CD4+ #	12.3[14]	12.6[15]	7.7	9.2	4.8
IQR	8.0-20.9	9.4-23.3	3.0-11.1	4.4-11.0	2.0-11.8
CD8+ #	4.9	5.1	5.5	5.8	4.1
IQR	2.4-13.1	2.9-13.5	2.3-8.1	1.8-12.5	2.0-7.2
CD28+ #	3.8	3.4	5.6	5.0	6.0
IQR	1.4-8.0	1.2-8.8	2.6-13.0	2.9-10.2	2.1-23.0
Interferon-□+ #	3.1	3.1	3.4	3.8	2.4
IQR	1.7-6.0	1.2-6.1	1.9-6.4	2.5-6.4	1.9-6.8
HLA-DR+ #	26.2	26.2[16]	35.2	37.8	22.9
IQR	15.3-35.0	17.5-34.4	18.6-43.0	31.0-43.1	13.7-41.2
CD1a+ #	3.6	5.9	4.2	5.3	2.1
IQR	0.9-8.6	1.6-9.3	2.1-7.0	3.3-7.0	1.2-5.7
CD45RA+ #	5.3	5.3	3.0	4.4[17]	1.7
IQR	2.5-15.1	2.5-11.9	1.3-6.4	2.9-7.8	0.1-3.5
CD45RO+ #	15.7	15.7	13.9	16.0[18]	5.7
IQR	13.1-23.2	13.3-22.5	7.3-17.5	13.8-23.8	1.2-11.7
CD62L+ #	3.5[19]	3.5[20]	0.7	0.7	1.5
IQR	1.2-6.1	1.2-6.1	0.6-3.5	0.5-3.4	0.7-3.1
Granulysin+ #	0.6	0.6	0.5	0.5	0.7
IQR	0.3-1.0	0.2-1.0	0.4-1.0	0.4-0.9	0.3-1.0
FoxP3+ #	0.3	0.3	0.5	0.7	0.2
IQR	0.1-0.8	0.1-0.6	0.2-0.8	0.3-1.0	0.1-0.8

* Median values of the percentages of cell area measured within the total section
# Median values of the percentages of positive cells within the cell area of the section

[11] p=0.045 by comparison with all HIV infected
[12] p=0.008 by comparison with unreactive HIV infected
[13] p=0.005 by comparison with all HIV infected
[14] p=0.022 by comparison with reactive HIV infected
[15] p=0.022 by comparison with reactive HIV infected
[16] p=0.037 by comparison with reactive HIV infected
[17] p=0.036 by comparison with unrecative HIV infected
[18] p=0.003 by comparison with unreactive HIV infected
[19] p=0.038 by comparison with all HIV infected
[20] p=0.046 by comparison with reactive HIV infected

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10 Curriculum vitae

Not available in public version.

11 Publications

Journal article:

Sarrazin H, Wilkinson KA, Andersson J, Rangaka MX, Radler, L, van Veen K, Lange C, Wilkinson RJ. "Relationship between tuberculin skin test reactivity, memory CD4 subset and circulating FoxP3 expressing cells in HIV infected persons." *The Journal of Infectious Diseases* 2008: in press. To be published in April 2009
The photograph of a TST reactive sample stained for CD45RO (see figure 3.6) will appear as front cover picture of the April 2009 issue of the Journal of Infectious Diseases.

Oral presentations:

Sarrazin, H.; Wilkinson, K.; Andersson, J.; Rangaka, M.; Radler, L.; van Veen, K.; Lange, C.; and Wilkinson, R.: "Lack of influx of memory T-cells results in non-reactive tuberculin-skin-test (TST) in HIV-infection"; *17th Annual Congress of the European Respiratory Society, Stockholm, September 15th-19th, 2007, Abstract #2976*

Poster presentations:

Sarrazin, H.; Rangaka, M.; Andersson, J.; Radler, L.; Wilkinson, R.; Lange, C.: "Lack of influx of memory T-cells results in non-reactive tuberculin-skin-test (TST) in HIV-infection"; *48th Annual Congress of the German Society for Pneumology and Respiratory Medicine, Mannheim, March 14th-17th, 2007; P270; Pneumologie 2007; 61:S1*

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* Recipient of Young Investigator Award

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Sarrazin, H., Lange, C.: “Die Tuberkulose bei HIV-Patienten – Wieso versagt der Tuberkulin-Hauttest?“; 2. Lübecker Doktorandentag, Juni 2008