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Analysis of mycobacteria-specific CD4+ T cell cytokine responses and memory differentiation in HIV patients and healthy controls

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Abstract

Among people living with HIV/AIDS, tuberculosis caused by *Mycobacterium tuberculosis* (MTB) is the main cause of mortality with 430 000 deaths in 2011. Multidrug-resistant tuberculosis is on the rise, and no effective vaccine is available.

HIV causes a depletion of CD4+ T helper cells, a cell subset that is of central importance in controlling mycobacterial infections. Although initiation of highly active antiretroviral therapy (HAART) in HIV patients results in increased CD4+ T cell counts, HIV patients still show, after treatment, increased susceptibility to mycobacterial infections. For unknown reasons, anti-mycobacterial T cell functions seem to be impaired in HIV patients despite onset of therapy. We hypothesize that in HIV patients the (poly)functionality of anti-mycobacterial CD4+ T cell responses as well as the memory T cell composition might be altered compared to healthy control subjects.

In this study we therefore established and applied methods to investigate anti-mycobacterial CD4+ T cell effector functions and memory T cell subsets from peripheral blood mononuclear cells (PBMCs) of HIV infected individuals and healthy controls. Although freshly isolated PBMC were of superior quality, cells were found to retain their cytokine-producing abilities if stored in liquid nitrogen but not at -80 °C. Isolated PBMCs were stimulated with antigens from *Mycobacterium tuberculosis* as well as *Mycobacterium avium* overnight. The next day the cells were analyzed by flow cytometry using a protocol with up to 11 colours per sample to simultaneously examine CD4+ T cell effector functions and memory T cell phenotype. Effector cytokine production was also analyzed by ELISA and real-time PCR.

We first examined the polyfunctionality of CD4+ T cells, which is the ability of T cells to respond to antigen-stimulation with simultaneous production of several T cell effector cytokines. Production of TNF, IL-2 and IFN-γ from CD4+ T cells was thus analyzed by flow cytometry.

Different memory T cell subsets in the total as well as the mycobacteria-specific (cytokine-producing) T cell population were next identified by flow cytometric staining with the markers CD27, CD28, CD45RO, CD57, HLA-DR, PD-1 and CCR7.

With the chosen study protocol we analyzed so far samples of five healthy controls and seven HIV patients. Our preliminary findings show that a low number of CD4+ T cells produce mycobacteria-specific effector cytokines in response to *M.tuberculosis* and *M.avium* antigens both in healthy individuals and HIV patients. The degree of polyfunctionality was lower in HIV patients; cells simultaneously producing all three cytokines of interest was almost absent from this group.

For memory T cell phenotyping there were no obvious differences for most markers when considering the total CD4+ T cell population, except a higher proportion of HLA-DR and PD-1 in HIV patients than in healthy donors. When the simultaneous expression of CD27 and CCR7 was analyzed, findings point to that the mycobacteria-specific CD4+ T cells of healthy individuals are late differentiated, at a stage where expression of both CD27 and CCR7 are lost. Mycobacteria-specific CD4+ T cells of HIV patients seem to be of an earlier differentiated memory phenotype, as cells expressing both CD27 and CCR7 are predominant.

In summary, we have established a study design to analyze mycobacteria-specific CD4+ T cells and memory T cells in blood samples from healthy individuals and HIV patients. A limited number of HIV patients and healthy control samples were analyzed and yielded some promising preliminary results. In the future, the protocol from this project will be used to analyze polyfunctional T cell responses against *M.avium* and *M.tuberculosis* as well as memory T cell composition in a larger cohort of HIV patients visiting the outdoor clinic at St. Olavs Hospital.

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1. Introduction

After HIV, no single infectious agent takes as many lives per year as *Mycobacterium tuberculosis* does (1). A total number of 8.7 million people were estimated to fall ill from tuberculosis in 2011, and 1.4 million estimated to die from it (1). Multidrug-resistant strains are on the rise, and no effective vaccine is available. Treatment is prolonged and expensive, and not easily available to all those who need it.

Tuberculosis is a bacterial infection caused by *Mycobacterium tuberculosis*. About one third of the world's human population is infected with this bacterium, but in 90 % of the infected individuals, the bacteria persists in a dormant stage where it does not cause disease (2). If left untreated, the disease is potentially lethal. Risk of active disease is far greater in those with compromised immunity than in healthy individuals, and this is the reason why tuberculosis is a huge problem in patients co-infected with HIV/AIDS. In fact, tuberculosis is the main cause of death among HIV/AIDS patients (3).

HIV (Human immunodeficiency virus) is a condition leading to immunosuppression as T cell numbers are lowered to a minimum level. This leads to increased risk of opportunistic infections, as T cells are of great importance to the immune system's ability to avoid challenges harmful to the human body. Tuberculosis is one of the opportunistic infections that can be acquired by HIV patients while T cell count is still relatively high, early in disease progression, and all people newly diagnosed with HIV should be tested for tuberculosis as soon as possible (3). With decreasing CD4 T cell count, the risk of active tuberculosis in HIV patients increases (4). HIV patients with latent tuberculosis are also at much higher risk for progressing to active tuberculosis disease than HIV negative individuals (3).

This part will focus on the background for the study, the immune system as a general, and mycobacterial infections in detail. Both innate and adaptive immune response against mycobacteria will be covered, but T cells will be in focus, as they represent the major weapon against tuberculosis and are depleted in HIV patients.

1.1 The immune system

The human immune system consists of two separate, yet intertwined, components; the innate immune system and the adaptive (acquired) immune system.

The innate immune system is fully developed already from birth, and represents the first line of defense as pathogens try to enter the human body. It is characterized by rapid response, lack of memory and germline receptors only, and plays a major part in fighting pathogens. The innate immune system even eradicates a large number of infections before they are even allowed to enter the body (5).

The innate immunity comprises both cellular and non-cellular components, among the latter are physical barriers like the gut flora, the mucus of the respiratory and gastrointestinal tract, hair/cilila, and antimicrobial components of tears and saliva (5). In addition, the complement system is said to be part of the innate immunity, although it can also be looked at as a link between the innate and adaptive immune system (reviewed in (6)). The complement system consists of many different small proteins circulating in the blood as precursor proteins, and they are activated by upon infection. The activation may follow one out of three different pathways, but they all lead to the same thing, namely pathogen lysis by the membrane attack complex (MAC). Small cleavage products from the complement activation may also opsonize pathogens for phagocytosis or recruit other immune cells by chemotaxis (reviewed in (7)).

The most important cells of the innate immunity are the monocytes/macrophages and the granulocytes, and their major task in pathogen eradication is phagocytosis. Phagocytosis refers to the engulfment of solid particles by 'eater' cells, and more specifically describes how especially macrophages, but also neutrophils, destroy microbes by recognizing their presence and then destroying them before they can exhibit microbial activity to damage the host (8). Macrophages are extremely important for immune response towards mycobacterial infections, a topic that will be covered later on in this section (1.3).

Cells of the innate immunity have no memory, and are dependent on recognizing pathogens and distinguishing self from harmful non-self. This is achieved through germline-encoded surface receptors on immune cells named pattern-recognition receptors (PRRs) that recognize

microbe-specific patterns named pathogen-associated molecular patterns (PAMPs). PAMPs are components of microorganisms that are essential for their survival, and therefore must be preserved during evolution (9). This, and the fact that these patterns are absent on human cells, ensures attack of pathogens only. Important PRRs for mycobacterial infections are the so-called Toll-like receptors (TLRs), discussed later in the text.

In response to infection, various antimicrobial peptides and proteins are also secreted. Antimicrobial peptides are effectors of the innate immune system and offer a rapid response to invading pathogens (reviewed in (10)). Examples of antimicrobial peptides are the so-called acute phase proteins like C-reactive protein (CRP) and complement factors that have opsonizing roles (reviewed in (11)), and lipocalin-2 (Lcn2), produced in response to bacterial stimuli to reduce bacterial growth by sequestering iron from bacteria (12). These effectors are secreted in response to TLR activation and cytokine secretion (11)(12).

When infection cannot be cleared by the innate immune system, the adaptive immune system needs to be activated. The adaptive immune system develops from birth and needs to learn how to eradicate infections by encountering pathogens throughout life. It has four characteristic attributes; it is specific, diverse, has memory and shows self-nonself recognition. Its response is slower than that of the innate immunity, but it shows a rapid response for secondary, repeated infections with the same infectious agent (reviewed in (13)).

Adaptive immune response depends on lymphocytes and antigen-presenting cells (APCs). Lymphocytes are a subgroup of leukocytes and comprise the B cells and the T cells. B cells produce antibodies (immunoglobulins) upon activation and these antibodies bind to antigens (*anti*body *gen*erator, Ag) on pathogens to facilitate their clearance from the body. Antibodies produced by B cells belong to the humoral branch of the immune system, a system that is highly effective to clear infections consisting of free antigens circulating in the body (reviewed in (7)). As they are believed to have limited impact on mycobacterial infections, B cells and immunoglobulins will not be discussed a lot in this text. It should however be noted that recent findings point to a possible role for B cells in immunity against tuberculosis (reviewed in (14) and (15)), as this population possibly may influence cytokine production.

T cell-mediated adaptive immunity, however, is considered central in defense against mycobacterial infections. T cells arise in the bone marrow and mature in the thymus before they migrate to lymphatic tissues where they become activated. T cells bear a unique antigen-binding T cell receptor (TCR) associated with a CD3 co-receptor, and this TCR determines to what specific antigen the T cell clone can mount a response. To become activated, T cells depend on encountering antigen-derived peptides associated with Major histocompatibility complex (MHC) molecules on the cell surface of so-called antigen-presenting cells (16). Examples of professional APCs are dendritic cells (DCs) and macrophages. The major T cell subsets are CD4+ and CD8+ T cells. CD4 T cells mainly recognize exogenously derived antigens presented in the context of MHC class II, whereas CD8 T cells mainly recognize endogenously derived antigens presented in the context of MHC class I. Upon activation by antigen contact, the T cell starts proliferating, producing an entire army of monoclonal T cells, thus with identical antigen specificity as the progenitor cell itself (17). In addition to the two main T cell subsets mentioned above, several unconventional T cell subsets exist, such as Natural killer T (NKT) cells (reviewed in (18)), γδ T cells (reviewed in (19)) and CD1-restricted T cells (reviewed in (20)).

Upon activation, differentiation into effector cell subsets also takes place. CD8 cells develop into cytotoxic T lymphocytes (CTLs) which possess direct cytotoxic activity, whereas CD4 T cells can develop into a variety of effector cells (see section 1.4.2). Both CD4 and CD8 T cells produce effector molecules called cytokines (17). Cytokines direct the immune response by exerting a variety of effects on all other cells of the immune system. CD4 T cells and their effector functions will be thoroughly covered in the rest of the text, as they represent one of the key components of this work.

1.2 Mycobacterial infections

The etiological agent causing tuberculosis was discovered by Robert Koch in 1882 (described in (21)). The disease is caused by *Mycobacterium tuberculosis* (MTB), an acid-fast and rod-shaped bacterium. The bacteria are slow-growing and surrounded by a thick and wax-rich cell wall (22). After the whole genome of the bacteria was published in 1998 (23) it was found that more than 250 of the bacteria's genes are involved in fatty-acid metabolism, producing substances of the cell wall. The most important components of the cell wall are different forms of mycolic acids. The presence of this characteristic cell wall is important for the

virulence of the bacteria. It prevents the attack of the bacteria by medical drugs, and also inhibits their dehydration. In addition, it slows the rate of nutrients entering the bacteria, and although this may seem as a drawback, it may actually also explain why the bacteria is such a strong pathogen and causes chronic infection (24)(25), as it results in prolonged exposure to a large diversity of antigens (reviewed in (26)).

The disease is very contagious and the bacteria are primarily transmitted through the respiratory route leading to pulmonary disease of the lungs (27), see figure 1.1. Droplets containing bacilli are expelled by infected individuals and inhaled by anyone in close proximity. The bacilli end up in the lung where they are engulfed by alveolar macrophages. Macrophages residing in this part of the lungs are especially active as they guard one of the main entrances to the body. As a response to the detection of invaders, cytokines are produced from macrophages and various inflammatory cells are recruited to the lungs. Macrophages are often unable to fully eradicate the pathogen. Instead, the bacilli are able to survive and replicate inside the host macrophage until macrophages burst and release large numbers of bacilli. As specific T cells are recruited a few weeks after infection, they induce the infiltration of macrophages and the formation of so-called granulomatous lesions, or tubercles. These lesions contain Th1 cells and infected macrophages and have a necrotic middle due to the release of lytic enzymes from the activated macrophages and the subsequent destruction of nearby cells. Due to a non-favorable environment and the presence of activated macrophages in the surroundings, the bacilli are not able to replicate within the granulomas, instead they slow down their growth and enter a latent stage where disease is not developed, this stage is called latent TB infection (LTBI) (28)(29). A dynamic balance develops between bacterial persistence and host defense, and this balance may be long-lasting, and even lifelong. Up to one third of the world's population have been infected with this infectious agent, but for 90 % of these individuals dormancy and latency maintains and they live their life without any obvious signs of disease. If they ever become immunodeficient, by for example an HIV infection, chance of reactivation is increased and they may experience active tuberculosis.

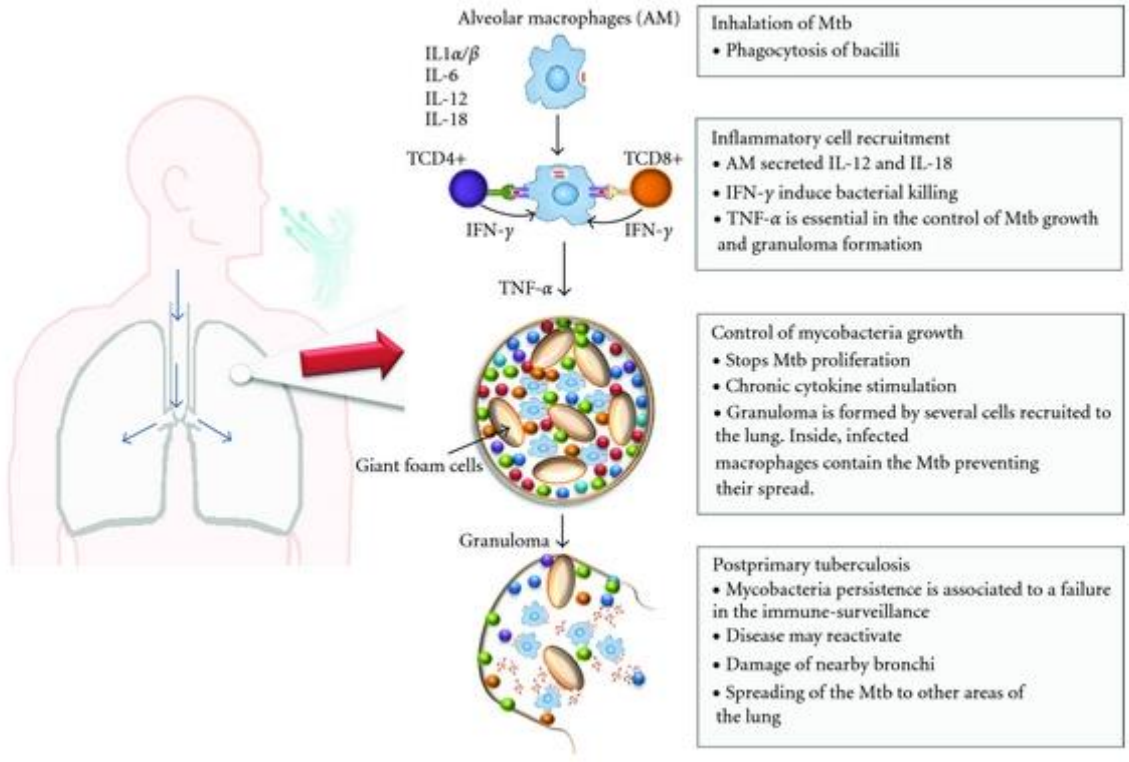


Figure 1.1: Pathogenesis of tuberculosis. Bacilli are inhaled through the respiratory tract and reach the lungs. They first encounter alveolar macrophages and become phagocytosed, leading to the subsequent production of proinflammatory cytokines. This attracts other immune cells in the formation of so-called granulomas where bacilli slow growth and enter dormancy. In granulomas, bacteria persist in a state where they do not cause active disease. However, if individuals become immunosuppressed, granulomas may burst and bacilli are spread to cause active disease. Figure from (30).

Mycobacterium avium is a close relative to *Mycobacterium tuberculosis*, also capable of causing human disease. *M.avium* is often grouped with *M.intracellulare* in formation of the *Mycobacterium avium* complex (MAC). *M.avium* is able to cause pulmonary disease in immunocompromised individuals, children and elderly people, and the disease is often hard to get rid of. MAC disease in patients with AIDS is a serious complication of HIV. However, the introduction of highly active antiretroviral therapy (HAART) for HIV patients has reduced the number of people at risk for the disease (described in (31)). Due to the fact that *M.avium* shares many properties with its better known relative, and its less stringent biosafety level needs, the bacteria is often used as a model organism for *Mycobacterium tuberculosis* (32).

1.3 Innate immunity and mycobacterial infection

As *Mycobacterium tuberculosis* first enters the body, the bacteria encounter alveolar macrophages that are part of the innate immune system. The containment of the bacteria is dependent on cooperation between the innate and adaptive immunity where macrophages, T cells and several cytokines play a central part. In the initial phase of the mycobacterial infection, antimicrobial proteins of the innate immune system, such as lipocalin-2 (Lcn2), may help limit extracellular growth of mycobacteria (33).

Macrophages are important effector cells for fighting intracellular pathogens, among them *M.tuberculosis*. The bacteria, in turn, have converted this attribute into exploting their key killer as their preferred habitat. The central role for eradication of pathogens by macrophages is dependent on their recognition of harmful non-self, usually by PRRs recognizing PAMPs, as explained earlier in the text. It is generally accepted that *M.tuberculosis* is engulfed by macrophages by conventional phagocytosis (34). The two processes of recognition and phagocytosis are facilitated by two different classes of macrophage surface proteins. For recognition of bacteria, Toll-like receptors (TLRs), NOD-like receptors (NLRs) and C type lectins (e.g DC-SIGN) are found, whereas complement receptors, mannose receptors and scavenger receptors are important for the uptake of bacteria (figure 1.2).

1.3.1 Immune recognition and macrophage uptake of *Mycobacterium tuberculosis*

Toll-like receptors (TLRs) were originally identified (and named Toll, meaning «great» in German) in Drosophila and found to control the antifungal response in flies (35), but similar molecules were later also discovered in the human species (36). To date, there are 12 known variants in humans (TLR1-TLR12) (reviewed in (37)), and their task is to recognize PAMPs. TLRs are membrane-spanning glycoproteins, some of them situated on the cell surface, and others in intracellular compartments. All TLRs specialize in recognition of conserved microbial patterns to sense pathogens and thereby inducing an immune response.

Not all known TLRs are believed to be involved in MTB recognition. To date, TLR2, TLR4, and TLR9 seem to be most important (reviewed in (24)). Upon recognition of MTB PAMPs, different signaling pathways are triggered, leading to the activation of an appropriate immune

response. Intracellular adaptor protein MyD88 seems to play an important part as it associates with β receptor-associated kinases (IRAK) in activation and nuclear translocation of nuclear transcription factor NF-κB. The end result of this is the production of proinflammatory cytokines like IL-1β (interleukin 1β), TNF (tumor necrosis factor), IL-6, IL-18 and several others (reviewed in (24)).

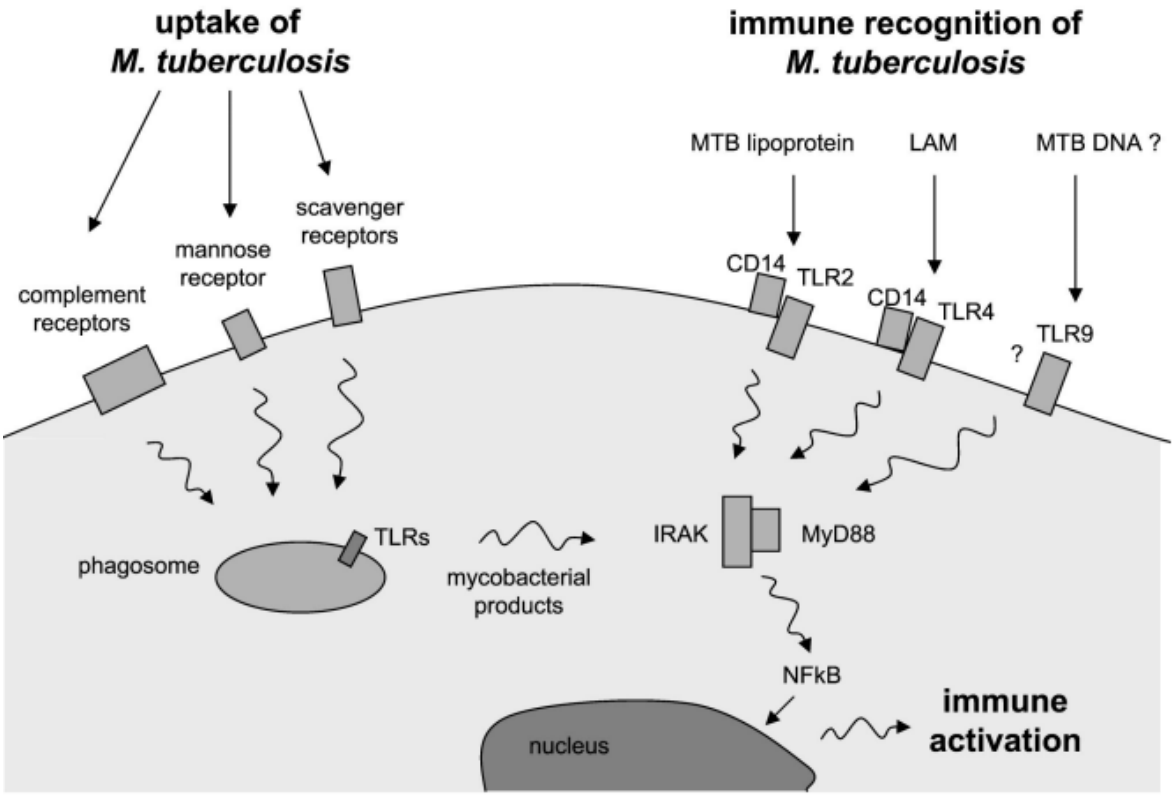


Figure 1.2: Immune recognition and uptake of *M.tuberculosis*. Different Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) of the mycobacterial cell, including lipoproteins (TLR2), lipoarabinomannan (LAM) (TLR4) and DNA (TLR9). This activates an intracellular signaling cascade in the host cell. Adaptor protein MyD88 is often involved, as is IL-1 receptor-associated kinases (IRAK), leading to the subsequent translocation of transcription factor NF-κB and the production of proinflammatory cytokines like IL-1β. Uptake of bacilli for phagocytosis is facilitated by different receptors on the host cell, including complement receptors, mannose receptors and scavenger receptors. Some of the receptors require bacilli are opsonized with complement split products, but they can also be taken up without these. After uptake, bacilli rest in phagosomes, where they inhibit immune activation by inhibition phagosome-lysosome fusion. Figure from (28).

In mycobacterial infections, TLRs activate the immune system by recognizing a key component of the mycobacterial cell wall; the glycolipid lipoarabinomannan (LAM), probably much like TLR4 is known to sense LPS of Gram negative bacteria. In addition, TLR4 may be activated by heat-shock proteins (hsp) produced by the bacilli. TLR2 are also able to recognize acylated mycobacterial lipoproteins, whereas TLR9 is believed to recognize unmethylated CpG motifs in mycobacterial DNA (reviewed in (24)).

In addition to TLRs, other macrophage molecules also help in mounting an immune response. NOD-like receptors (NLRs) are involved in the inflammasome formation and the subsequent production of IL-1β. C-type lectins like Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and dectin-1 may also be involved as PRRs when macrophages encounter *M.tuberculosis* (reviewed in (24)).

Macrophages will try to eliminate MTB in several ways; among them are the production of reactive oxygen and nitrogen species (ROS and RNS, respectively), acidification of the phagosome and phagosome-lysosome fusion (reviewed in (28)). First, however, the bacilli need to be engulfed by the macrophage, a process that is mediated not through the TLRs themselves, but by other mycobacteria-binding receptors on the macrophage surface.

Complement receptors (CR) present on the surface of immune cells are able to promote engulfment of particles opsonized with complement components like C3 (34). It was shown that CR1, CR3 and CR4 mediate uptake and phagocytosis of *Mycobacterium tuberculosis*, and it is possible even for non-opsonized bacilli to bind to some of the complement receptors. Still, the favorable mechanism for uptake of non-opsonized bacteria is through the mannose receptor recognizing terminal mannose residues (38), one example of a ligand being LAM, discussed earlier as a TLR ligand as well. There are also other molecules facilitating the uptake of *M.tuberculosis* (reviewed in (28)), among them scavenger receptors. It has even been proposed that the mechanism by which the bacilli are taken up in to the macrophages, decides their fate (reviewed in (39)).

1.3.2 Life inside the macrophage

As bacteria are taken up by macrophages and macrophages are activated, bacterial killing by phagocytosis is performed by the macrophages. The material taken up is enclosed within a membrane-bound phagosome, and the ingested material is degraded through the action of a series of hydrolytic enzymes (reviewed in (40)).

Mycobacterium tuberculosis is one of the most successful pathogens when it comes to escaping from the immune system, and is therefore able to survive within macrophages for a long period of time. As the phagosome presents a harsh environment for the bacilli, *M.tuberculosis* arrests phagosome maturation and inhibits phagosome-lysomsome fusion. In this way, the bacilli prevent the digestive activity of acidic hydrolases that would otherwise threaten their existence (41).

Although the bacilli enter a state of slow growth and dormancy within the macrophages, they are still dependent on a steady supply of iron for their survival. Ironically, the iron is also essential for some host-defense mechanisms, and as the bacilli successfully competes for iron with the host, it promotes its own survival and suppress host-defense. The arrest of phagosome maturation give the bacilli free access to iron as iron is taken up bound to transferrin through transferrin receptors and then trafficked to early endosomal compartmens (reviewed in (39) and (42)). Mycobacteria have also developed a type of iron-binding molecules, so-called siderophores, that transfer iron molecules from host proteins to the mycobacterial cell wall (43). The host's answer to the bacterial siderophores is lipocalin-2 (Lcn2), an iron sequestering innate immune protein that is able to bind iron with higher affinity than most bacterial siderophores and thus can starve bacteria for iron (12). Lcn2 is readily produced and stored for secretion in neutrophils, and production can be induced in other cell types such as hepatocytes in infected liver tissue (Haug *et al*. 2013, submitted). However, if infected macrophages take up Lcn2 from the surroundings, it is directly degraded by the endosomal pathway, whereas iron bound to transferrin ends up in the recycling compartment where the mycobacteria resides (33). Thus, Lcn2 seems to have no effect on intracellular bacteria like mycobacteria.

If IFN-γ is present in the environment, macrophages become fully activated (44). This, in turn, increases the production of reactive oxygen and nitrogen species, it promotes acidification of phagosomes and the fusion of phagosomes to lysosomes. IFN-γ may be

produced in response to IL-12 and IL-18, from Natural killer (NK) cells early during infection, or T cells after adaptive immunity has been activated (45). Even activated macrophages are unable to fully eradicate *M.tuberculosis* infection (described in (22)), but this activation and antimicrobial activity is essential for the containment of bacteria and the prevention of active disease.

The bacilli are able to persist in the host in a dormant stage and stay this way until the host defenses are down, like they are in for example HIV infection (46). They are then able to reactivate and cause active tuberculosis.

1.4 Adaptive immunity and mycobacterial infection

Mycobacterium tuberculosis is an intracellular bacterium, and host protection therefore mainly relies on cell-mediated immunity, and primarily T cells.

The bacteria are taken up by macrophages by phagocytosis (as described in 1.3.1), but may also enter dendritic cells (DCs) in the lungs and reside within them. Interactions between bacilli and DC are poorly understood, but seem to have an important impact on the initiation of a protective immune response (47)(48)(49), as DCs have an important role as APCs.

Normally, exogenously derived antigens such as bacterial antigens are taken up and trafficked to a phagosome. Presentation with MHC class II is the natural choice, this attracts CD4 T cells and leads to the subsequent activation of CD4+ T cells and differentiation into various effector subsets. Phagolysosomal arrest by *M.tuberculosis* affect the macrophage's ability to process and present mycobacterial antigens to CD4+ T cells in a MHC class II context and thereby infers with the whole adaptive immune response, as CD4+ T cells are dependent on antigen-presenting cells such as macrophages and DCs to become activated. For exogenously derived antigens to be presented on APC surfaces, the internalized antigens need to be processed and associated with MHC class II molecules. This involves fusion of intracellular vesicles, a process inhibited by mycobacteria. Due to this, it is conceivable that the Ag presentation is decreased in mycobacterial infections (50). It was also described that a 19 kDa lipoprotein antigen of *M.tuberculosis* downregulate MHC class II due to intraphagosomal TLR2 stimulation (51).

Despite this, mycobacterial antigens are presented in both a MHC class I and a MHC class II context (reviewed in (22)). Presentation to CD8+ T cells is made possible as infected APCs release extracellular vesicles as they undergo apoptosis. The vesicles contain mycobacterial antigens and are taken up by bystander APCs, leading to the subsequent presentation with MHC class I and the activation of CD8+ T cells (52).

Activation of adaptive immune cells is accomplished despite the bacteria's evasion strategies of phagolysosome maturation arrest and MHC II downregulation in innate immune cells. The activation of CD4+ Th1 cells are assumed most important in defense against mycobacteria, as will be highlightened in the following sections.

1.4.1 T cells

T cells are a key component of the human immune protection. Their importance is indisputable; they activate B cells and macrophages, they recruit granulocytes to site of infection, they orchestrate the entire immune system through their cytokine production, and some even exhibit direct cytotoxic activity. One striking example of their importance can be seen among HIV patients that show a depletion of their CD4 T cell population. These patients are extremely prone to opportunistic infections, and the risk of infection is inversely proportional to the CD4 T cell count.

T cells arise from the hematopoietic stem cells in the bone marrow and migrate to the thymus where they mature as so-called thymocytes. In the thymus they go through a series of developmental steps including the rearrangement of their T cell receptor to achieve a broad range of specificities towards different antigens. The thymocytes also experience two selection steps to ensure they are able to mount a response towards their specific non-self-antigen, but not towards self-antigens. When the naïve T cells (i.e. cells that have not yet encountered antigen) leave the thymus, the main subsets express one out of two characteristic surface molecules; CD4 or CD8, the choice directing their future activities (reviewed in (53)). T cells continuously recirculate through the blood and lymph to lymphoid organs like the spleen and lymph nodes. T cells stay in the blood for only 30 minutes, but spend 12 hours in the lymph nodes before circulating further (54).

The next step for naïve T cells is to meet the one antigen to which the T cell can mount a response. The antigen encountering happens in secondary lymphoid organs like the lymph nodes, where professional APCs present processed antigens associated with MHC on their surface. T cell activation requires two signals; peptide from processed antigen (matching its T cell specificity) presented in the context of MHC class I (CD8 T cells) or MHC class II (CD4 T cells) on antigen-presenting cell (APC) and a costimulatory signal from the interaction between CD28 (on the T cell) and B7 (on the APC). Upon activation, a signaling cascade is initiated in the T cells, resulting in the translocation of transcription factors and the subsequent changes in gene expression and the production of cytokines and other characteristic markers (reviewed in (55)). For four to five days the cell divides two to three times every day, creating a monoclonal population of progeny cells. These cells differentiate into different subpopulations; effector T cells and memory T cells. The effector T cells are characterized by a profound cytokine production and they express markers affecting their recirculation pattern. Effector cells are derived from naïve T cells, but also from memory T cells after antigenic activation. Memory T cells are antigen-experienced, long-lived and responsible for the secondary response (see 1.4.4).

Naïve T cells migrate preferentially to the tissues where they will encounter antigen, a process named *homing*. This increases the chance of a naïve T cell meeting its antigenic counterpart. It is the presence of adhesion molecules like selectins (e.g CD62L) on the T cell surface that controls the homing of cells (reviewed in (56)). Antigen-experienced T cells display different migration patterns, and this is reflected by different sets of surface markers. Upon activation, effector T cells downregulate the expression of CD62L and CCR7, two of the most important molecules for homing to secondary lymphoid organs (reviewed in (57)). At the same time, they upregulate homing molecules that target them to infected non-lymphoid tissues. They recognize inflamed vascular endothelium and chemokines, and exert their effector functions in the same non-lymphoid tissue region as the one where they were first activated (reviewed in (57)).

Memory T cells are antigen-experienced and have less stringent requirements for activation than do naïve T cells. In 1999, a pivotal study classified them as either central memory cells, T_{CM}, or effector memory cells, T_{EM} (58). Central memory cells express CCR7 and thus home to secondary lymphoid organs. They secrete IL-2 and can give rise to effector T cells that

migrate to site of infection (59). Effector memory cells lack CCR7 and thus the ability to home to secondary lymphoid organs. Instead, they home to non-lymphoid tissue and can rapidly secrete a variety of cytokines. There is still some controversies and much uncertainty about the memory T cell population (reviewed in (60)), and everything about it is still not uncovered. There is also another part covering memory subsets later in the text (1.4.4).

Mycobacteria reside in vesicles within macrophages or DCs, and are therefore most likely to be presented with MHC class II, and activate CD4+ T cells. For this to happen, T cells depend on the activity of professional antigen-presenting cells (APCs), like macrophages or DCs. It is believed that DCs play an important role in activating the adaptive immune system in mycobacterial infections. DCs encounter mycobacterial antigens in the lungs and then migrate to draining lymph nodes where they present antigen to naïve T cells (reviewed in (61)). Naïve CD4 or CD8 T cells, activated by antigenic peptides presented in the context of MHC class II or MHC class I, respectively, undergo proliferation and traffic back to the lung (reviewed in (61)). In the lung, T cells produce a wide variety of cytokines that influence the outcome of mycobacterial infection by affecting the macrophage bactericidal capacity (IFN-γ, TNF), granuloma formation and maintenance (TNF, IL-1), activation of Th1-responses (IL-12), recruitment of effector cells (IL-8), increased (IL-6) and decreased (IL-10) effector responses in target T cells and macrophages (reviewed in (62), (63) and (64)).

As mycobacterial antigens are most likely to be presented in association of MHC class II, CD4 T cells are despite phagolysosomal arrest activated in mycobacterial infections. As IFN-γ producing Th1 cells are considered central in antimycobacterial immunity, CD4+ T effector cell subsets will therefore be more closely described in the next paragraph (1.4.2). However, CD8 T cells can also be activated by the mechanisms earlier mentioned and also have a role in fighting *M.tuberculosis* infection (65)(66), as well as unconventional γδ T cells (reviewed in (39)). CD8 T cells play an important part in clearing virus-infected cells, and they are also capable of producing a set of cytokines, including IFN-γ, TNF and IL-2 (reviewed in (67)).

1.4.2 CD4 T cell subsets

The CD4 T cell population can be further divided into several subpopulations and the development of the different subsets is directed by the cytokine milieu in the environment during activation. The differentiation of one lineage often suppresses the other sub-

populations, for example IFN-γ produced by Th1 cells inhibits development of Th2 cells (68). A summary of the differentiation of the CD4 T cell population can be found in figure 1.3.

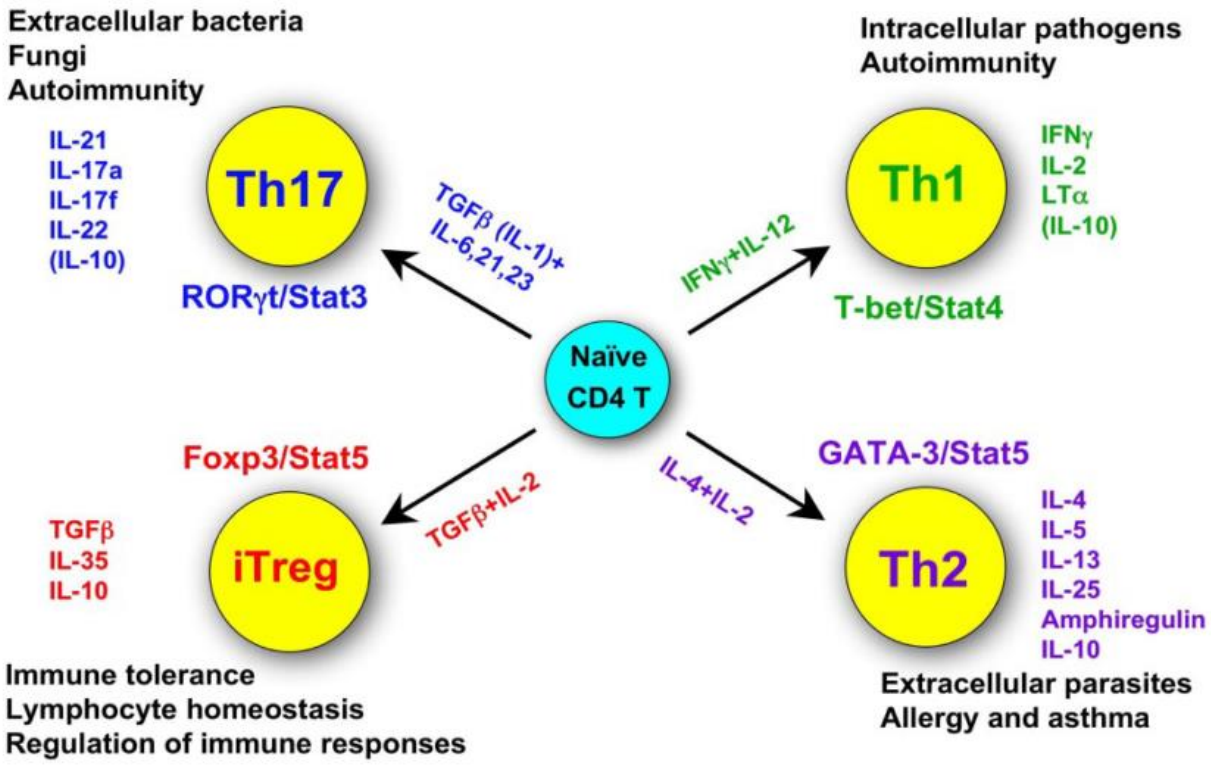


Figure 1.3: Differentiation of naïve T cells. Based on the cytokine environment, naïve T cells can differentiate into four major subsets; Th1, Th2, Th17 and iTreg. IFN-γ and IL-12 induces differentiation to the Th1 lineage, as well as transcription factors T-bet and Stat4. The Th1 cells produce cytokines that are important in infections with intracellular pathogens and autoimmunity. Th2 cells are induced by cytokines IL-2 and IL-4 and transcription factors GATA-3 and Stat5. Th2 cells are involved in the eradication of extracellular parasites, as well as in allergy and asthma. Cytokines TGF-b, IL-6, IL-21 and IL-23, as well as transcription factors RORγt and Stat3 induce differentiation to Th17 cells that are involved in immunity against extracellular bacteria and fungi. Last, in the presence of TGF-b and IL-2 and transcription factors Foxp3 and Stat5, regulatory Tregs are induced. These cells produce anti-inflammatory cytokines and are involved in the negative regulation of other T cell subsets. Figure from (69).

The first two subsets discovered were the Th1 and the Th2 subsets in 1986 (70). The two groups were distinguished based on their cytokine production; Th1 cells produced IFN-γ as their signature cytokine, whereas Th2 produced IL-4. It was later concluded that the cytokine profile of the subsets defined their role in the immune system, as Th1 cells were found to fight intracellular pathogens, as opposed to Th2, playing an important role in immunity against extracellular pathogens and in allergy and asthma (reviewed in (67) and (71).

Th17 is a newly identified class of effector T cells (72)(73) (74) producing IL-17 among others, this subset being involved in eradication of extracellular bacteria and fungi.

The fourth major CD4 T cell subset are the (induced) regulatory T cells, iTreg (75). These cells display immune-suppressing activity, and produce anti-inflammatory cytokines like IL-10 and TGF-β (76).

Th1 are perhaps the most important T cell subset for mycobacterial infections, due to their role as potent IFN-γ producers. As already mentioned, IFN-γ are powerful macrophage activators, and this activation is crucial for mycobacterial containment. People with defects in interferon γ or its receptor show an increase in susceptibility towards mycobacteria (reviewed in (77)), proving how IFN-γ is required for host defense and survival. More recent indications show that IFN-γ alone is not enough for host protective immunity against *M.tuberculosis*, and that other cytokines need also be considered. Polyfunctional T cells, cells simultaneously producing more than one cytokine, has received a lot of interest lately. The important role for IFN-γ and other cytokines in mycobacterial infections is explained in more detail in 1.4.3.

1.4.3 Cytokines

Cytokines are secreted low-weight protein signaling molecules acting in an autocrine or paracrine manner in the body. They regulate the intensity and duration of the immune response by directing the activity of all other immune cells. Cytokines may be either proinflammatory, promoting systemic inflammation, or anti-inflammatory, inhibiting inflammation (reviewed in (78)). Each cytokine has a matching cytokine receptor on the surface of the cell it is supposed to regulate, and this receptor and the subsequent intracellular signaling cascade makes it possible for the cytokine to induce a variety of biological effects in nearby cells, like upregulation or downregulation of genes. Four recognized and important cytokine attributes are; pleiotropy, redundancy, synergy and antagonism, and they often form complex cytokine networks. Due to their widespread activity, cytokines need to be tightly regulated, and any disturbance to this regulation may seriously interfere with the immune balance (79).

This part will focus on three main cytokines that are associated with defense against mycobacteria and can be produced from CD4+ Th1 cells; IFN-γ, TNF and IL-2. Th1 cell

subsets can produce one, two or all three cytokines simultaneously (polyfunctionality of T cells). Additional cytokines IL-1β and IFN-β will also be discussed in brief, as they were chosen as targets during PCR.

IFN-γ is important in the control of MTB infection (80), as it is a key activator of macrophages (figure 1.4). IFN-γ is mainly produced by Th1 cells after activation, but can also be produced by natural killer (NK) cells, APCs and CD8 T cells. IFN-γ importance was confirmed in 1993 as it was found that mice with IFN-γ knock out (GKO) could not control even small doses of *Mycobacterium tuberculosis* (81). Instead, the mice experienced widespread tissue destruction and necrosis.

Interferon γ is the only type II interferon, and it is essential for intracellular pathogens, virus control and tumor immunity. IFN-γ secretion by NK cells and APCs are believed to be of great importance early in infection, but T cells become the major source in adaptive immunity. Production of IFN-γ is controlled by the cytokine environment. As macrophages sense the presence of for example *M.tuberculosis* through their PRRs, the production of proinflammatory cytokines are upregulated, among them IL-12 and IL-18. These cytokines induce the IFN-γ production in for example T cells. The IFN-γ then affect the macrophages again, making them active and increase the antimicrobial activity. Anti-inflammatory cytokines IL-4, Il-10 and TGF-β function as negative regulators of IFN-γ. IFN-γ primarily signals through the Jak-Stat pathway (82).

In T cells, IFN-γ mRNA is detectable shortly after T cell stimulation, as it belongs to the group of «early» genes expressed in activated T cells. mRNA expression is detectable as soon as 30 minutes post antigen encounter (83).

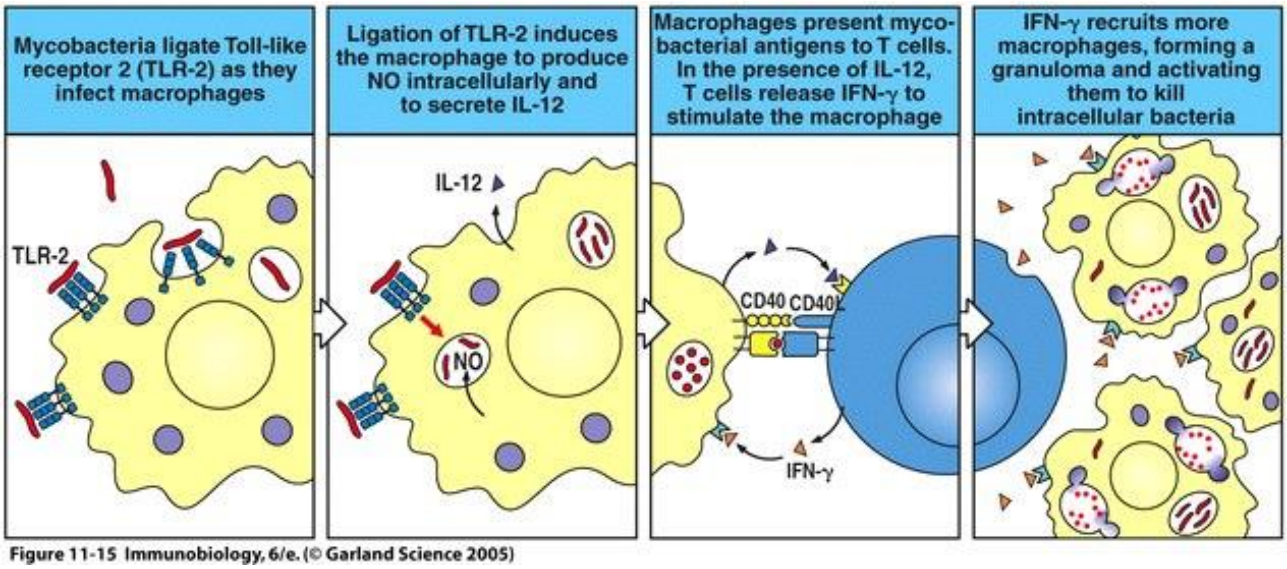


Figure 1.4 Activation of macrophages by cytokine IFN-γ. Mycobacterial pathogen-associated molecular patterns (PAMPs) activate macrophage TLRs during early stages of mycobacterial infections. This activation leads to the production of proinflammatory cytokines such as IL-12 from the infected macrophage. Macrophages present processed mycobacterial antigens associated with MHC class II to CD4+ T cells. In addition to the presence of IL-12 in the surroundings, this leads to the production of IFN-γ by activated CD4+ T cells. IFN-γ then stimulates and activates nearby macrophages that answer by upregulating their microbial activity. Figure from (84).

However, IFN-γ is not the only Th1 specific cytokine important for mycobacterial infections, and other cytokines have proved their importance as well, among them TNF and IL-2 (reviewed in (85)).

TNF was in 1995 associated with survival and control of *Mycobacterium tuberculosis* infection in mice (86). In the absence of a receptor for TNF, mice succumbed on average 22 days postinfection, while the control mice lived throughout the whole experiment. TNF is an inflammatory cytokine that induce a broad spectrum of biological effects, including apoptosis and translocation of NF-κB to increase production of cytokines and the expression of adhesion molecules. TNF can also contribute to the activation of macrophages, as reviewed by (87). TNF is ineffective alone, but synergizes with IFN-γ in induction of NO production and RNIs. The cytokine is also important in granuloma formation, perhaps due to its influence on chemokines and chemokine receptors (88).

TNF is also believed to accumulate early in immune response. Its mRNA was reported to peak between two and four hours after LPS stimulation in lymphocytes (89), whereas others

(83) reported the TNF-β mRNA to be detectable 1-3 hours post stimulation. It has been shown by other members in the research group that during ELISA, TNF is the first detectable cytokine in supernatant from stimulated PBMCs, even before IFN-γ. However, TNF levels decrease rather rapidly and seem to be almost gone when IFN-γ peaks (unpublished observations).

IL-2 is discussed as the third Th1 cytokine important in mycobacterial infections. The cytokine describes the status of the T cell population, as it is necessary for proliferation and differentiation of T cells to effector memory cells, and thus the generation of long-term protective immunity (90). Expression of IL-2 also upregulates the expression of IL-2 receptor (IL-2R = CD25) and synthesis of IFN-γ in human T cells (91). The simultaneous expression of IL-2R on T cells also makes it possible for the IL-2 produced to act as an autocrine signaling molecule on the population from which it arose.

Polyfunctional Th1 cells, meaning Th1 cells that produce all three cytokines, have been proposed to be particularly potent in controlling MTB infection (92)(93). This finding was first recognized in the protozoa *Leishmania major*(94), but the effect is likely to apply to tuberculosis as well. Polyfunctional T cells (PFT) are often targets in immunoassays when considering mycobacterial infections (95)(96), as it has been shown to correlate with long-term protective immunity against tuberculosis (97). Others, however, claim that this polyfunctionality alone fails to predict protective immunity (98).

Proinflammatory IL-1β is first produced by alveolar macrophages upon encountering *M.tuberculosis* and the subsequent signaling through Toll-like receptors (TLRs) (99). IL-1β induce fever, the acute phase response and the stimulation of neutrophil production. As TLRs sense mycobacterial PAMPs, a signaling cascade is initiated resulting in the translocation of NF-κB and the subsequent production of pro-IL-1β. A so-called inflammasome is assembled as a response to intracellular danger-associated molecular patterns (DAMPs). Through the action of the protease caspase-1, the inflammasome converts pro-IL-1β to its active form, IL-1β, that is later secreted. The role for IL-1β in host defense against *M.tuberculosis* was demonstrated as mice deficient in IL-1 receptor type I (IL-1RI) suffered fatal consequences upon infection with the bacteria (reviewed in (99)).

Fibroblasts are the main source of interferon beta (IFN-β), but they can also be produced by dendritic cells (DCs). IFN-β mediate a variety of immunoregulatory effects, including an increase in NK cytotoxic activity. IFN-β may also enhance IFN-γ production (reviewed in (100)).

1.4.4 T cell memory status

Memory T cells are derived from naïve T cells after they encounter antigen and from effector T cells after antigenic activation and differentiation. They are antigen-experienced and long-lived and respond with a strengthened reactivity towards repeated infections with the same infectious agent, known as the secondary response. This small population remains for a long time after the primary response has declined, and circulate in the body looking to reencounter the same antigen once more. Memory T cells seem to have less stringent needs for activation than do naïve T cells. They may be activated by a broad range of cells, this in contrast to naïve cells that need to be activated by professional APCs. This is believed to be due to the heightened expression of surface markers like numerous adhesion molecules (101).

Several surface markers help identify the differentiation status of the T cell population, but a lot is still unknown about the memory T cell population, their markers and their effector functions. Memory T cells express many of the same surface markers as effector T cells, and no set of markers clearly defines them as memory T cells. The memory population is very heterogeneous and surface markers expression gradually change over time during differentiation. This part will look into what is already known in this field, focusing on a set of memory markers believed to be important in this context, namely CD45RO, CD27, CD28, CD57, PD-1, HLA-DR and CCR7. Part of this thesis work includes the investigation of how expression of memory surface markers differs in HIV patients and healthy controls.

Markers like CD45RO (or its counterpart, CD45RA), and CCR7 are typically used to describe memory differentiation, but CD27 and CD28 may be beneficial in further subdividing the major memory populations. It may also be that the phenotype resulting from expression of CD27 and CD28 overlap with the populations characterized based on CD45 and CCR7 expression (102). It can generally be said that the expression of CD27, CD28, and CCR7 are lost as differentiation proceeds, whereas CD45RO expression is increased, before it seems to be lost again at terminal differentiation. CD57 and PD-1 are both useful when describing cells at the end of the differentiation pathway, at replicative senescence.

A short description of some memory markers follows.

- CD45 (Cluster of differentiation 45) is perhaps the oldest known epitope on T cells reflecting their memory phenotype. Naïve T cells bear a variant called CD45RA on their surface, but as they encounter antigen, they differentiate to a CD45RO phenotype (103) (104). This is made possible from the alternative splicing of exon 4/5/6 (A/B/C). Activation leads to a programmed shift in splicing, resulting in the decrease of the high molecular weight isoform (CD45RA) and a subsequent increase in the low molecular weight isoform, the null form (CD45RO) (described in (105)). Terminal effector cells may lose the expression of CD45RO (106).

- CD27 is a costimulatory signal and a member of the tumor necrosis factor receptor (TNFR) family. It is expressed on all naïve T cells and on most memory cells, but seem to be lost during memory differentiation (107)(108).

- CD28 is perhaps the most important costimulatory molecule for T cell activation, and it is expressed on all mature thymocytes. Loss of CD28 expression has been coupled to T cell replicative senescence (109). It has been proposed that an increased proportion of CD28- cells in HIV patients explains their increased risk of aging-associated diseases (110).

- CD57 is a surface marker that defines replicative senescence in CD4+ and CD8+ T cells (111).

- PD-1 (Programmed cell death protein 1), also known as CD279, negatively regulates T cell responses, and is in HIV associated with T cell exhaustion and disease progression (112). In healthy individuals, the marker is preferentially expressed on effector memory cells (113).

- HLA-DR is a MHC class II cell surface receptor that is part of the human leukocyte antigen (HLA) system. Its main task is to present antigenic peptides to other cells of the immune system. Expression on T cells is often increased in response to stimulation, and HLA-DR is therefore also recognized as an activation marker (114). It is proposed that this makes the T cells capable of presenting Ag to other cells (described in (115)).

- CCR7 (C-C chemokine receptor 7), or CD197, is a chemokine receptor that has an important role in recirculating and homing of lymphocytes (116). CCR7 is expressed by all naïve T cells, and some memory cells. In fact, CCR7 is believed to divide the memory cell population in two distinct subpopulations (58).

Naïve T cells express CD27, CD28, CD45RA and CCR7.

Central memory T cells, T_{CM}, express CCR7 (and $CD62L^{hi}$) and are able to home to lymphoid organs. They lack the ability to immediately start producing cytokines (they do produce some IL-2), but they divide rapidly upon an antigen reencounter and can then even differentiate further to effector cells (58). Central memory T cells are described as CD45RO+CD28+ phenotype (85)(117), additionally CCR7 (and $CD62L^{hi}$) might be used to describe them. Central memory T cells are a population that is directly derived from naïve T cells after they encounter antigen (before naïve T cells develop into effector CD4+ subsets). T_{CM} are believed to be maintained during infection, and their ability to turn in to effector cells upon secondary stimulation make them important mediators of long-term protective immunity (118).

Effector memory T cells, T_{EM}, show a significant production of for example IFN-γ and other cytokines but lack the CCR7 surface marker (and are $CD62L^{lo}$) (58). These cells express recepors for homing to inflamed tissues and are also believed to express CD45RO+CD28+, like the T_{CM} population. Effector memory T cells are surviving T cells that are derived from naïve T cells after primary antigen contact and after subsequent differentiation into different effector CD4+ T cell subsets.

Both T_{CM} and T_{EM} are found in the blood, however, T_{CM} are predominant in the CD4 population (reviewed in (59)).

Terminally differentiated T cells lack CD27 and CCR7 (described in (85)).

The cytokine production seems increase profoundly as cells differentiate to a CCR7- phenotype (119)(102). A model has been proposed for the CD4+ T cell differentiation, explaining the correlation between memory status (represented by the expression of CCR7) and effector functions (represented by the production of IFN-γ, TNF and IL-2), see figure 1.5.

The long-term memory potential is greatest among the T_{CM} population, and effector function peaks as CCR7- expression is lost and CD4 T cells simultaneously produce all three effector cytokines.

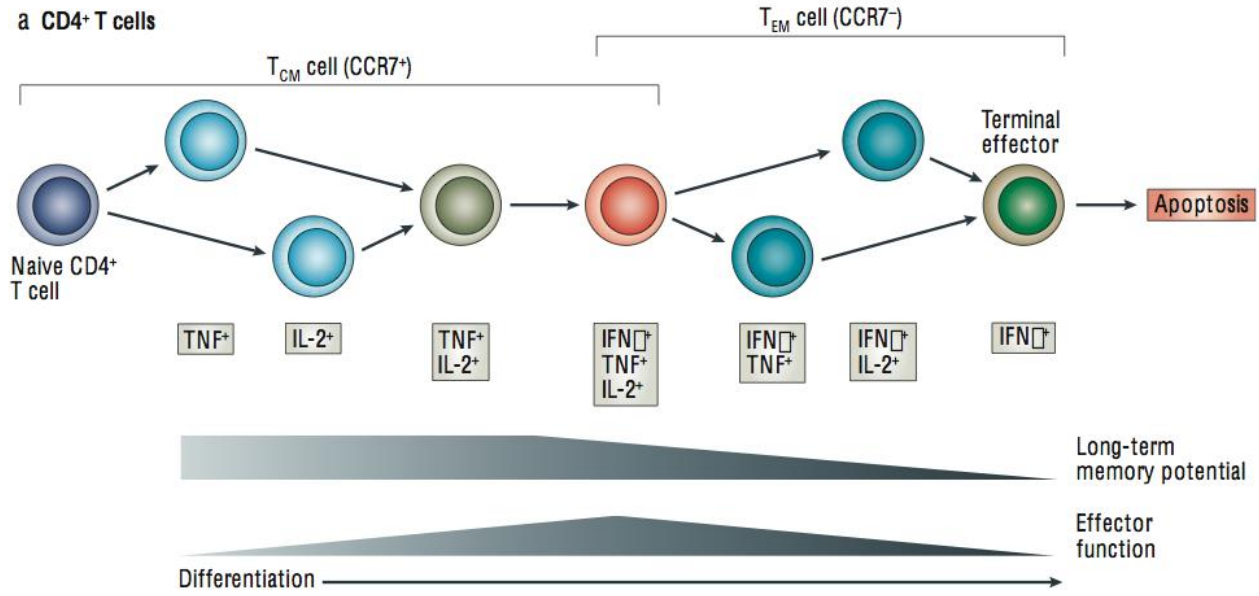


Figure 1.5 Model for CD4+ T cell differentiation and correlation with cytokine production. Upon antigenic stimulation, naïve CD4+ T cells differentiate and progressively gain effector functions until they reach a peak where they simultaneously produce all three cytokines IGN-g, TNF and IL-2. Up until them, cells are CCR7+ and inhibit a central memory phenotype where the long-term memory potential is high. From this point, the cells lose CCR7 expression and are characterized by an effector memory phenotype. Cytokine production and long-term memory potential is gradually lost and cells are finally terminally differentiated (producing only IFN-γ) before they undergo apoptosis. Figure from (120).

1.5 HIV

HIV (human immunodeficiency virus) is a retrovirus affecting the immune system. It is transmitted primarily through sexual contact, but also during sharing of needles or blood transfusions. Mother-to-child transmittance also occurs.

Even though antiretroviral treatment may slow down disease progression, no cure is yet available, and since its discovery early in the 1980s (121)(122), it still remains a huge global health problem. According to WHO (123), 34 million people are living with HIV (2011), and in the same year, a total number of 1.7 million deaths were registered worldwide due to AIDS (123). In addition, there were a significant number of 2.5 million individuals newly infected

with HIV. Although this is recognized as a global problem, the majority of the individuals infected are located in Sub-Saharan Africa.

Cell entry by virus particles is mediated through interactions between a viral envelope protein and CD4 via the chemokine receptor CCR5. HIV RNA and enzymes are transferred to the host cell (e.g. CD4 T cell or DC), making the virus able to replicate within the host. Virus particles are later released as virions that are able to infect new cells (124). The increase in blood-borne virus particles is associated with a depletion of the CD4 T cell population. In addition, cytotoxic CD8 T cells are activated. The reason for the rapid and progressive CD4 T cell depletion is not well understood, although the killing of infected cells by CTLs may contribute to this. Other direct or indirect cytopathic effects are also proposed as possible explanations (125). HIV disease also cause an increase in the T cell turnover (126).

Although HIV cannot be cured, the introduction of highly active antiretroviral therapy (HAART) has had a significant impact in HIV medicine. HAART inhibits HIV replication and is associated with a rapid increase in CD4 numbers in patients with advanced disease (127).

HIV positive individuals are more susceptible to tuberculosis than HIV negative, most likely because HIV causes a depletion of the CD4 T cell population, a statement supported by the fact that patients with a low CD4 count are more prone to tuberculosis infection than patients with a higher count (128). There is also a correlation between the CD4 T cell count and the reactivation of LTBI (reviewed in (129)). The increased susceptibility remains, even after treatment with HAART (130). It is speculated that the quality of the restored CD4 T cell population does not resemble that of healthy individuals. Polyfunctionality of the T cells, i.e. the ability to produce more than one mycobacteria-specific cytokine in response to stimulation (see 1.6.1) or the memory phenotype of the T cells (see 1.6.2) may be altered, leading to an impaired immune response towards tuberculosis.

1.6 HIV- MTB co-infection

The HIV/tuberculosis combination is potentially lethal and the risk of active tuberculosis is increased more than a 100-fold in those with HIV. 14 million people worldwide are believed to suffer from this co-infection (131), and it kills 430 000 of these individuals every year (1). Knowledge on how these two diseases are intertwined and affect each other in a negative manner, is essential to understand how they can be controlled, and how death due to tuberculosis can be avoided in HIV patients.

Co-infection accelerates the deterioration of the immunological functions leading to subsequent death if left untreated. However, the reasons this happens are debated (reviewed in (132)). This part focuses on the two most important aspects for this work; how mycobacteria-specific cytokine production is affected in HIV positive individuals, and how HIV alters the T cell memory phenotype and how this can influence the host response against mycobacterial infections.

1.6.1 CD4+ T cell effector functions in HIV infection

As was earlier elucidated (section 1.4.3), IFN-γ, TNF and IL-2 are important CD4+ T cell effector cytokines to defend the host against mycobacterial infection. Polyfunctional CD4 T cells producing two or more of these are believed to be of great importance to the immune system's ability to contain tuberculosis infection. However, the ability of the CD4 T cells to produce two or more cytokines seem to decrease with increasing viral load (95). The same study found that in HIV disease, IL-2 seemed to be lost first, followed by TNF and at last, IFN-γ production.

Studies have revealed that the proportion of cytokine-producing mycobacteria-specific CD4 T cells is smaller in HIV positive individuals than in HIV negative individuals (133). This in contrast to other studies, claiming that HAART reconstitutes the mycobacteria-specific response of CD4 T cells (134)(135). Others again claim the IFN-γ secreting capacity is impaired during HIV infection (136)(137).

Polyfunctionality is also believed to be impaired during HIV infection (133)(138), however, some claims it can be restored during HIV therapy (106).

There has been reported that during therapy there is a dynamic relationship between the cytokines being produced (IFN-γ and IL-2 are the ones investigated), and shifts between what cytokine is the prominent one may occur (139).

1.6.2 Memory T cells in HIV infected individuals

Memory T cell populations can be phenotypically characterized using a combination of surface markers, like described earlier in this section (1.4.4). The chosen parameters were CD45RO, CD27, CD28, CD57, PD-1, HLA-DR and CCR7. Earlier studies have shown that IFN-γ producing CCR7- T cells are highly concentrated in the pleural fluid of tuberculosis patients (140). Not much is known about whether or not, and how, co-infection with HIV alters the memory phenotype.

Therapy in HIV patients is believed by some to increase the polyfunctionality of the CD4 T cells. The newly generated CD4 T cell population consists of mainly effector memory cells simultaneously producing TNF and IFN-γ. This implies that HIV therapy should reduce susceptibility to MTB. This is not always the case, however (see 1.5). Central memory CD4+ T cell responses have been found not to be restored by HAART (141). In SIV infection (the HIV equivalent in non-human primates) it was found that memory CD4+ T cells are lost (142).

Not much is known about the memory population in HIV infected individuals and how this affects the immune response towards tuberculosis. It is reason to believe that therapy skews the memory phenotype, and that this leads to impaired response in HIV patients (112)(133)(143)(144)(145)(146)(147). This study hopes to investigate these matters.

2. Aim of study

Onset of antiretroviral therapy can stop the decline of CD4+ T cells in HIV-patients and restore the CD4+ T cell population. But for unknown reasons, HIV-patients show, even at relatively normal T cell counts, increased susceptibility to mycobacterial infections (section 1.5). We hypothesize that polyfunctionality (simultaneous production of several effector cytokines, 1.4.3 and 1.6.1) of anti-mycobacterial CD4+ T cell responses as well as changes in the memory T cell subset composition (1.4.4 and 1.6.2) might be involved in this impaired response against mycobacteria of HIV-patients.

The main aim of this study was therefore to establish and apply protocols to investigate anti-mycobacterial CD4+ T cell effector functions and memory T cell composition HIV-infected individuals and compare them with healthy controls.

Specific objectives were:

- To establish a protocol for isolation of PBMCs from heparinized peripheral blood samples from healthy control subjects as well as HIV positive patients at the indoor clinic at St. Olavs hospital under a BL3 protocol.

- To test the effect of long-term cryopreservation of PBMCs on the quality of anti-mycobacterial T cell effector functions.

- To design a multicolor flow cytometric staining panel allowing analysis of mycobacteria-specific CD4+ T cell effector responses as well as memory T cell phenotyping from the same sample.

- To establish, optimize and apply an *in vitro* assay to stimulate PBMCs from HIV patients and healthy donors with mycobacterial antigens from *M.avium* (heat-killed *M.avium* and *M.avium* PPD) and *M.tuberculosis* (MTB PPD). After overnight stimulation, T cells should be analyzed by multicolor flow cytometry for:

    - Frequency of *M.avium* and *M.tuberculosis*-specific CD4+ T cells in PBMCs from HIV patients and healthy controls

    - Polyfunctionality of this T cell response (simultaneous production of the three cytokines IFN-γ, TNF and IL-2).

    - T cell phenotyping of mycobacteria-specific as well as total CD4+ and CD8+ T cells for memory T cell status markers.

- Cytokine production of PBMCs stimulated with mycobacterial antigens should be verified using additional methods such as PCR and ELISA.

3. Materials and methods

In short, this project comprises the analysis of host effector mechanisms in healthy donors versus HIV positive patients towards infection by *Mycobacterium tuberculosis*. HIV patients are immunodeficient and are prone to opportunistic infections by agents such as *M.tuberculosis*. Study of CD4 T cells were the main focus, as they are depleted in HIV patients, and at the same time are the main weapon against mycobacterial infections. Such analysis is best performed using fluorescent antibodies and detection by flow cytometry. In addition to analysis by flow cytometry, PCR and ELISA were performed.

This part of the work describes how analysis of blood samples from healthy donors as well as HIV patients was performed. Included is the build-up of the study, the processing of samples, and descriptions of methods used (flow cytometry, PCR and ELISA) as well as a discussion regarding the ethical issues of using patient samples. Focus will be on flow cytometry, as this was the main technique used.

3.1 Study build-up

As a start for the whole project, healthy donors were recruited from the staff at IKM, NTNU. All donors except one had received a BCG vaccine in their late childhood. None of them had ever experienced any signs of tuberculosis, however none had been tested for the disease. They were all assumed to be HIV negative, although never tested.

The contributors donated a volume of between 15 and 30 mL of heparinized blood. Peripheral blood mononuclear cells (PBMCs) were isolated using ficoll density gradient centrifugation as described in Appendix 1.

Some of the isolated cells were used immediately and were stimulated with three different mycobacterial agents, as described in 3.2.2. After stimulation, they were stained for extracellular markers (see 3.3.5) and intracellular for cytokine production (see 3.3.6), and it was proceeded with flow cytometry. Additional methods used to describe cytokine profile were PCR (Polymerase chain reaction) and ELISA (Enzyme-linked immunosorbent assay).

The rest of the white blood cells were cryopreserved as described in 3.2.2, some in -80 °C and some in liquid nitrogen. At a later stage, some were thawed and stimulated to compare results of frozen cells with those of fresh cells.

For the patient part of the study, HIV positive patients were recruited from the indoor clinic at St. Olavs Hospital, Trondheim, where they visit on a regular basis for controls and follow up. Professor Jan Kristian Damås coordinated the recruitment of HIV positive donors.

A total number of seven patients agreed to each grant between 15 and 25 mL heparinized blood.

Isolation of white blood cells from HIV patients was performed as described in 3.2.1 (exact protocol in Appendix 1), and they were stimulated as described in 3.3.2. They were analyzed based on the same criteria as the healthy donors and by the same methods (only flow cytometry and PCR were used for the HIV patients).

A biobank was established based on the blood samples from the HIV patients. White blood cells were isolated, and preserved in cryotubes in liquid nitrogen. Samples were anonymized but linked to a spreadsheet containing information on age, sex, ethnicity, CD4 T cell count, viral load, medicine information and so on. At another location on the hospital, plasma and serum samples from each patient were also stored. The purpose of the biobank was to establish a starting point for further analysis of HIV patients' immune effector mechanisms.

Table 3.1 includes some vital facts about the recruited HIV patients.

Table 3.1: HIV patient characteristics

ID	Amount blood received	PBMCs isolated (• 10^6)	Age	Sex	HIV-RNA (copies/mL)	CD4 count (cells/µL)
H001	18 mL	14	33	M	120	70
H002	15 mL	10,6	26	F	70	370
H003	15 mL	5,4	43	M	<20	220
H004	20 mL	14,4	33	F	<20	450
H005	20 mL	20	34	F	<20	550
H006	25 mL	13	42	M	<20	570
H007	20 mL	9	37	M	<20	340

3.2 Processing of blood samples

3.2.1 Isolation and counting of peripheral blood mononuclear cells (PBMCs)

Sodium heparin tubes were used to acquire blood samples. Heparin is an anticoagulant that prevents clotting of the blood from sampling to further processing. As samples are processed within short time from sampling, using the mild anticoagulant heparin is sufficient.

The principle behind the isolation of PBMCs from whole blood is the separation of components through a density gradient. For a complete description of the protocol, refer Appendix 1. The protocol is an modification of a protocol routinely used in the research group for isolating PBMCs from buffy coats. A modification is necessary because heparinized blood contains much less cells than a buffy coat, and therefore requires less washing steps.

The blood samples were diluted at a 1:1 ratio with PBS (Sigma), and 30 ml of blood/PBS were carefully layered on top of 15 mL Lymphoprep solution (Axis-Shield PoC). Lymphoprep is a reagent developed especially for the purpose of isolating white blood cells, and it contains polysaccharid and sodium diatrizoate (148). It has a density that just exceeds water (namely 1.077 g/mL), and centrifugation at 1800 rpm for 20 minutes yields a solution where heavy erythrocytes pass through the medium and are collected at the bottom of the tube. Plasma has a low density and will stay in the top of the tube, over the Lymphoprep. At

the plasma/Lymphoprep interface, a ring of white blood cells is formed. This ring can easily be harvested by a sterile pipette and transferred to a new tube for washing and purification.

After washing the isolated white blood cells twice (800 rpm for 8 minutes, then 1400 rpm for 6 minutes) in HANKS balanced salt solution (Sigma), the cells were resuspended in 5 mL culture medium. As culture medium, RPMI (Sigma) is supplemented with human serum as a source of nutrients for the living cells. 2 % serum is sufficient for short-term handling of the cells, whereas 10 % is required for overnight stimulation of T cells. L-glutamine is used for amino acid supplement in culture medium (149), and HEPES is a buffer that makes the medium exhibit an effective buffering in the physiological range (150).

As cells are adequately purified, they need to be counted before further work is continued. Several methods are available for quantification of cells, among the most widely used in this lab is the coulter counter based on the electrical resistance of the cells, counting in a burker chamber in a microscope and the Countess automated cell counter (Invitrogen) based on a dye exclusion principle exhibited by trypan blue. Using the Countess automated cell counter is a fast way to determine cell concentration in solution, and it is believed to be both accurate and precise (151). Based on this information, this was the preferred method of counting cells in this project.

The Countess automated cell counter utilizes the fact that trypan blue is selectively able to distinguish between live and dead cells and combines this with an accurate image analysis to provide information on cell concentration and cell viability in a sample. Live cells have intact cell membranes, and therefore exclude dyes like trypan blue. Dead cells, however, are not able to exclude the dye, and the color is taken up in the cell. For counting, 10 μL trypan blue is mixed with 10 μL sample. The two are mixed and 10 μL transferred to a glass counting chamber. As the chamber is inserted into the device, an image is constructed where live cells appear with a colorless cytoplasm, whereas dead cells have a dark appearance. As fine adjustments are made, the machine is then able to analyze the picture and elucidates information on the sample.

After counting, cells are stimulated or frozen for long-term storage, see below.

3.2.2 Biobanking of WBCs

Long-term storage of patient samples may be beneficial for more reasons than one. This will make it possible to establish a biobank that can be utilized for several research purposes now and later. Several patient samples may be analyzed simultaneously to reduce the possibility of variations between independent experiments. In this project, cryopreservation up to three months of healthy donor samples was performed to decide whether or not this is a suitable method for storing of HIV patient samples.

Cryopreservation of cells is a method for long-term storage with minimal damage to intracellular structures and effector functions. At sub-zero temperatures, intracellular activity is paused, and cells are believed to retain their functionality at thawing.

For this project specifically, the most critical aspects of cryopreservation is the viability after freezing and the effect of cryopreservation on cytokine production. As a limited number of cells are available, it is essential that viability does not drop too far. Additionally, good and careful freezing and thawing protocols will assure that cells are affected as little as possible from cryopreservation and that characteristics such as cytokine production in response to stimuli are similar as for fresh cells. Although live cells are distinguished from dead ones in the flow cytometer based on a live/dead stain procedure, optimal analysis of cells and their effector functions depend on a great proportion of cells being alive and healthy.

Earlier studies have shown that viability does not decrease much even after cryopreservation up to 12 years (152). Others report on a decrease in viability (153), and some points to that high viability depends on an optimal freezing/thawing protocol (154).

To ensure the best possible conditions for the cells during cryopreservation, it is critical that the freezing and thawing are performed in a controlled way. Freezing rate is ideally kept at one degree/minute, to avoid too much intracellular ice crystal formation (155). The rate-controlled freezing device Mr Frosty (Nalgene) was used in this project. Isopropanol alcohol inside the box ensures the correct rate of cooling as the device is stored in -80 °C. After 24-72 hours, individual cryotubes can be moved from Mr Frosty to containers of liquid nitrogen, keeping the temperature at -196 °C. Thawing should be rapid, again to avoid intracellular ice crystal formation.

20 % dimethyl sulphoxide (DMSO) in fetal calf serum (FCS) was used as freezing medium. Up to 7.5 • 10^6 PBMCs were resuspended in 0.5 ml pure FCS. Then 0.5 mL of the 20 % DMSO/FCS freezing medium was carefully added, resulting in a final concentration of 10 % DMSO. Cryotubes were immediately transferred to Mr. Frosty and stored a -80 °C freezer until moving to liquid nitrogen (for a complete description of the protocol, refer Appendix 2). DMSO is known to be toxic to the cells, but is nevertheless used as a cryoprotective agent. At cooling to low temperatures, DMSO is integrated into cell membranes and thereby inhibit the formation of ice crystals (156). To avoid DMSO from exerting toxic effects to the cells, and hence creating stress to the cells, mixing of the freezing medium and the cells must be gentle.

Different studies have proposed varying views on the effect of cryopreservation on cytokine secretion (156)(157)(158). Some results indicate that cytokine production is not at all affected by freezing and thawing, whereas others report on a four to fivefold decrease. This verifies how a careful protocol for freezing and thawing is absolute necessary to have the PBMCs retain their effector function after cryopreservation.

After samples are frozen, they are either preserved for long-term storage or they are thawed after a while and analyzed for effector functions after stimulation, see below.

3.2.3 Stimulation of WBCs

Upon encountering antigen, the T cells specific for the antigen present will be activated and start to divide. This leads to a population of T cells that is able to exert specific effector functions that aim at fighting infection. This project was mainly performed using short (overnight) stimulation. Total percentage of specific T cells is therefore low.

Facilities for handling live *Mycobacterium tuberculosis* are not available at IKM as of now. Instead, *M.avium* is often used as a model organism for *M.tuberculosis* (32). *M.avium* itself may be pathogenic, but is not considered to be as harmful as *M.tuberculosis* and can be handled under BL2 conditions.

Stimulation is therefore performed using heat-killed *M.avium*. In addition, cells are stimulated with purified protein derivative (PPD) from *M.avium* and *M.tuberculosis* (kind gift from Ingrid Olsen, Norwegian Veterinary Institute, Oslo), respectively. PPD consists of purified parts (i.e. antigens) of bacteria that function as immunogens, they are thus able to mount an

immunologic response in host cells. PPD can be prepared by growing bacteria in medium, and then collecting the supernatant. Proteins are present in the supernatant and may be further purified using for example chromatography (159)(160). Even though results using a heat-killed close relative and PPD from the close relative and the tuberculosis bacteria itself may not be completely realistic, it is hope that the results may later be confirmed using live *M.tuberculosis*.

Stimulation for later flow cytometric assays was performed in flat bottom 96-well plates. To each well is added 0,5 • 10^6 cells, to a total volume of 200 µL per well (RPMI 10 % A+ is used as culture medium). Tests with different numbers of PBMCs per well were performed (see 4.1.3), and in the low range it is hard to obtain satisfying number of cells when aiming for the count of total 50 000 cells per analysis in the flow cytometer. Stimulation for PCR and ELISA was carried out in flat bottom 48-well plates (1,25 • 10^6 cells – 500 µL).

Stimulating agents are distributed to the cells as described in Appendix 2. Stimulation was always performed in duplicates, and negative and positive controls were always included. As negative control, some wells were unstimulated. As positive control, Cell stimulation cocktail 500x (eBioscience) was used. Cell stimulation cocktail consists of several substances, including phorbol 12-myristate 13-acetate (PMA) and ionomycin that stimulate the production of cytokines. PMA is a structure analogue to diacylglycerol, a substance known to allosterically activate protein kinase C (PKC). Activation of PKC triggers a cascade of events, resulting in T cell activation (161). PMA is known to downregulate CD4 expression (162). Ionomycin is an ionophore that synergizes with PMA in the activation of PKC, by raising the intracellular levels of calcium ions (163).

Costimulatory signal was also added to the wells prior to stimulation. Costimulatory molecules are one out of two required signals for T cell activation, and are usually present on antigen-presenting cells that deliver the other essential signal for activation, association between MHC molecules and antigen-derived peptides. To ensure activation of antigen-specific T cells, anti-CD28 (eBioscience) and anti-CD49d (BioLegend) are added (1 µg/mL).

Cells are stimulated in 37 °C overnight and to a total of approximately 16 hours. When cells are to be used for flow cytometric assays, Protein transport inhibitor cocktail 500x (eBioscience) is distributed to the cells. This cocktail contains a mixture of substances that

inhibit the transport of newly produced cytokines from the ER to the Golgi and ultimately the transport out of the T cell into the surroundings. This is essential for the analysis of each individual T cell and the cytokines produced from it. As manufacturer's guidelines is vague on how the transport inhibitor should be distributed to the cells, experiments were conducted, results can be read later on in this thesis (see 4.1.2). Unless otherwise stated, protein transport inhibitor cocktail was added after 16 hours and left to work for four hours.

The next step involves extracellular and intracellular fluorescent staining (for flow cytometric assays) or cell lysis and supernatant harvesting for PCR and ELISA, respectively.

3.3 Flow cytometric analysis

3.3.1 Introduction to flow cytometry

Flow cytometry refers to the technique of measuring physical and/or chemical characteristics of cells or other particles while they flow past a laser one by one. Utilization of fluorescence in cell quantification, and the subsequent development of flow cytometers accelerated in the 1960s, and one of the first reports describing fluorescence flow cytometry was written by van Dilla and collegues in 1967 (164).

In today's instruments, a sample in solution is inserted to the flow cytometer and hydrodynamic focusing produces a stream of single particles. Light scattering from each cell is measured and provides important information about the cell's properties. Light that is scattered in the forward direction, that is at low angles from the laser beam's axis, is focused by a lens and detected by a forward scatter detector (FSC). This detector supplies information about the size of the cell, and can be used to distinguish between for example T cells (relatively small) and macrophages (big in comparison). Light scattered at an angle of 90 degrees is detected by the side scatter detector (SSC) and provides an understanding of the cell's granularity. Granulocytes have a higher SSC signal than lymphocytes that are agranular. The principles behind the flow cytometer optics are shown in figure 3.1.

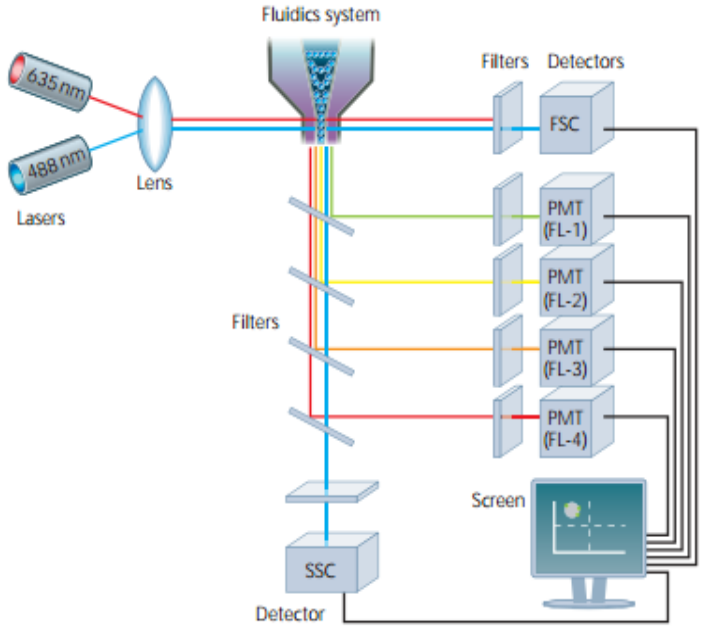


Figure 3.1: The principle behind the optics of a flow cytometer. Cells flow in a stream of single particles, and are illuminated with light from different lasers. Light scattered in the forward direction is focused by a lens and detected by a forward scatter detector (FSC), whereas light scattered at 90 ° is detected by the side scatter detector (SSC). The flow cytometer can also detect fluorescence as cells are illuminated with laser light and fluorochromes emit light at different wavelengths. Detectors and filters sense the emitted light and signals are sent (via photomultiplier tubes) to a computer and a software that interpret them and give an output characterizing the sample. Figure from (165).

Fluorescence measurement is a useful and important application of flow cytometry. Antibodies to cell components (intracellular or extracellular) may be labeled with a fluorescent tag and different detectors inside the flow cytometer detects emitted light from these fluorochromes as they are illuminated with light from a laser. Different channels with filters letting through light only of a specific wavelength make it possible to detect light from several different fluorochromes at once, as long as they are matched not to overlap in emission spectra. Multicolor flow cytometric assays are the main subject for this project.

For this project, a BD LSR II flow cytometer was used. This bench-top flow cytometer has four lasers and different filter combinations makes detection of up to 16 fluorochromes at the same time possible. The different lasers and possible combinations are listed in table 3.2.

Table 3.2: Lasers and corresponding filter combinations for the BD LSR II flow cytometer.

Laser	Filter combination
Violet, 405 nm	780/60
	705/70
	610/20
	525/50
	450/50
Blue, 488 nm	685/35
	585/42
	525/50
Yellow/green, 561 nm	780/60
	710/50
	670/14
	610/20
	585/20
Red, 640 nm	780/60
	710/50
	670/14

11 out of 16 filter combinations were used simultaneously in this project (see 3.3.5/3.3.6).

3.3.2 Compensation

When it is necessary to measure more than one cell characteristic, and thus more than one fluorochrome, at once, an investigation of the spectral overlap between the different fluorochromes is important. Each individual fluorochrome emits light over a fairly broad range of wavelengths, and there is a risk of two or more fluorochromes overlapping in their emission spectra. This creates a spillover in the neighboring channels, thus making the true measurement from each signal difficult. Spectral overlap between two fluorochromes A and B detected in the two channels FL-1 and FL-2, respectively, is illustrated in figure 3.2 below. It can be seen from the figure that a measurement of the signal from FL-1 mostly consists of signal from fluorochrome A, but a small part of the total signal is due to fluorochrome B as well.

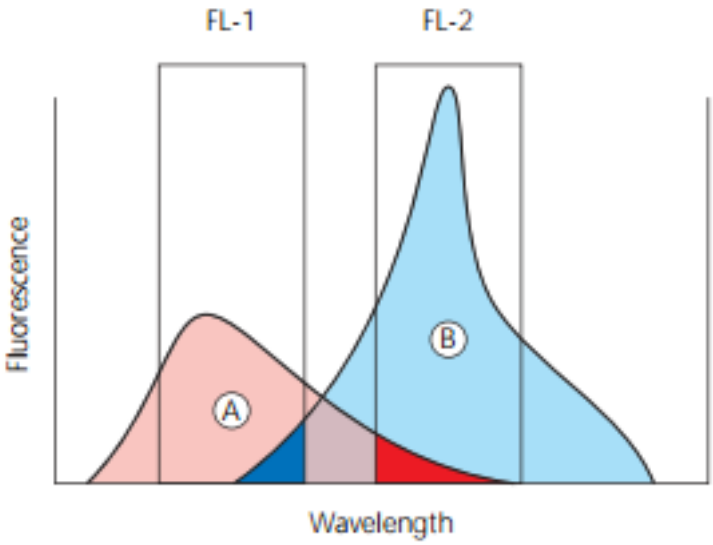


Figure 3.2: Illustration of spectral overlap between two fluorochromes and spillover into neighboring channels. A is measured in the FL-1 channel and B in the FL-2 channel. Dark blue shade represents the proportion of B that overlaps into the FL-1 channel. Red shade represents the proportion of A that interferes with FL-2 channel measurements. Compensation corrigates for this. Figure from (165).

To minimize errors due to spillover into neighboring channels, it is essential to compensate for these overlaps. Compensation is ultimately performed inside the software, and is a calculation of the percentage interference a fluorochrome will give in all other channels except the one where it is assigned to be measured.

To be able to calculate the overlap, each fluorochrome was run through the flow cytometer alone, meaning with no other fluorochromes present. Instead of using a cell-based compensation, bead-based compensation was performed. Beads (BD CompBeads) are spherical particles that bind fluorescently tagged antibodies, resulting in beads labeled with a single colored antibody bound.

When beads are run for all fluorochromes, the FACS Diva Software calculates a compensation where the spillover from any other channel is subtracted from each color.

3.3.3 Controls

Including relevant controls is essential for multicolor flow cytometric assays, and especially when staining for intracellular cytokines that are usually present in small amounts. Well-

separated populations are not always obtained, and distinguishing between negative and positive populations may be hard. So-called isotype controls are often included as negative controls. These controls are fluorochrome-specific and isotype control samples contain cells and isotype controls for the fluorochrome of interest. The isotype control antibody does not recognize an epitope that is expressed by the cells in the sample, but is coupled to the same fluorochrome and can in this way help decide if a positive signal from the stained sample is a true positive signal or just the result of unspecific binding between cells and fluorochrome. It is important that the isotype controls have the same characteristics as the specific antibodies in analysis; from the same species, in the same concentration, and of the same immunoglobulin class (isotype). The latter is due to avoid signal from unspecific binding between antibody and Fc receptors.

It is claimed that isotype staining may not be the best way to identify gating boundaries (166), especially when considering intracellular staining, and that other controls should be included as well. One possibility is to use a method called Fluorescence minus one (FMO), where all fluorochromes in the panel except one is included for each control (167). Then, the difference in signal between the actual sample and the FMO control represents the positive events, and the information can be used to set the gating. As controls ideally are chosen changing one parameter at a time, FMO may be a better choice than isotype controls.

Like earlier mentioned, controls were also included for the stimulation itself. Unstimulated samples will show signal from all the extracellular markers, but they should not have any cytokine-producing populations. As positive controls, cell stimulation cocktail with PMA upregulate the cytokines, and these controls show great cytokine production if the experiment is successful. As a control, samples were also run in duplicates.

3.3.4 Colors

Colors were chosen based on a number of criteria. The total number of fluorochromes that can be detected simultaneously in a multicolor flow cytometric assay depends on the number of lasers and detectors and filters in the flow cytometer. The BD LSR II flow cytometer used in this project has four different lasers (blue, red, violet and yellow/green), and various filter combinations makes detection of up to 16 colors possible (refer chapter 3.3.1).

What was important for this study, can be summed up in three points:

- The possibility of distinguishing CD4 T cells from the rest of the cell population
- The detection of cytokines produced from these CD4 T cells
- The presence of surface markers that determine the differentiation of CD4 T memory cells

As all T cells express CD3, this marker was included to identificate the T cell population from the rest of the PBMCs. CD4 and CD8 are also stained to identify CD4+ and CD8+ subsets. A viability dye (eBioscience Fixable viability dye eFluor 780) was included that irreversibly stains dead cells to exclude them from analysis. The principle behind the protocol is that compromised membranes of dead cells are permeable to the viability dye and cause a strong fluorescence.

Detection of IFN-γ, TNF and IL-2 effector cytokine production from T cell subsets in response to stimulation with mycobacterial antigens were chosen as read-out to detect mycobacteria-specific T cell frequencies. These cytokines are assumed major effectors of mycobacteria-specific CD4 T cells, and are well-documented (see 1.4.3) choices for T cell analysis in tuberculosis-related research.

In addition, a set of surface markers allowing for memory T cell differentiation status analysis were chosen; CD27, CD28, CD45RO, CD57, PD-1, CCR7 and HLA-DR.

The actual choice of fluorochromes was established at the beginning of the project and optimized during the work. Ideally all fluorochromes in a panel should be stable, bright and stain the markers effectively. This is not always achievable in a multicolor assay, and compromises must often be made. For example, it may be a good idea to choose the brightest and most stable colors for the markers that are least expressed and have the least difference between their positive and negative populations.

Brightness can be defined using a so-called staining index. Using the same antibody conjugated to different fluorochromes and comparing their stain indices, their relative brightness may be considered. For example, PE has an average stain index of 302, being one of the brightest fluorochromes. APC is next with 278, whereas Alexa Fluor 700 has a staining index of 61, and FITC has 56 (168).

To stain for the effector cytokines TNF, IFN-γ and IL-2, fluorochromes like APC, eFluor450, BV421 and PE were obvious choices as these colors cause a bright fluorescent signal. For identification of major populations like CD3, CD4 and CD8, less bright colors are usually sufficient, since these epitopes are highly expressed on the cells (168). When stability is considered, most single dyes are stable and retain their fluorescent activity for quite some time after staining. However, to increase the maximum number of detectable markers, tandem dyes have been developed. Tandem dyes utilize the advantages of two separate dyes and when the first dye is excited, all energy is transferred to the second dye that causes the fluorescence from its emission. Such dyes are often sensitive to photoinduced degradation and light exposure and exposure to paraformaldehyd (fixating agent) must be minimized. It would be ideal to avoid the use of unstable dyes, but it is sometimes necessary.

Careful consideration was made not to choose colors that were non-compatible with one another regarding spectral overlap. Several online web tools are available for this purpose, for example BioLegend Multicolor Panel Selector. This tool allows the selection of compatible colors and gives a notice when colors cannot be combined.

The BioLegend Fluorescence Spectra Analyzer even illustrates the emission spectra overlap, and figure 3.3 below shows the emission of a selection of the colors used in the study (BV421, BV510, BV605, BV785, Alexa Fluor 700, eFluor450, APC, PE).

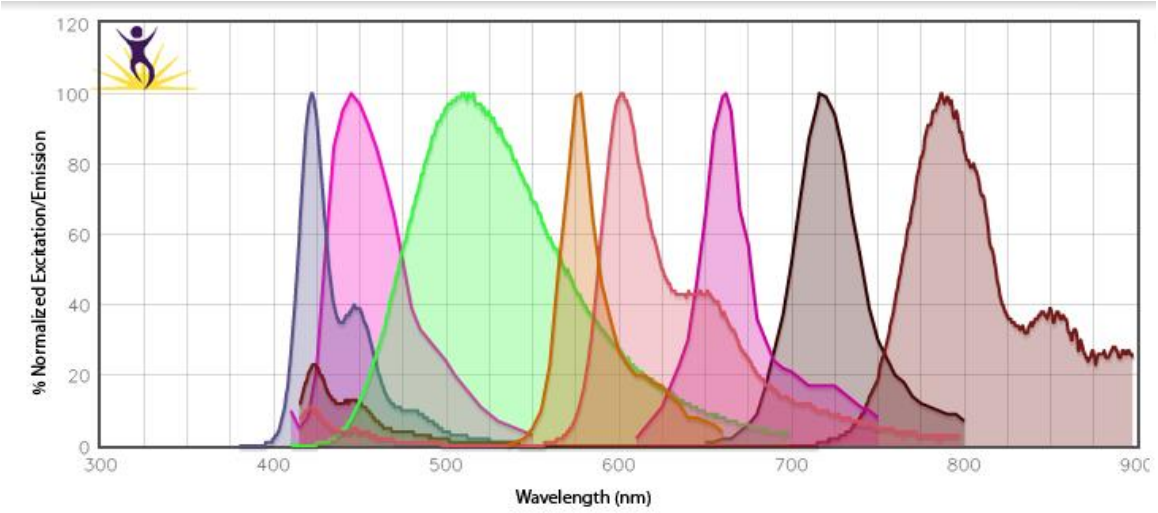


Figure 3.3: Emission spectra of fluorochromes BV421, BV510, BV605, BV785, Alexa Fluor 700, eFluor450, APC and PE. Image constructed using BioLegend Spectra Analyzer that may be utilized to ensure selected fluorochromes are compatible. Figure from (169).

The figure shows that emission maximum peaks at different wavelengths, and these colors are therefore compatible and can all be used in the same multicolor assay. The fact that there is some overlap between the spectra is almost inevitable, and can be corrigated for using software compensation (see 3.3.2).

3.3.5 Extracellular staining of PBMCs

A more detailed protocol of how the extracellular and intracellular staining was performed, can be read in Appendix 2.

After overnight stimulation, cells are transferred to 12 × 75 mm polystyrene tubes and extracellular antibodies conjugated to fluorescent dyes are distributed to the cells at a concentration of 5 µl/1 mL (meaning 1 µl was added to each well in the 96-well plate containing 200 µL of sample) and are left in the dark to work for 15 minutes. For the HIV study, the staining was performed using fluorochromes as described in table 3.3. Surface markers like these, especially CD3, CD4 and CD8, are relatively easy to identificate, and they were therefore analyzed using less bright colors (refer 3.3.4). It should also be noted that fluorochromes BV421 and eFluor450 are non-compatible, meaning CD57 and CCR7 cannot be stained for simultaneously.

Table 3.3: Extracellular staining. The epitopes, fluorochromes, manufacturers and laser and filter combinations are shown.

Epitope	Fluorochrome	Manufacturer	Laser	Filter combination
CD3	BV785	BioLegend	Violet, 405 nm	780/60
CD4	BV711	BioLegend	Violet, 405 nm	705/70
CD8	Alexa700	eBioscience	Red, 640 nm	710/50
CD27	BV510	BioLegend	Violet, 405 nm	525/50
CD28	PE/Cy7	eBioscience	Yellow green, 561 nm	780/60
CD45RO	FITC	eBioscience	Blue, 488 nm	525/50
CD57	eFluor450	eBioscience	Violet, 405 nm	450/50
CCR7	BV421	BioLegend	Violet, 405 nm	450/50
PD-1	BV605	BioLegend	Violet, 405 nm	610/20
HLA-DR	APC	eBioscience	Red, 633 nm	670/14

Dyes were washed away, and a live/dead stain followed. Fixable viability dye eFluor780 (eBioscience) penetrates the damaged membranes of dead cells and in this way they can be excluded from further analysis.

After extracellular staining, cells were fixed in a 2 % solution of paraformaldehyd (PFA) in PBS. Fixation terminates every biochemical reaction going on in the cells, and thereby preserves them in a fixed state. Several fixation methods are available, among them paraformaldehyd that fixate cells by crosslinking proteins.

3.3.6 Intracellular staining of PBMCs

For the intracellular staining, cells need to be permeabilized. The protein transport inhibitor used, prevents cytokine release from the cells, but the stain needs to enter the cells and bind to cytokines there. As a permeabilizing agent, 0,5 % saponin in PBS was used. Treatment with saponin detergent creates small pores in the cell membrane where dyes are able to pass.

Intracellular stain was added to the cells in the same concentration as extracellular dyes, 1 µl/test (2 µg/mL). As an experiment, it was tested to modify this dose, and results showed that this concentration should not be lowered (see 4.1.4).

All HIV samples were divided in two sets after stimulation, and one set was stained intracellularly with all three cytokine antibodies using the same fluorochrome (PE). PE is a very bright fluorochrome, and therefore a good choice for identification of cytokines from a small number of mycobacteria-specific T cells (refer 3.3.4). By staining all three cytokines with the same fluorochrome, cytokine-producing T cells can be identified in one single channel and this allows for the use of other channels for additional T cell markers.

To see if cells produce one, two or all three cytokines, the second set of cells from HIV patients were stained with different colors (table 3.4) and less surface markers. All chosen fluorochromes are very bright and well-suited for identification of cytokines.

Table 3.4: Staining protocol for identification of CD4 T cell polyfunctionality

Epitope	Fluorochrome	Manufacturer	Laser	Filter combination
IFN-γ	PE	BioLegend	Yellow green, 561 nm	585/20
TNF	BV421	eBioscience	Violet, 605 nm	450/50
IL-2	APC	eBioscience	Red, 633 nm	670/14

The exact protocol and staining and stimulation panel for extracellular and intracellular staining can be found in Appendix 2 and Appendix 3, respectively.

After these staining procedures, cells were ready for flow cytometric analysis.

3.3.7 Flow cytometric analysis

Cells were analyzed using a BD LSR II Flow Cytometer and BD FACSDiva software.

The flow cytometer was connected to a computer and FACSDiva software opened. For each sample, 50 000 (or 80 000 – 100 000 for HIV samples) cells were analyzed, and the FCS threshold was set to 20 000.

When starting an experiment with new colors, compensation was required (see 3.3.2), and the software calculated the degree of spectal overlap. When the same colors were used again in another experiment, the compensation controls were applied from a catalogue, and did not have to be calculated again.

3.3.8 Analysis of results

For result analysis, FlowJo v. 7.6.5 (TreeStar Inc.) was used.

One of the main tasks was to analyze the CD4 T cell effector functions (cytokine production). It is desirable to look at CD4 T cells only, and it is necessary to gate for these cells. Gating of live CD4 T cells is illustrated in figure 3.4. First, the main population is chosen based on the dotplots of SSC and FSC. From this, SSC against CD3 dotplot is chosen, and the CD3 positive population is gated for. From a dotplot of CD8 versus CD4, the CD4 positive CD8 negative cells are gated for (on figure 3.4 CD8 was not stained for, and gating was performed using a SSC/CD4 dotplot) and from these, the live CD4 T cells can be further analyzed.

After the CD4 T cells are selected, cytokine production may be analyzed. When all three cytokines are pooled using one single cytokine, the analysis is rather straight forward. The cytokine channel is chosen as x axis in a dotplot, and SSC may be chosen as the y axis. Proper controls are used in determination of gating boundaries, and the positive population is detected, and may even be further analyzed, for example for memory differentiation.

When the three cytokines are stained in different channels, analysis is a bit more complicated. In a dotplot, analysis is restricted to two characteristics at once, while it is desirable to consider all three cytokines simultaneously. To achieve this, a tool in the FlowJo software called 'combination gates' is used. Boolean combination gates can be used to create a gate that is a combination of any other existing gates, where Boolean operators AND, OR and NOT are used. Creating combination gates from IFN-γ+, TNF+ and IL-2+ populations provides new gates that show all possible combinations between these three. One single triple positive population is created, three double positive, three single positive and one triple negative. These calculations and percentages can be exported to Excel to create pie charts representing the relative proportions of single, double and triple producers. The importance of this is obvious, as triple producers represent the more healthy CD4 T cells that are best suited to fight mycobacterial infection (refer section 1.4.3).

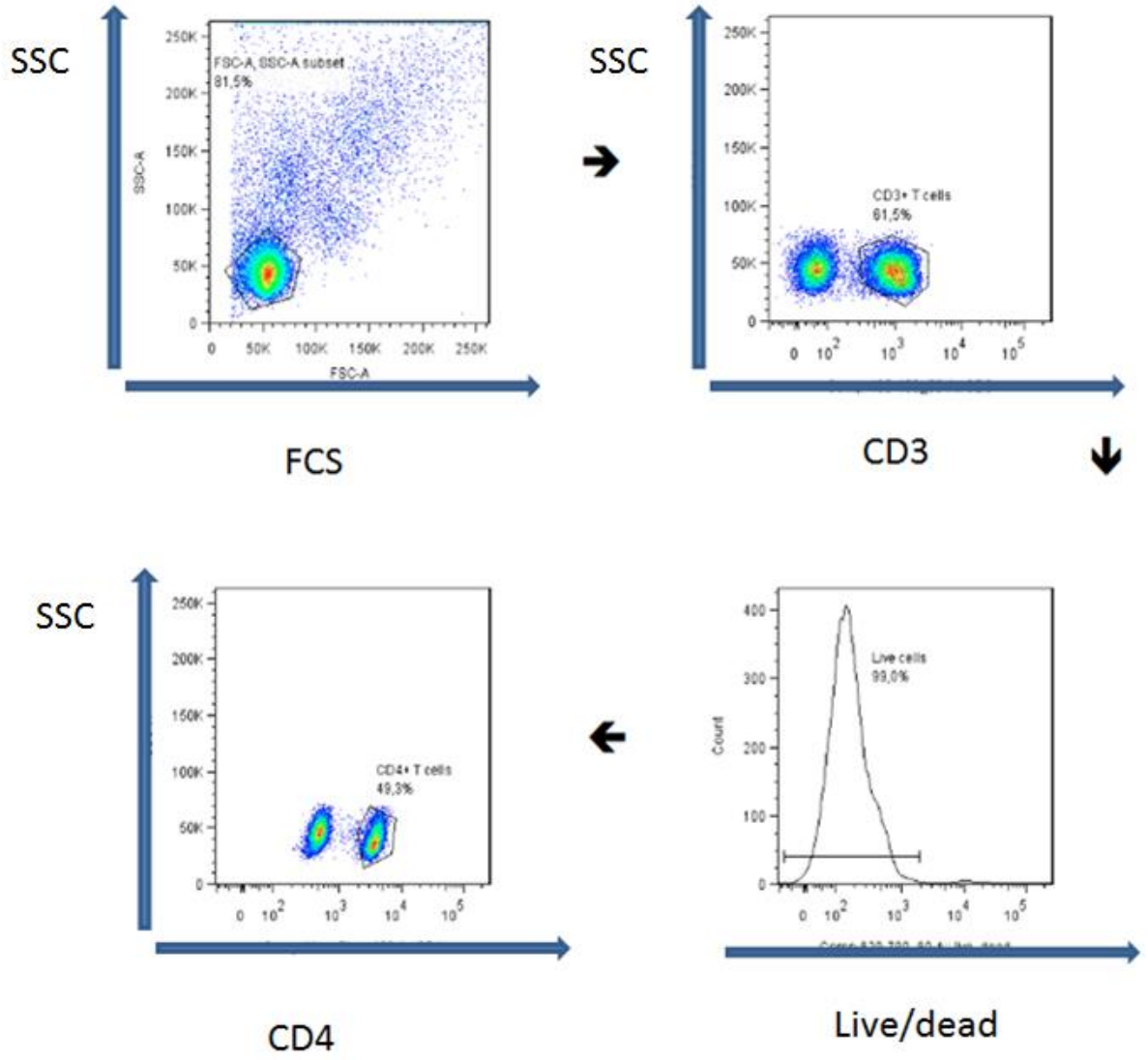


Figure 3.4: Gating of CD4 positive T cells. The main population is gated for from the SSC and FSC dotplots. The CD3 positive population is gated for from the SSC/CD3 dotplots. Dead cells are excluded by the gating of the negative population from the live/dead (eBioscience Fixable viability dye e780) channel. CD4 positive T cells are identified from the SSC/CD4 dotplots.

3.4 Expansion of mycobacteria-specific T cells

One experiment was performed using an expanded population of mycobacteria-specific T cells from a HIV patient. Expansion of the T cell population greatly increases the proportion of specific cells, as specific T cells in culture will divide.

For this experiment, isolated PBMCs were diluted in RPMI 10 % A+ to a working concentration of 3,5 • 10^6 cells/mL. Cells were seeded in a 24 well flat bottom plate, 1 mL per well. For stimulation, heat-killed *M.avium* was added at MOI (multiplicity of infection) 5:1.

At day two, 20 U (units) IL-2 was added to the T cells. IL-2 is a potent growth factor for T cells and causes T cell proliferation. As T cells encounter their specific antigen presented in a MHC context, the cells start dividing. For four to five days the cells divide two to three times a day, creating a monoclonal T cell population (as mentioned in 1.4.1).

In parallel, autologous monocyte-derived macrophages (MDM) were generated. PBMCs were diluted in RPMI 10 % A+ to a working concentration of 2 • 10^6 cells/mL and seeded in 96-well flat bottom plates. 100 μL (0,2 • 10^6 cells) were added to each well. Cells were incubated at 37 °C for 1 hour, just enough time for the monocytes to selectively adhere to the bottom of the plate.

Monocytes are the cell type with the greatest affinity for the culture substrate and therefore the first to adhere, often already after 30 minutes (155). The rest of the cells were eliminated from culture by removing the supernatant and washing the monocytes three times with warm HANKS buffer. For the generation of macrophages, monocytes were cultured in RPMI medium 30 % A+ serum for nine days in 37 °C.

At day nine, the MDMs were stimulated using heat-killed *M.avium* and PPD from *M.avium* and *M.tuberculosis* and co-cultured with the expanded T cells for 16 hours. This induces cytokine production in the expanded population of specific T cells.

After stimulation overnight, cells were treated with protein secretion inhibitor and extracellular and intracellular staining were performed as described in 3.3.5 and 3.3.6. T cells were analyzed for effector functions on a flow cytometer.

3.5 PCR

PCR is a method used in biological sciences to amplify short pieces of DNA into large amounts. Real time PCR, as utilized in this study, is mainly used for quantification of DNA

segments. Quantification may be absolute, as in number of copies, or relative, when normalizing agents such as housekeeping genes are used.

This study used quantitative real time PCR to determine the relative amounts of cytokine mRNA made by PBMCs stimulated with PPD from *M.avium* and *M.tuberculosis* and heat-killed *M.avium*. Upon such stimulation, signaling cascades (e.g. from PRRs) induce activation of transcription factors, a process that leads to the transcription of cytokine genes. This leads to accumulation of cytokine specific mRNA inside the cells, these levels can be measured by real time PCR after RNA isolation from lysed cells. After 16 hours of stimulation in round bottom 48-well plates, plates were centrifuged and the supernatant removed (and used for ELISA, see below). The cells were lysed to access the mRNA. To achieve lysis, cells are treated with RLT buffer (QIAGEN) containing guanidine-thiocyanate. β-Mercaptoethanol is added to degrade RNases. mRNA is then isolated and transcribed to cDNA. This cDNA was loaded to a PCR machine that amplifies the DNA strand and quantifies the original amount of mRNA present in the sample.

The PCR was performed using probe pairs for three genes: TNF, IFN-β and IL-1β. As normalizing agent, the housekeeping gene GAPDH was used.

3.5.1 Assay procedure

RNA was isolated using the RNeasy Mini Kit (QIAGEN), a kit designed to extract high-quality RNA from small amounts of animal cells. The process was fully automated using QIAcube (QIAGEN). The principle behind the process is the binding of RNA (as samples are already lysed) to silica membranes in so-called spin-columns in an environment containing ethanol to provide optimal binding conditions. RNA is later eluted in a new tube. A DNase digest step was included as a RNA clean-up step to eliminate genomic DNA contamination.

To determine the RNA concentration, a NanoDrop 1000 spectrophotometer (Thermo Scientific) was used. This device provides information on both the concentration and the quality of the extracted RNA.

RNA need to be converted to complementary DNA (cDNA) before PCR, and the High capacity RNA-to-cDNA kit (Applied Biosystems) was utilized. cDNA is produced by

enzymes reverse transcriptase and DNA polymerase in the presence of nucleotides. To control the temperature, the C1000 thermal cycler (Bio-Rad) was programmed in the following way to ensure optimal conditions for cDNA synthesis: 25 ºC for 10 minutes, 37 ºC for 120 minutes, 85 ºC for 5 minutes and hold at 4 ºC.

As cDNA synthesis is complete, quantitative real-time PCR can be performed. In a reaction tube, cDNA is mixed with TaqMan probes for the genes of interest and a mastermix. The samples are loaded onto StepOnePlus™ Real-Time PCR System (Applied Biosystems) that analyzes the gene expression from the detection of fluorescent light as the TaqMan probes are inserted to the DNA strand and later destroyed.

The samples were processed using the quantitative ΔΔCT program at the following settings: 50ºC for 2 minutes, 95 ºC for 20 seconds and 40 cycles of 95 ºC for 1 second and 60 ºC for 20 seconds.

3.5.2 Analysis of results

The software calculates the gene expression of the target sample normalized to the endogenous control; the housekeeping gene GAPDH and a calibrator (the unstimulated sample). This yields the value relative quantification (RQ). The RQ value is the fold change in samples compared to the calibrator. The calibrator gets a value of 1, and all samples are compared to this control.

3.6 ELISA

ELISA is a biochemical assay that detects the presence of a given substance in a sample. The substance is often an antigen, but is in this case a cytokine, TNF. This ELISA was performed as a 'sandwich' ELISA, meaning an ELISA plate is first coated with an antibody specific for the cytokine of interest, before sample is added and the cytokine binds to the antibody. Yet another antibody, specific for another epitope on the cytokine is added and binds the cytokine. The second and so-called detection antibody is later conjugated via streptavidin to an enzyme (HRP) that causes a color change in the wells upon addition of enzyme substrate. This color change is measured optically and represents the amount of cytokine present in the sample.

Upon stimulation, the antigen-specific cells in the cell population will start producing cytokines and release them into the surroundings. ELISA cytokine analysis was performed from the cell culture supernatant using the samples where mRNA was analyzed by real time PCR (see 3.5).

The ELISA is performed using Human TNF-α DuoSet kit provided by R&D Systems. The principle behind the method is to make a standard curve using known concentrations of the cytokine of interest. From this standard curve it is possible to determine the concentrations of TNF in the unknown samples.

Samples were analyzed both undiluted and in a 1:10 dilution. The stimulation was performed in duplicates, and for the ELISA, duplicates were analyzed for every sample, meaning each biological replicate was analyzed four times. Unstimulated cells and PMA-stimulated cells were included as negative and positive controls, respectively. Each known concentration in the standard curve was analyzed in triplicates.

3.6.1 Assay procedure

The assay was performed according to the user manual provided by R&D Systems. In brief, a flat bottom 96-well ELISA half area plate (NUNC) was coated with mouse anti-human TNF-α and left overnight to have the capture antibody bind to the plate. Washing buffer is used to remove unbound antibody. The ELISA wash buffer contains tween 20, a detergent that helps prevent unspecific antibody binding. The plate is later blocked using a solution containing bovine serum albumin (BSA). BSA will bind to sites in the well not already bound by capture antibody to prevent unspecific binding later on.

Standard solutions and samples are added to individual wells and are left to react so TNF-α in samples are able to bind to the capture antibody in the well. After washing, the second antibody is distributed to the wells and bind to a second epitope on the cytokines in the sample. As a streptavidin-HRP (horseradish peroxidase) conjugate is later added to the samples, streptavidin binds to multiple sites on the detection antibody. The last assay step is the addition of a mixed substrate solution, a substance that reacts in the presence of HRP enzyme to produce a color change. Stop solution N_2SO_4 inhibits the enzyme catalyzed

reaction, and the optical density (OD) can be measured in a spectrophotometer at wavelength 450 nm.

3.6.2 Determination of sample concentrations

For OD measurement and data analysis, the software Bio-Rad Microplate Manager was used. From the measured OD (after a blank was subtracted), a standard curve was constructed from plotting OD against known concentrations. The concentration range went from 15,6 pg/mL to 1000 pg/mL. To customize a regression for the data set, a so-called «4-Parameters Fit» was used. This regression represents y as follows in equation (3.1).

$$y = (A-D)/(1+(X+C)^B)+D \quad (3.1)$$

where A, B, C and D are fixed constants.

The measured OD for the unknown samples are inserted as y values in (3.1), and the TNF-α concentration in pg/mL for the different unknown samples can be calculated by solving for X.

3.7 Ethical issues

Blood from HIV positive patients and healthy donors was used in this study. All patients gave their written and informed consent to participate in research related to their HIV disease. The study was approved by the Regional Ethics Committee (ID: 2012/153/REK midt).

Handling of HIV blood was performed under BL3 conditions, and all samples and results were anonymized.

4. Results

The project was divided in two parts. The first part consisted of optimization of methods to best preserve peripheral blood mononuclear cells (PBMCs) and analyze their effector functions. For this purpose, blood from healthy donors was utilized.

The second part was the patient study that took use of blood from HIV positive patients and healthy controls.

The first part of the results section describes what was discovered during the analysis, and method optimization in the preliminary study. This includes flow cytometry and tests to determine whether analysis of HIV samples may be performed using frozen specimen, how to distribute protein secretion inhibitor to the cells, dosage controls of cells per well and intracellular stain, the effect of short-term storage on stained and fixed cells. These optimized protocols were then applied in a patient study focusing on cytokine production and memory differentiation phenotype in HIV patients. At last, results from experiments using PCR and ELISA to study total cytokine production will be presented.

4.1 Preliminary studies

4.1.1 Influence of cryopreservation on cytokine production from CD4+ T cells

For a study using patient samples, long-term storage of samples may be of great importance. Few patient samples are available each day or week, and for reproducibility and comparability reasons it may be beneficial to analyze several samples simultaneously. It was therefore investigated if PBMC quality is impaired when cells are kept at sub-zero temperatures.

White blood cells were isolated from healthy donors' heparinized blood using ficoll-hypaque density gradient (see 3.2.1). After blood cell isolation, 3-7.5 • 10^6 PBMCs were transferred to NUNC (Nalgene) cryotubes containing a DMSO-based freezing medium and duplicate samples were stored in liquid nitrogen or a -80 °C freezer (see 3.2.2).

Cells were thawed after approximately three months, and their viability examined using Countess automated cell counter (Invitrogen). Cryopreservation did not seem to have any effect on the viability prior to stimulation, as it remained stable at around 90-95 % for both fresh and frozen specimen (data not shown). There was no significant difference between cryopreservation in liquid nitrogen or -80 °C.

Effector functions were compared between fresh PBMCs and frozen samples from the same donor, preferably stored both in liquid nitrogen and in -80 °C. Each sample was stimulated (described in 3.2.3) and stained for identification and cytokine production (3.3.5/3.3.6). All three cytokines (IFN-γ, TNF and IL-2) were stained using the same fluorochrome (PE). Analysis was performed using flow cytometry (see section 3.3).

Results for one representative donor are shown in figure 4.1 below. Other donors were analyzed with similar outcomes. The figure shows the percentage of live CD3+CD4+ T cells that can produce any of the three cytokines (TNF, IFN-γ or IL-2) in response to stimulation with mycobacterial antigens. PBMCs have been cryopreserved for three months, and cells are gated using isotype controls. Gating strategy for detection of cytokine-producing live CD4+ T cells is shown in figure 4.1 A. The cytokine-positive population from the last dotplot represents the red column corresponding to *M.avium* PPD in figure 4.1 B. Surface markers seem not to be influenced by cryopreservation (data not shown). Stimulation with the aforementioned agents gives rise to mycobacteria-specific CD4+ T cells that are able to produce cytokines above background signal. Fresh cells provide the strongest signal and show the largest proportion of mycobacteria-specific CD4+ T cells (figure 4.1 B).

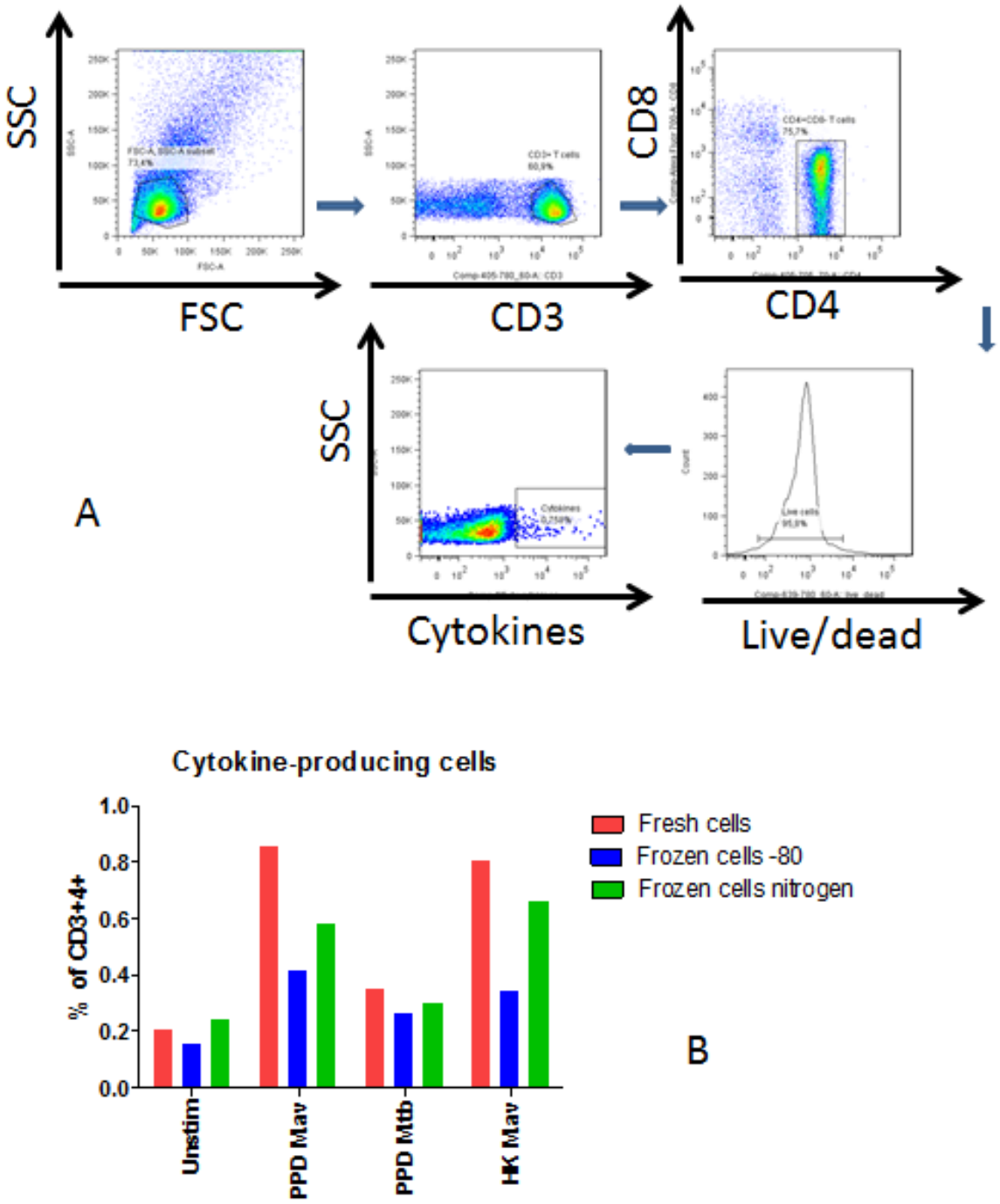


Figure 4.1: Cytokine production from fresh or cryopreserved CD3+CD4+ T cells in response to stimulation with mycobacterial antigens. A. Gating strategy. PBMCs from the same donor were stimulated overnight before adding protein secretion inhibitor. Cells were stained with CD3, CD4 and CD8 on the surface, and a live/dead staining procedure based on dye exclusion was included. After fixation and permeabilization, cells were intracellularly stained for three effector cytokines (TNF, IFN-γ and IL-2) in the same channel (using PE fluorochrome). Cells were gated for live CD3+CD4+CD8- T cells. B. Analysis of cytokine production (cytokine-positive populations from figure A) as shown by the percentage of CD4+ T cells that produce any if the three effector cytokines. Fresh cells are compared to cells that have been stored in liquid nitrogen and -80 °C for three months. Unstimulated cells are included as controls.

Cells that have been frozen and thawed showed the same pattern as the fresh cells although a decrease in total cytokine production was seen. The tendency was found for both storage methods, but is more profound for PBMCs stored at -80 °C compared to liquid nitrogen. Results indicate that PBMCs can be stored in liquid nitrogen and still retain most of their effector functions and yield results that are comparable to fresh cells. Long-term storage of PBMCs in liquid nitrogen therefore seems to be possible for analyzing cytokine production from CD4+ T cells. However, as the proportion of specific cells is low after stimulation overnight, decrease in cytokine production may be significant and using fresh cells should be considered. Fresh PBMCs was used in this project, but PBMCs from HIV patients were stored in liquid nitrogen as a back-up for repeating experiments.

Difference between cells that were kept in liquid nitrogen and those kept in -80 °C is also seen when taking viability post stimulation (using eBioscience Fixable Viability Dye) in to consideration. For cells stored in a regular -80 °C freeze container, viability drops compared to fresh cells, whereas cells that have been stored in colder conditions in a liquid nitrogen tank, show the same viability as fresh cells (data not shown).

4.1.2 Inhibition of secretion of mycobacteria-specific effector cytokines produced by CD4+ T cells

This project seeks to analyze the cytokine production from CD4+ T cells which normally are secreted from these cells and released to the extracellular space. Cytokines need therefore be accumulated within the cells, and this is achieved by treatment with a protein transport inhibitor cocktail (eBioscience 500x) that inhibits transport pathways out of the cell.

Manufacturer (eBioscience) recommends leaving the protein transport inhibitor on cells for five to 18 hours. Within the research group, regular practice has been to stimulate PBMCs for 16 hours, before adding protein transport inhibitor and incubating four new hours. However, waiting 16 hours from stimulation to inhibition of secretion may lead to loss of some of the early produced cytokines, as they are known to possess varying kinetics (83)(89). To investigate this and conclude on what time frame is the ideal to achieve best possible signal in flow cytometric assays, two different approaches were tested: The one just described, and one where the protein transport inhibitor was added one hour after stimulating agents and left on cells for 16 hours.

Cells were stimulated and stained as described in 3.2.3 and 3.3.5/3.3.6, respectively.

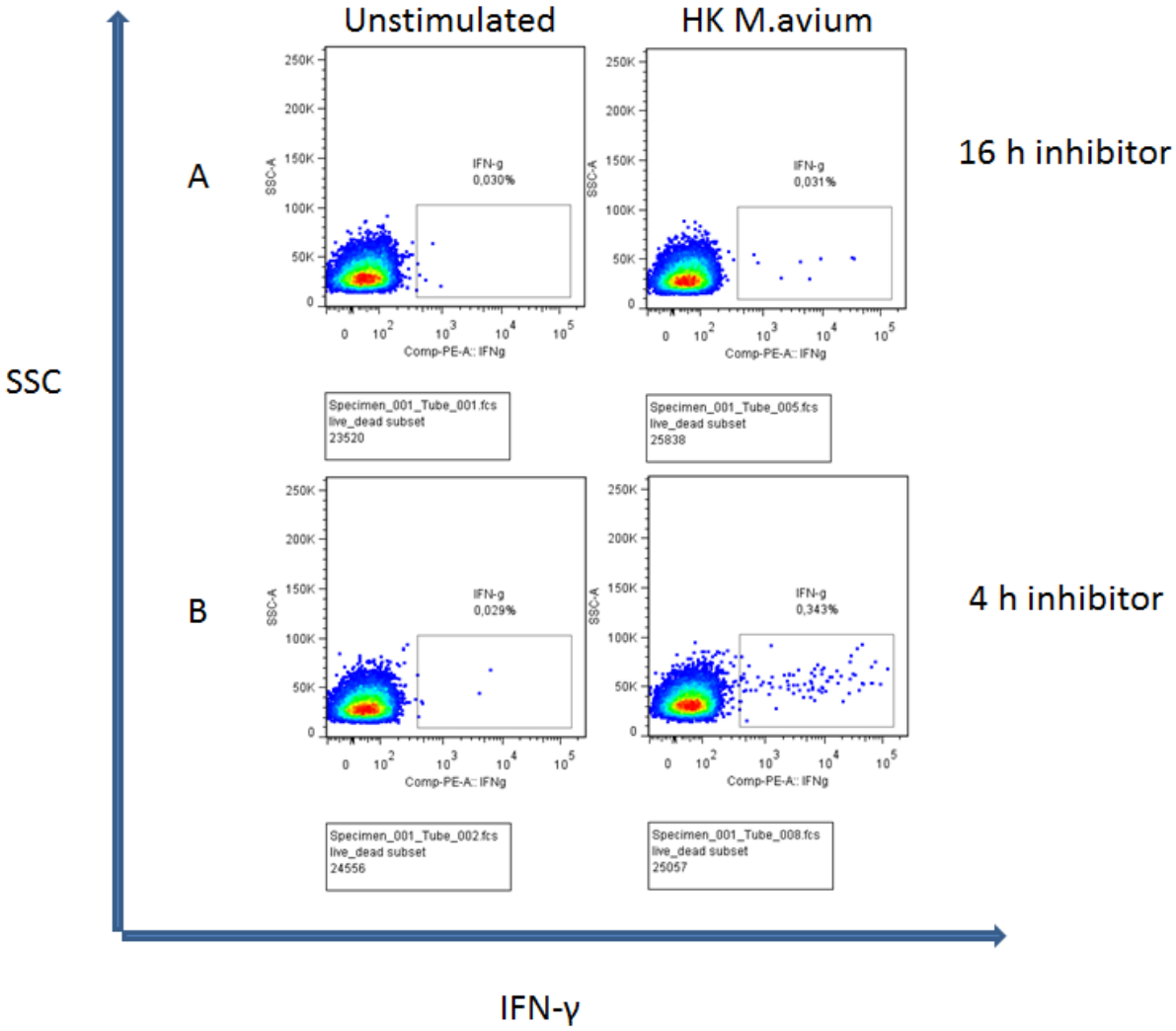


Figure 4.2: Optimal time interval for inhibition of cytokine-secretion to detect IFN-γ production in mycobacteria-specific T cells. A. Cells were stimulated using heat-killed *M.avium* and transport inhibitor cocktail was added one hour post stimulation and left on cells for 16 hours. B. Cells were stimulated with heat-killed *M.avium* overnight (~ 16 hours) and transport inhibitor cocktail was distributed to the cells for another four hours. For both methods, cells were stained for CD3, CD4 and CD8, fixed, permeabilized and stained for effector cytokines in three different channels. Cells were gated CD3+CD4+CD8- and analyzed for production of IFN-γ (x axis). Unstimulated controls are shown on the left, HK *M.avium* - stimulated cells on the right.

Results show marked differences between the two methods. When protein transport inhibitor is added just one hour after stimulation, cells show low to moderate production of IFN-γ and IL-2, and moderate to high production of TNF. When added 16 hours after stimulation, large

amounts of IFN-γ are produced, while TNF and IL-2 levels decline compared to the first method. Figure 4.2 shows how the two different methods influence the production of IFN-γ. No cytokine production above background signal is detected when transport inhibitor is added one hour post stimulation, whereas large amounts are produced after 16 hours. On the basis of these results, it was decided to add protein transport inhibitor after 16 hours of stimulation and for another four hours.

4.1.3 Optimal cell numbers for the mycobacteria-specific T cell stimulation assay

Experiments were carried out to optimize the flow cytometric assay in order to determine what conditions are the best possible when investigating T cell effector functions. As the number of PBMCs are often scarce, especially when receiving blood from HIV patients, it is necessary to use the least number of cells possible while still being able to execute the experiments in a satisfactory manner. To test this, PBMCs were seeded in the range from 0,2 • 10^6 to 10^6 per well. During flow cytometry, 50 000 cells were acquired. Results are shown in figure 4.3.

The figure shows no tendency for differences between the different dosages and the small variation can probably be attributed well-to-well variations. This means that the whole range of cell numbers may be utilized. However, obtaining the desired number of at least 50 000 cells per flow-analysis may be difficult if initial PBMC concentration is too low. As the proportion of specific T cells is small, increasing the number of events to record from 50 000 to 100 000 in the flow cytometer may be beneficial, which provides even more stringent requirements for initial cell concentration. Based on this, PBMCs were normally seeded in a concentration of 0,5 • 10^6 cells per well.

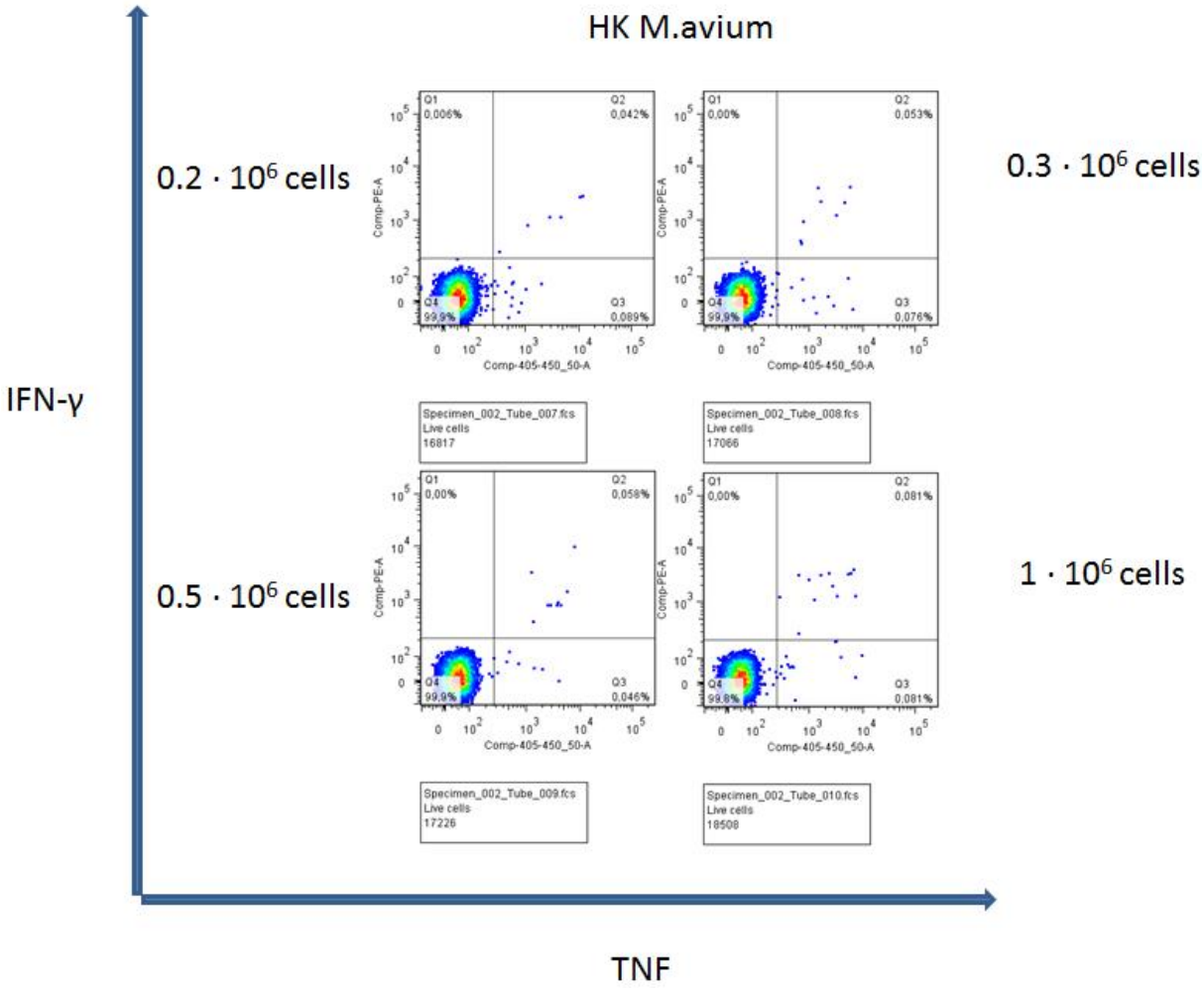


Figure 4.3: Analysis of effect of different numbers of cells per flow cytometric sample. Cells were stimulated with heat-killed *M.avium* overnight. On the surface they were stained for CD3, CD4 and CD8. Cells were then fixed and permeabilized and stained intracellularly for the three effector cytokines (TNF, IFN-γ and IL-2). Live cells were gated CD3+CD4+CD8-, analysis of production of IFN-γ (y axis) and TNF (x axis) are shown. Cell number per well varied and is indicated on the figure.

4.1.4 Antibody titration for optimal fluorescence staining of effector cytokines

The dosage of fluorescent dye for intracellular staining was also tested. Manufacturers often recommend using a specific volume of dye per test which should give sufficient staining for example for 10 • 10^6 cells. However, it is recommended that the optimal concentration of each antibody should be tested for each application. Finding the correct amount of fluorescent dye is one of the most important issues in immunophenotyping, as deviations from the ideal volume may affect the results. Too much antibody will increase unspecific binding and thereby reduce the signal-to-noise ratio. If the amount is not high enough, fluorescent staining

will be incomplete, a critical problem for an assay like this (170). As we only have low cell number (a maximum of $0,5 \cdot 10^6$ cells per well) and low numbers of cytokine-producing cells (usually less than 1 %), it is important to specifically detect these cells with a good signal and as little background as possible at the same time. It was therefore chosen to use bright fluorochromes with a high staining index for staining of effector cytokines (refer section 3.3.4).

In addition, experiments were performed where the amount of fluorescence-labeled effector cytokine antibodies was titrated to find the optimal concentration for the assay.

PBMCs were stimulated (3.2.3) using heat-killed *M.avium* and stained for CD3, CD4 and CD8 for identification. For the intracellular stain, a five-point range from 0,2 to 5 µL (0,4 - 10 µg/mL) dye per test was used, the three cytokines (TNF, IFN-γ and IL-2) were all tested simultaneously. Measured fluorescent signal for cytokines TNF (x axis) and IFN-γ (y axis) are shown in figure 4.4. Signal increases as amount of dye is increased. For simplicity (using the same volume of dye for the extracellular and intracellular staining) and cost efficiency, it was chosen to use 2 µg/mL for future analysis.

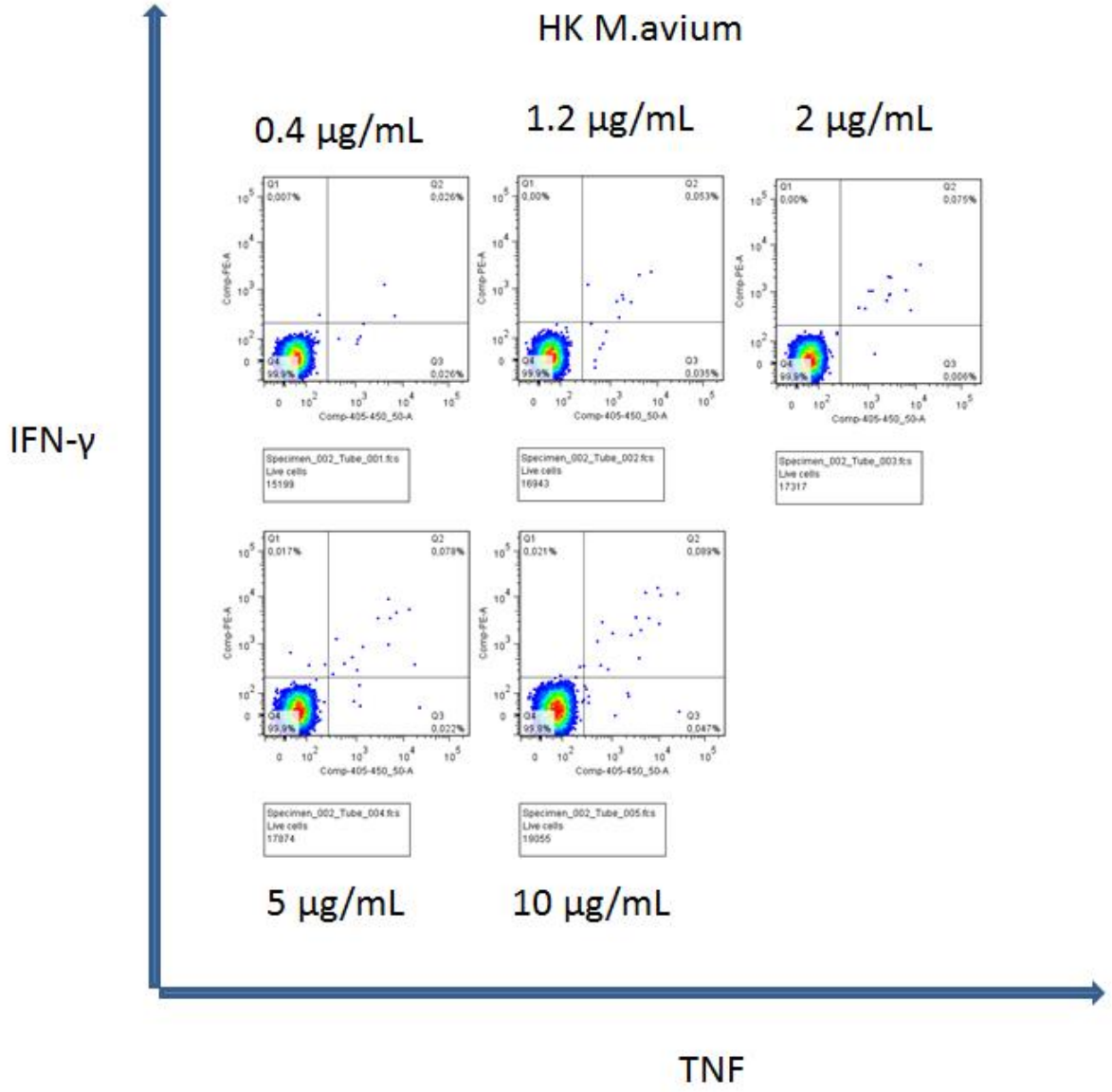


Figure 4.4: Antibody titration for staining of intracellular cytokines. Cells are stimulated overnight using heat-killed *M.avium* and stained for extracellular and intracellular markers. Live CD3+CD4+ cells are shown. TNF is shown on the x axis, IFN-γ on the y axis. Fluorochrome concentration varied and is indicated on the figure.

4.1.5 Influence of short-term storage of fixed and stained cells before analysis of effector cytokine production by flow cytometry

The day after stimulation, cells are extracellularly stained for surface markers and fixed in a paraformaldehyde (PFA) solution. PFA crosslinks the proteins and makes it possible to store the cells in 12 × 75 mm polystyrene tubes at 4 °C without any damage to intracellular structures. Intracellular staining and flow cytometry is usually performed the next day.

Analyzing the samples before their stability is compromised is essential, as fluorochromes may bleach in light or degrade (for example for tandem dyes), thus influencing the results.

To examine the effect of short-term storage of stained cells on the end result of flow cytometry, 12 × 75 mm polystyrene tubes («flow tubes») were analyzed on day zero after finishing intracellular staining procedure and flow cytometry was repeated on the same tubes on day five (after storage at 4 °C). Results are shown in figure 4.5 for one representative tube. The results indicate that storage of stained and fixed cells for at least five days have little, if any, effect on the results. All samples were analyzed on day five, yielding the same result as below, meaning with no significant change in signal from day zero to day five. Despite this, samples were normally analyzed on the same day as intracellular staining was performed.

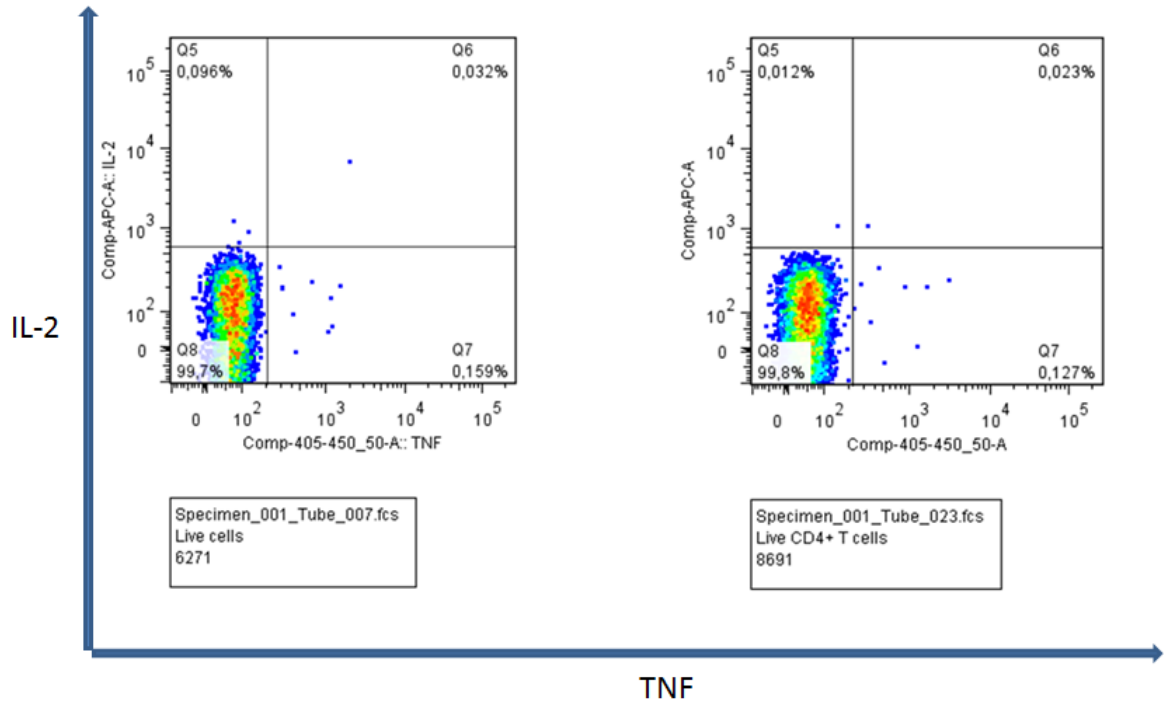


Figure 4.5: Effect of storage of stained and fixed cells on detection of effector functions. PBMCs are stimulated overnight using heat-killed M.avium. Cells are then stained extracellularly, fixed, permeabilized and stained intracellularly. Cells are live and CD3+CD4+, and cytokine production is represented by TNF (x axis) and IL-2 (y axis). Left is shown flow cytometric analysis at day zero, right is flow cytometric analysis performed on the same tube at day five, after storage at 4 °C.

4.1.6 Multicolor flow cytometry protocol for phenotyping of effector cytokine production and memory status of CD4+ T cells

The previous sections (4.1.1 to 4.1.5) describe how the method for analysis of mycobacteria-specific CD4+ Th1 cells using flow cytometry was optimized. This method was then used for analyzing cytokine polyfunctionality and memory differentiation in HIV patients and healthy donors.

It was found that fresh cells are the preferred choice for studying mycobacteria-specific cytokine production, due to a decrease in the percentage of specific cells after cryopreservation. However, cells stored in liquid nitrogen for up to three months are possible options if necessary. For this study, fresh cells were normally the choice, and all PBMCs from HIV patient samples were analyzed fresh. PBMCs from HIV patients were also stored in liquid nitrogen, meaning they can be analyzed at a later stage of the research project.

The distribution of protein secretion inhibitor was found to influence the cytokine production, as cytokines inhibit varying kinetics. TNF seems to accumulate early after stimulation, and yields best results in the subsequent flow cytometric assay if protein transport inhibitor is distributed to the cells one hour post stimulation, as TNF levels seem to decrease relatively early. IFN-γ is secreted at a later stage, and for this cytokine, it is preferred to wait 16 hours after stimulation before adding secretion inhibitor, leaving it for four hours. Leaving the protein transport inhibitor on the cells for too long may influence the cells in a negative manner and should thus be avoided. It was decided to always wait for 16 hours after stimulation before adding protein transport inhibitor. Deviations from the protocol should be avoided for as long as the study is ongoing.

It was also decided on always seeding cells at a concentration of $0{,}5 \cdot 10^6$ cells per well, as this amount was necessary to obtain the desired 50 000 – 100 000 events per flow cytometric analysis. Both extracellular and intracellular antibodies were distributed to the cells at a concentration of 2 μg/mL.

Based on this, it was decided on a protocol where PBMCs were isolated (3.2.1) and seeded fresh in flat bottom 96-well plates (12-13 wells per donor). Cells were stimulated (3.2.3) using three mycobacterial antigens; *M.avium* PPD, *M.tuberculosis* PPD and heat-killed *M.avium*.

All stimulating agents were distributed in duplicates, and in addition to cells stimulated with mycobacterial antigens, two wells were unstimulated as negative controls, and two wells were stimulated using cell stimulation cocktail for positive control. Some extra wells were stimulated with heat-killed *M.avium* for controls. Cells were then incubated in 37 °C overnight to a total of approximately 16 hours, before adding protein transport inhibitor, leaving this on for another four hours.

Half the panel was stained extracellularly and intracellularly (3.3.5/3.3.6) for the purpose of investigating the mycobacteria-specific CD4+ T cell cytokine production and polyfunctionality. This means the three cytokines IFN-γ, TNF and IL-2 were stained using three different, bright fluorochromes. In addition, identification markers (CD3, CD4 and CD8), a live/dead stain and some memory markers were included. The other half was stained for the purpose of examining memory differentiation in CD4+ T cells and mycobacteria-specific CD4+ T cells. To be able to stain as many surface memory markers as possible, the three cytokines were stained using the same fluorochrome. Identification markers (CD3, CD4 and CD8), live/dead stain and six memory markers (CD27, CD28, CD45RO, CCR7, PD-1, HLA-DR) were also included. For some donors, an additional tube was stained using CD57 instead of CCR7 (the two antibodies occupied the same channel). This means 11 of the 16 possible filter combinations on the flow cytometer was used during analysis. Controls always included isotype controls for all memory markers and all cytokines. When compensation was required, one tube was prepared for all fluorochromes, with one drop of compensation beads and 1 μL of the fluorochrome of interest.

Bright and stable colors were chosen for the effector cytokines and memory markers, less bright colors for the major populations CD3, CD4 and CD8 (refer 3.3.4). Use of unstable tandem dyes was limited.

Figure 4.6 shows an example of how samples were analyzed using the final protocol. FSC, SSC and identification markers CD3, CD4 and CD8 are used to identify the CD4+ T cell population of interest, and a live/dead stain is included to exclude dead cells from further analysis. From the live population, cytokine production and expression of surface memory markers may be identified.

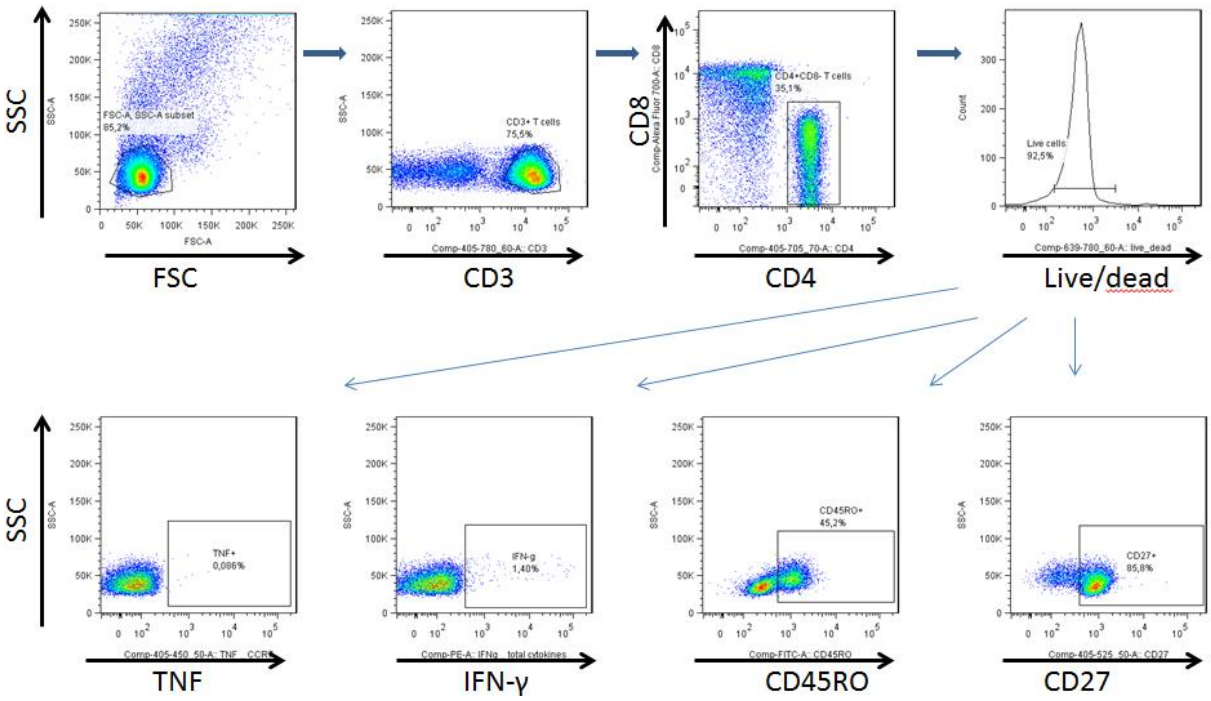


Figure 4.6: Gating strategy for patient study. PBMCs from healthy donors or HIV patients were stimulated overnight using *M.avium PPD*, *M.tuberculosis* PPD as well as heat-killed *M.avium* before protein secretion inhibitor was added for four hours. Cells were stained with CD3, CD4 and CD8 on the surface for identification, as well as some memory markers. A live/dead staining procedure based on dye exclusion was included. After fixation and permeabilization, cells were intracellularly stained for effector cytokines. Cells were gated for live CD3+CD4+CD8- T cells, before marker of interest (cytokine or memory marker) was investigated.

4.2 Patient study

4.2.1 Mycobacteria-specific cytokine production by CD4+ T cells in healthy individuals and HIV patients

To determine to what extent healthy individuals can mount a response towards mycobacterial antigens, T cell effector functions were examined using cytokines TNF, IFN-γ and IL-2 as markers for T cell specificity. These cytokines are main targets in assays investigating infections caused by mycobacteria (refer section 1.4.3). Polyfunctional T cells (PFT) are able to produce several of these effector cytokines simultaneously and are considered particularly important in controlling tuberculosis infection (92)(93)(97). Not much is known about their importance in *M.avium* infection, but it was desirable to analyze polyfunctionality of CD4+

Th1 responses from healthy individuals and HIV patients in response to antigens from *M.tuberculosis* as well as from *M.avium*.

Effector cytokine production from healthy donors (4.2.1.1 and 4.2.1.2) as well as HIV patients (4.2.1.3) were analyzed with the optimized protocol described in section 3.2 and 3.3 and in the first part of the results (final protocol in 4.1.6). Bright fluorochromes with high staining index were used to detect the effector cytokines IFN-γ, TNF and IL-2 from different channels. Colors used were PE, BV421 and APC, respectively. This enables to determine the relative proportions of single producers (producing any of the three cytokines), double producers (any two cytokines) or triple producers (all three cytokines). To determine the relative proportions, Boolean gating was used (section 3.3.8).

For information about the HIV patients that were recruited to the study, see section 3.1.

4.2.1.1 Mycobacteria-specific CD4+ T cell effector cytokine production in healthy individuals

Fresh PBMCs from healthy individuals were isolated (section 3.2.1), stimulated (section 3.2.3) and stained for T cell effector cytokine production (3.3.5/3.3.6). Three different mycobacterial antigens were tested; *M.avium* PPD, *M.tuberculosis* PPD and heat-killed *M.avium*, as well as unstimulated negative controls and PMA-stimulated positive controls. Results from some donors were rejected, as their handling procedures deviated from the final protocol described in 4.1.6. Samples were also eliminated if the signal-to-noise ratio was low or if the positive control did not work. A positive response was defined as twice the signal from the unstimulated negative controls. For the final result, four healthy BCG-vaccinated donors were included, men and women ranging from 30 to 55 years old.

All healthy donors responded to mycobacterial antigens.

Effector cytokine production of CD4+ T cells in response to overnight stimulation with mycobacterial antigens from four different healthy donors is shown in figure 4.7. Relatively large donor variability was detected, as can be seen from figure 4.7 A, where percentages of total mycobacteria-specific T cells (producing one, two or three cytokines) are plotted for stimulation with *M.avium* PPD, *M.tuberculosis* PPD and heat-killed *M.avium*. Generally,

responses ranged from 0,2 to 0.7 % for overnight stimulation with mycobacterial antigens. All donors have been vaccinated with Bacille Calmette-Guérin (BCG), a vaccine meant to provide long-term protective immunity against tuberculosis. Although earlier believed to greatly decrease the risk of tuberculosis (171), the vaccine was later found to be ineffective against adult pulmonary disease (172).

The degree of polyfunctionality also varies among the different donors, as seen in figure 4.7 B, indicating different degrees of polyfunctionality for the donors against *M.avium* PPD. For the majority of donors, single producers secreting either TNF, IFN-γ or IL-2 are predominant (34-93 %). For one of the donors, the majority of the mycobacteria-specific cells was found to be triple producers. The three donors with single producers as the predominant cells correspond to the three highest percentages of total cytokine-producing cells (4.7 A). IFN-γ is the predominant cytokine among the monofunctional cells, with TNF second. IL-2 is almost absent among monofunctional cells (data not shown).

When results from all donors are combined, monofunctional T cells are still predominant, accounting for approximately two thirds of all cytokine-producing cells (fig. 4.7 C). The rest of the cells are divided almost equally between double producers and triple producers. Among the double producers, IFN-γ+TNF+ cells are most predominant.

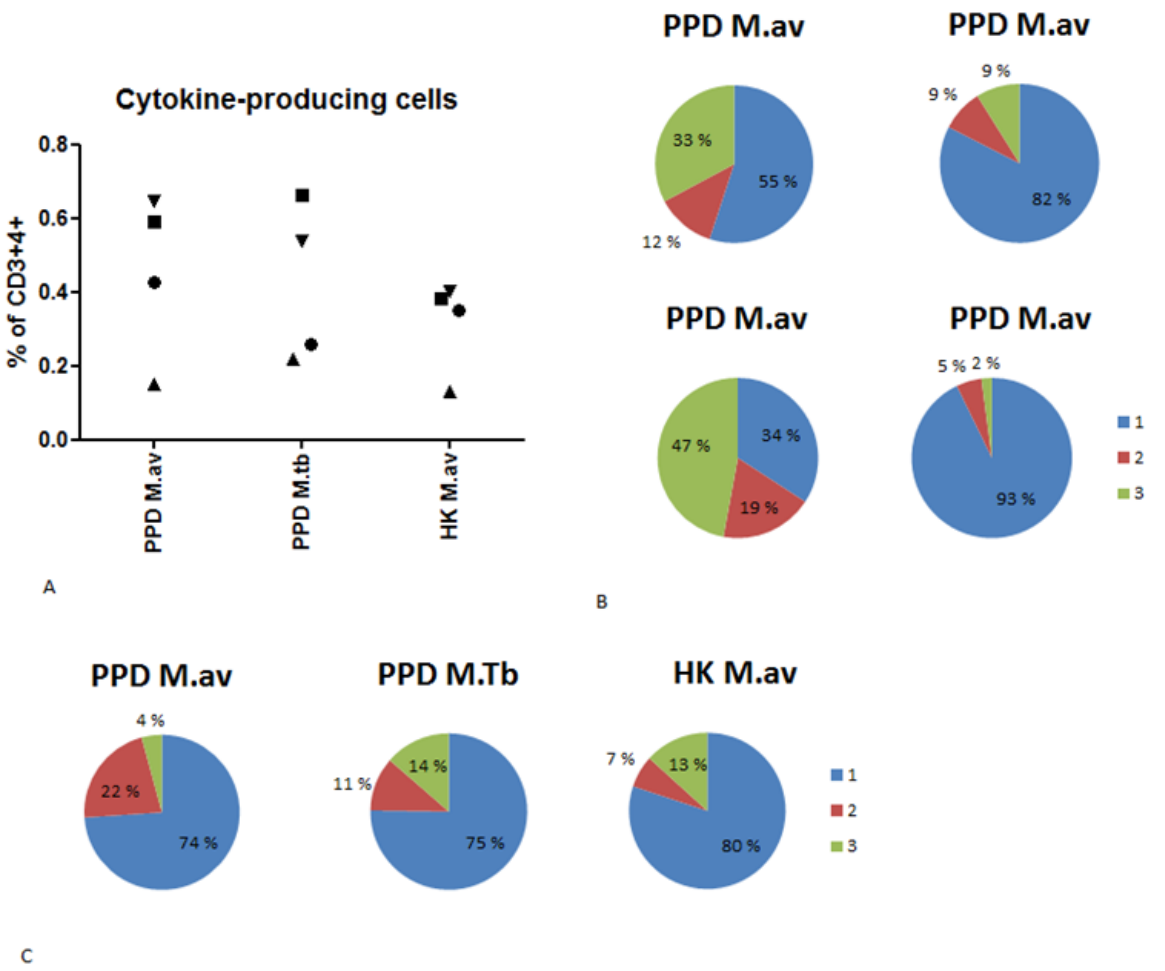
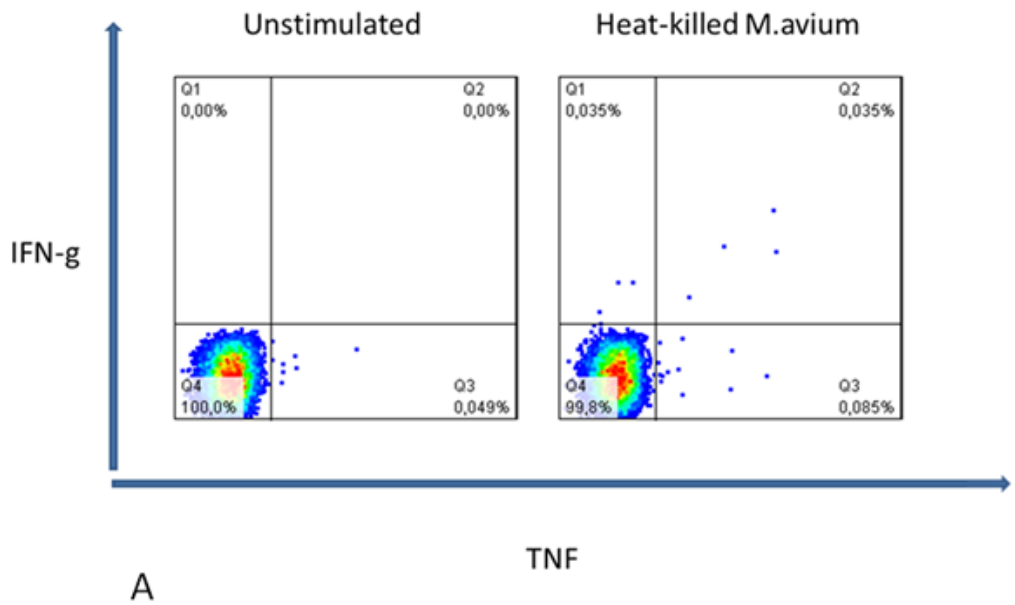


Figure 4.7: CD4+ T cell cytokine production from healthy individuals in response to antigens from *M.avium* and *M.tuberculosis*. PBMCs are stimulated overnight with three different stimulating agents originating from mycobacteria. Cells are stained with CD3, CD4 and CD8 on the surface, and a live/dead staining procedure based on dye exclusion is included. After fixation and permeabilization, cells were intracellularly stained for three effector cytokines (TNF, IFN-γ and IL-2) in three different channels (eFluor450, PE and APC, respectively). Cells were gated for CD3+CD4+CD8- T cells and cytokine production analyzed. T cell response of four different healthy and BCG-vaccinated donors was analyzed. A. Percentage of total mycobacteria-specific T cells producing one, two or three cytokines in response to PPD from *M.avium* and *M.tuberculosis* as well as heat-killed *M.avium*. B. The total effector cytokine response of the four healthy donors towards *M.avium* PPD from figure A was split into cells producing one, two or three cytokines to analyze polyfunctionality. Blue zones represent single producers, red zones double producers and green zones triple producers. C. Cytokine response results of all four donors from A were combined and polyfunctionality analyzed. The average of single, double and triple cytokine production from the four donors towards *M.avium* PPD, *M.tuberculosis* PPD and heat-killed *M.avium* is shown.

4.2.1.2 Mycobacteria-specific CD4+ T cell effector cytokine production in a non-BCG vaccinated healthy donor

One of the healthy donors recruited to the study was not BCG vaccinated. Following the exact same experiment set-up as described for the rest of the healthy donors, her PBMCs were analyzed, and results can be seen in figure 4.8. The number of specific T cells were low (ranging from 0.15 to 0.22 for the three different stimulating agents), quite below the mean for the four BCG vaccinated donors. However, there was a clear population of mycobacteria-specific CD4+ T cells found in response to stimulation with all three mycobacterial antigens that was not found for the unstimulated control (example shown in figure 4.8 A for heat-killed *M.avium*). The polyfunctionality resembles that of the other donors, although the proportion of single producers is a bit higher, ranging from 74-80 % against the three antigens (figure 4.8 B).



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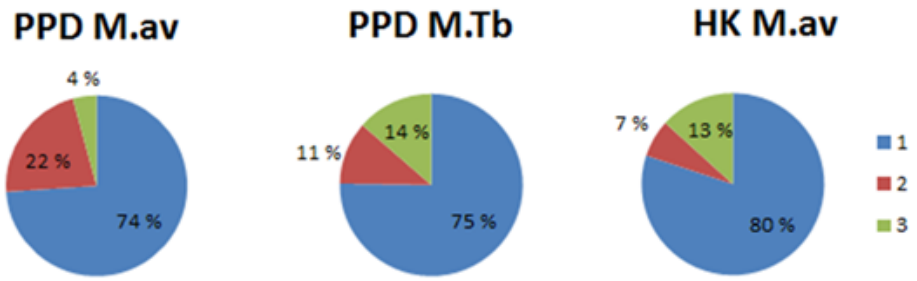
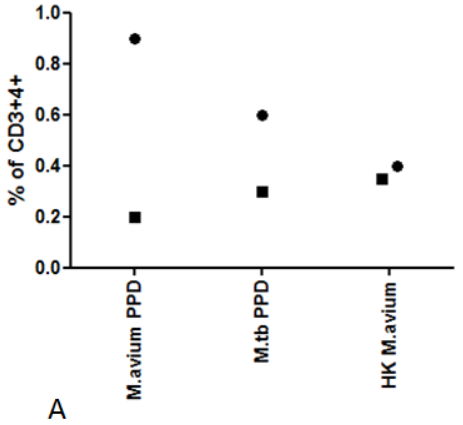


B

Figure 4.8: CD4+ T cell cytokine production from a non-BCG vaccinated healthy donor in response to antigens from *M.avium* and *M.tuberculosis*. A. Response from donor, unstimulated (left) and towards heat-killed *M.avium* (right), represented by production of IFN-γ (y axis) and TNF (x axis). B. Polyfunctionality of the non-BCG vaccinated donor in response to PPD from *M.avium* and *M.tuberculosis* as well as heat-killed *M.avium* The average of single, double and triple cytokine production from the four donors towards *M.avium* PPD, *M.tuberculosis* PPD and heat-killed *M.avium* is shown. Blue zones represent single producers, red zones double producers and green zones triple producers.

4.2.1.3 Mycobacteria-specific CD4+ T cell effector cytokine production in HIV patients

Cytokine production in HIV patients was also analyzed. However, obtaining satisfying results was a challenge. First and foremost, patient sampling and approval of the study by the regional ethics committee delayed the process. It was therefore possible to recruit only seven HIV patients to the study. Some samples were rejected due to very low CD4 count, making the subsequent analysis of cytokine production in mycobacteria-specific CD4+ T cells almost impossible. For other samples, there was unfortunately a problem of no cytokine production above background signal. This could be due to for example a problem with one of the cell culture reagents such as the medium or serum, as the cells otherwise looked healthy and showed no abnormal results from analysis of surface markers (4.2.2). Therefore it could also be possible that these HIV patients were non-responders without detectable numbers of mycobacteria-specific CD4+ T cells. It could in this study not be distinguished if the patients did not respond or if the negative results were due to a problem in cell culture. But as aliquots of PBMCs from patient samples were stored in liquid nitrogen, the analysis can be repeated as analysis from cells cryopreserved in liquid nitrogen was found to result in comparable results as from fresh cells (4.1.1). The re-analysis with cryopreserved cells was not performed by the end of this study, but it is recommended that this is done in the near future. After excluding all samples with very low CD4 count and doubtful experiments, samples from two donors could be analyzed for mycobacteria-specific CD4+ T cell frequency and polyfunctionality, and the results can be seen in figure 4.9. One of the donors show a moderate mean response towards the antigens, the other show relatively little response (figure 4.9 A). It is obvious from the results that a great majority of mycobacteria-specific cells are single producers (figure 4.9 B). Triple producers are almost absent among these two HIV patients. IFN-γ is the most predominant cytokine among the single producers, with TNF second.



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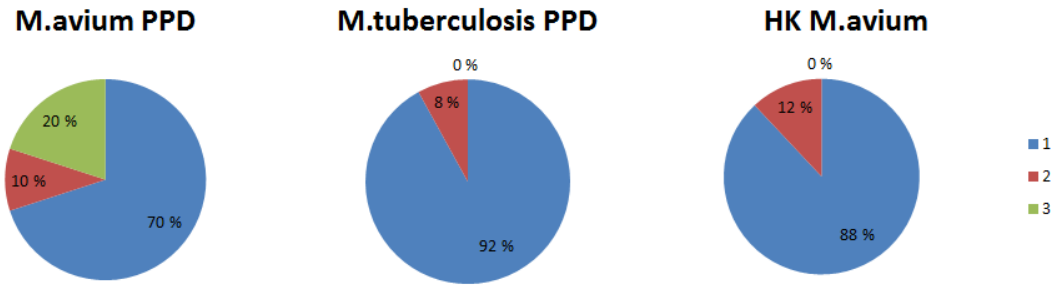


B

Figure 4.9 Polyfunctionality of HIV patients in response to PPD from *M.avium* and *M.tuberculosis* as well as heat-killed *M.avium* A. Frequencies of mycobacteria-specific CD4+ T cells towards the different antigens in the two HIV patients, given in percentage of CD3+CD4+ T cells. B. The average of single, double and triple cytokine production from the four donors towards *M.avium* PPD, *M.tuberculosis* PPD and heat-killed *M.avium* is shown. Blue zones represent single producers, red zones double producers and green zones triple producers.

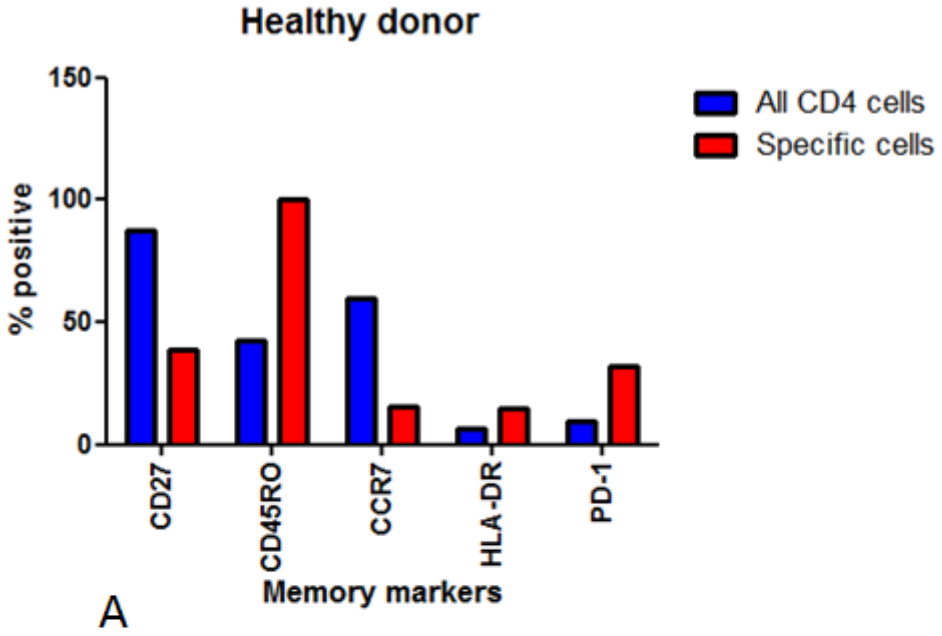
4.2.2 CD4+ T cell memory status

T cell memory phenotype reveals information about the T cell population status. Memory cells are long-lived and small populations that are able to exert a rapid response towards repeated infections with the same infectious agents (secondary response). The memory phenotype is hard to characterize, as no set of markers definitely defines them. They change continuously and lose and gain expression of a wide range of markers throughout their life span (see section 1.4.4). Additionally, not much is known about the T cell differentiation in HIV patients compared to healthy individuals.

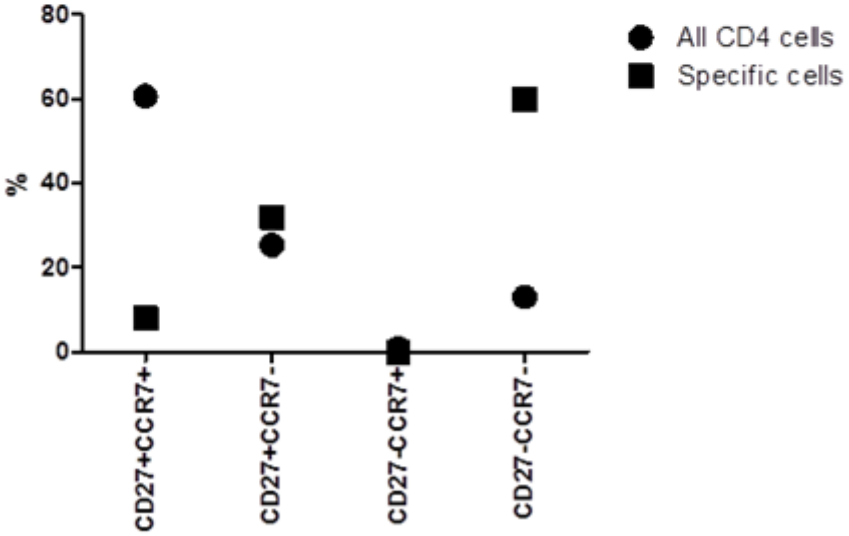
This project aimed at revealing information about T cell memory phenotype in healthy individuals and to compare to that of HIV patients. Seven surface memory markers (CD27, CD28, CD45RO, CD57, HLA-DR, PD-1 and CCR7) in addition to T cell identification markers (CD3, CD4, CD8) and effector cytokine staining (where all three effector cytokines IFN-γ, TNF and IL-2 were stained using the same fluorochrome) were analyzed using flow cytometry (see also 4.1.6).

4.2.2.1 CD4+ T cell memory phenotype in healthy individuals

The first experiment analyzed memory phenotype in healthy individuals. PBMCs were stimulated overnight and stained for identification markers and memory markers extracellularly, as well as cytokine production (all three cytokines were stained in the PE channel). All CD4+ T cells were analyzed, as well as specific cells only (cells producing any of the three cytokines in response to stimuli). Results are presented in figure 4.10. Expression of CD27 and CCR7 decrease in the specific cells compared to the whole CD4+ T cell population, whereas expression of CD45RO, HLA-DR and PD-1 increase (figure 4.10 A). There was no significant difference in expression between the different stimulating agents (data not shown). Two parameters (CD27 and CCR7) were analyzed in greater detail. These markers have been used to describe different T cell memory populations (58)(119)(85)(138). Using Boolean gating, their simultaneous expression was elucidated in all CD4 cells and specific cells (Figure 4.10 B). It can be seen from the figure that the majority of all CD4 cells are CD27+CCR7+, but that CD27-CCR7- cells dominate among the specific population. CD27-CCR7+ phenotype is absent from both populations. In addition to analysis of the CD4+ T cell memory phenotype, the CD8+ T cell population was also investigated (figure 4.10 C). This population shows the same trends as the CD4+ T cell population when first considering the CD8+ T cell population as a whole, before looking only at the mycobacteria-specific CD8+ T cells, but there are differences in expression of some of the markers between the CD4+ and CD8+ T cell populations. Results in 4.10 are from one representative donor.


Healthy donor
150
100
50
0
% positive
All CD4 cells
Specific cells
CD27
CD45RO
CCR7
HLA-DR
PD-1
Memory markers

A


80
60
40
20
0
%
All CD4 cells
Specific cells
CD27+CCR7+
CD27+CCR7-
CD27-CCR7+
CD27-CCR7-

B

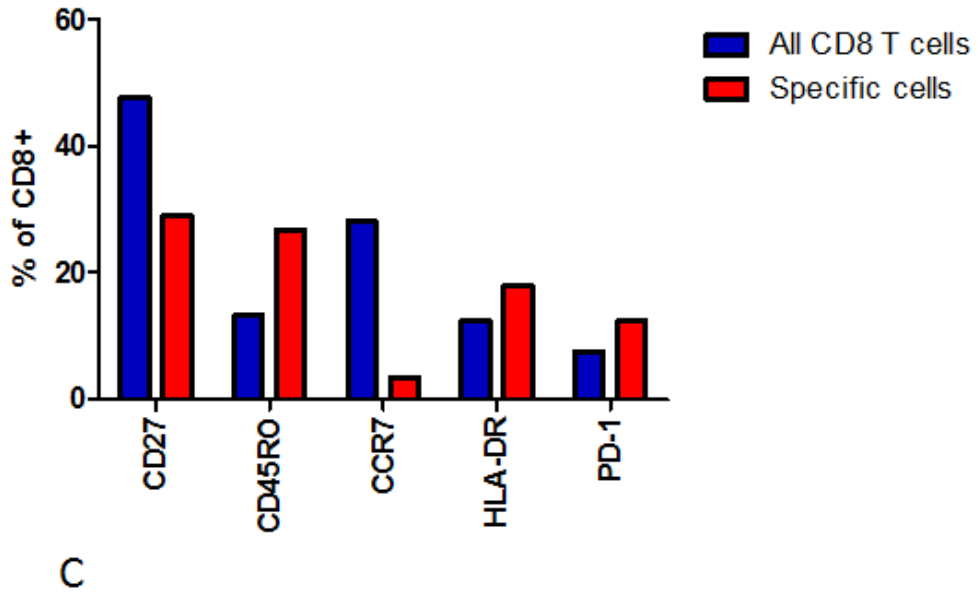
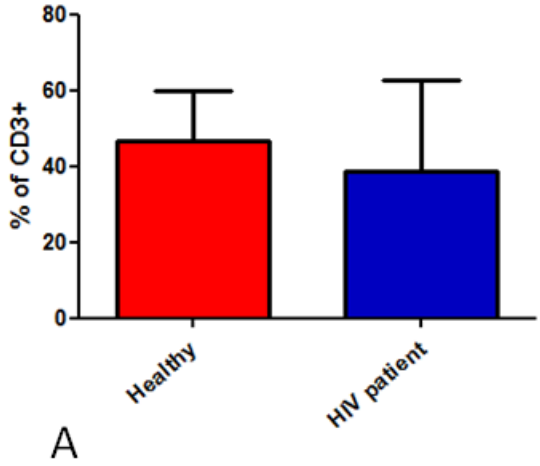


Figure 4.10: CD4+ T cell memory differentiation in one healthy donor. A. Shift in CD4+ T cell memory phenotype from the entire CD4+ T cell population to mycobacteria-specific CD4+ T cells only. The x axis shows five different memory markers, and the y axis shows their expression in the two different CD4+ T cell populations. B. Diagram showing the simultaneous expression of memory markers CD27 and CCR7 in all CD4+ T cells and mycobacteria-specific CD4+ T cells only. Circles represent all CD4+ T cells, whereas squares represent mycobacteria-specific CD4+ T cells. C. Shift in CD8+ T cell memory phenotype from the entire CD8+ T cell population to mycobacteria-specific CD8+ T cells only.

4.2.2.2 CD4+ T cell memory phenotype in HIV patients

To investigate differences in memory phenotype among healthy controls and HIV patients, PBMCs from HIV patients were characterized as described for healthy donors (4.2.2.1). PBMCs from HIV positive patients were stimulated overnight and stained for identification markers and memory markers extracellularly, as well as cytokine production. Due to a low number of HIV patients showing reliable results for cytokine production, and the low total number of specific CD4+ T cells among them, the memory characterization among the specific cells is difficult. Therefore, it was first looked at the CD4+ T cell population as a whole, and figure 4.11 shows the difference between healthy individuals and HIV patients when all CD4+ T cells are considered. A representation of the difference in CD4+ expression among the healthy individuals and HIV patients are included in figure 4.11 A. Healthy individuals showed a greater expression of CD4+ T cells among the CD3+ population, but the

differences are not that big. For markers CD27, CD45RO and CCR7, there is not much difference between healthy controls and HIV patients. However, HLA-DR and PD-1 seem to have an increased expression in HIV patients as compared to healthy individuals (figure 4.11 B). Results are based on analysis of three healthy individuals and five HIV patients.



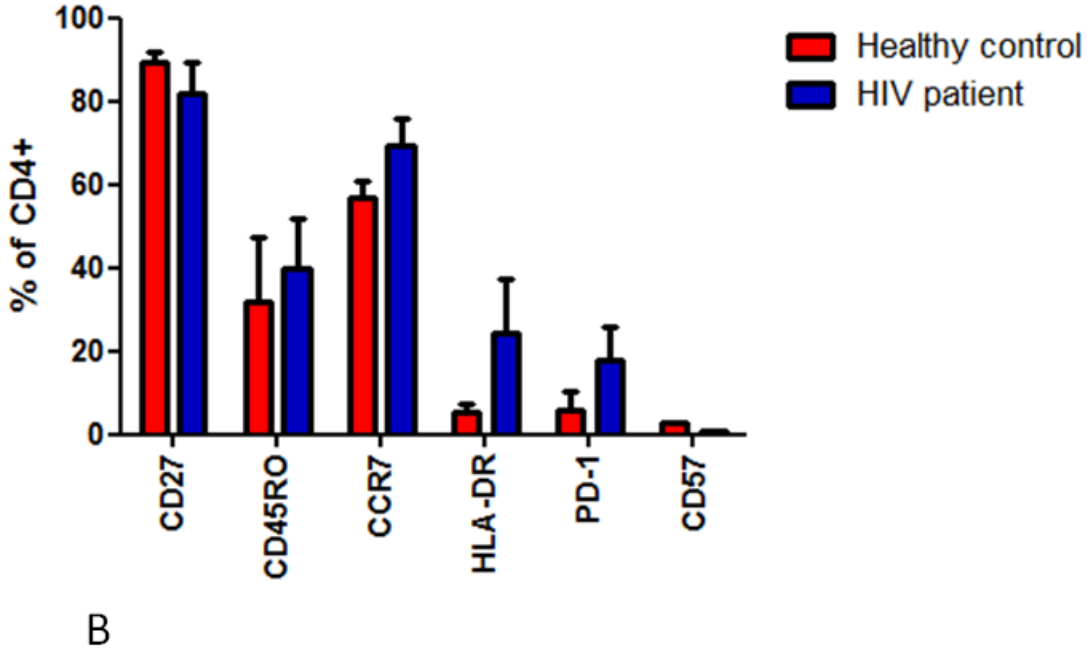


Figure 4.11: CD4+ T cell memory differentiation in healthy individuals compared to HIV patients when all CD4+ T cells are considered. A. Expression of CD4+ T cells in the CD3+ population of healthy individuals and HIV patients. Results plotted together with bars indicating the standard deviation (SD). B. Differences in the expression of five memory markers (x axis) in the entire CD4+ T cell population among healthy individuals and HIV patients. Results plotted together with bars indicating the standard deviation (SD).

Results from the analysis of all CD4+ T cells compared to specific cells only (cells producing any of the three cytokines in response to stimuli) are presented in figure 4.12. It was earlier stated that the analysis of cytokine production in some of the HIV patients was difficult (see 4.2.1.3). This also proposes a problem when analyzing memory differentiation in mycobacteria-specific CD4 T cells. Distinguishing between cytokine-producing negative and positive populations is sometimes ambiguous despite the use of appropriate controls. This should be kept in mind when studying the results. Results in figure 4.12 are from two HIV patients.

For many of the markers, if not all, there is no great increase or increase from all CD4+ T cells to specific cells only (figure 4.12 A). The trend for all of the markers, however, follows that of the healthy controls, except HLA-DR, being more expressed in the whole CD4+ T cell population than the specific population for the HIV patients.

When analyzing the joint expression of CD27 and CCR7 (figure 4.12 B), the trend for the whole CD4+ T cell population resembles the results from the healthy controls. For the specific cells, deviations can be seen. The majority (almost 50 %) of the cytokine-producing cells express both CD27 and CCR7 memory markers. Few (around 15 %) are double negative for these markers. For the HIV patient samples, there seem to exist a CD27-CCR7+ population.

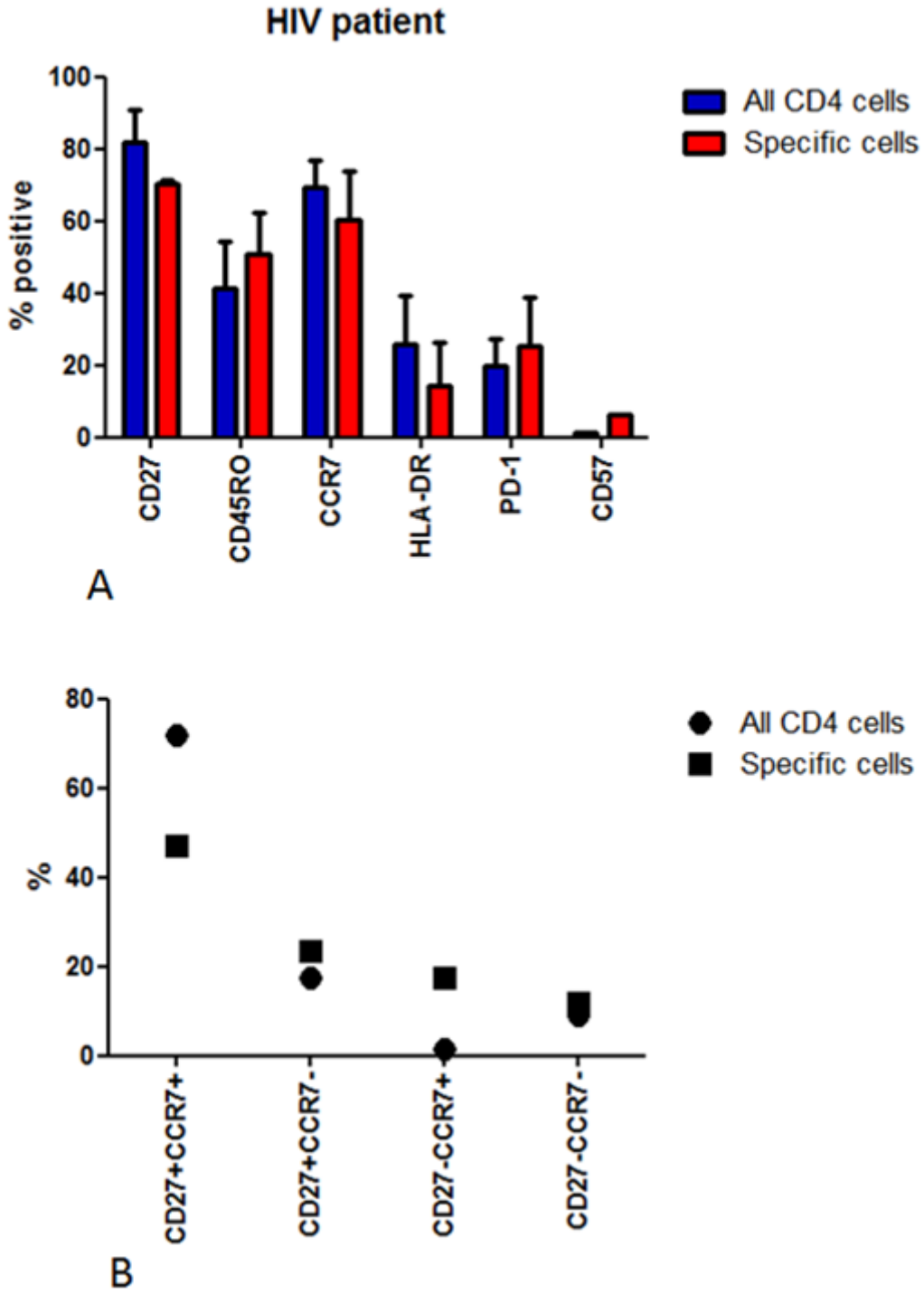


Figure 4.12: CD4+ T cell memory differentiation in HIV patients. A. Shift in CD4+ T cell memory phenotype from the entire CD4+ T cell population to mycobacteria-specific CD4+ T cells only. The x axis shows five different memory markers, and the y axis shows their expression in the two different CD4+ T cell populations. Results plotted together with bars indicating the standard deviation (SD). B. Diagram showing the simultaneous expression of memory markers CD27 and CCR7 in all CD4+ T cells and mycobacteria-specific CD4+ T cells only. Circles represent all CD4+ T cells, whereas squares represent mycobacteria-specific CD4+ T cells.

4.2.3 Expansion of mycobacteria-specific CD4+ T cells and co-culture with MDMs for effector cytokine production

When T cell population is expanded, specific cells proliferate, and this will increase the proportion of mycobacteria-specific T cells in culture compared to staining after just 16 hours of stimulation. As the proportion of specific cells after overnight stimulation is low, usually below 1 %, T cell expansion can make the cytokine-producing population easier to detect and investigate. An experiment was conducted to examine what happens if HIV patient samples are expanded, as that had not earlier been done in the research group. PBMCs were stimulated using heat-killed *M.avium* to activate mycobacteria-specific T cells. Simultaneously, autologous monocyte-derived macrophages (MDMs) were cultured. T cells and stimulated MDMs were co-cultured after ~ 10 days and for ~ 16 hours (refer section 3.4). It should be noted that the expanding population of T cells were handled in parallel with samples that did not seem to produce any cytokines above background when running flow cytometry after just 16 hours of stimulation (refer section 4.2.1.3). There is reason to believe results would have turned out differently if cytokine production was evident after overnight stimulation. Expansion significantly increased the proportion of mycobacteria-specific CD4+ T cells, but due to the aforementioned problems, the proportion of specific cells was much lower than would be expected from such an experiment. Still, results are promising for the future, and it is hope this can be successfully repeated with other HIV patients in the future, as cells otherwise looked healthy after 10 days in culture. Figure 4.13 shows a representative figure from another experiment of expanding mycobacteria-specific CD4+ T cells. The proportion of specific cells increased ten-fold after expansion.

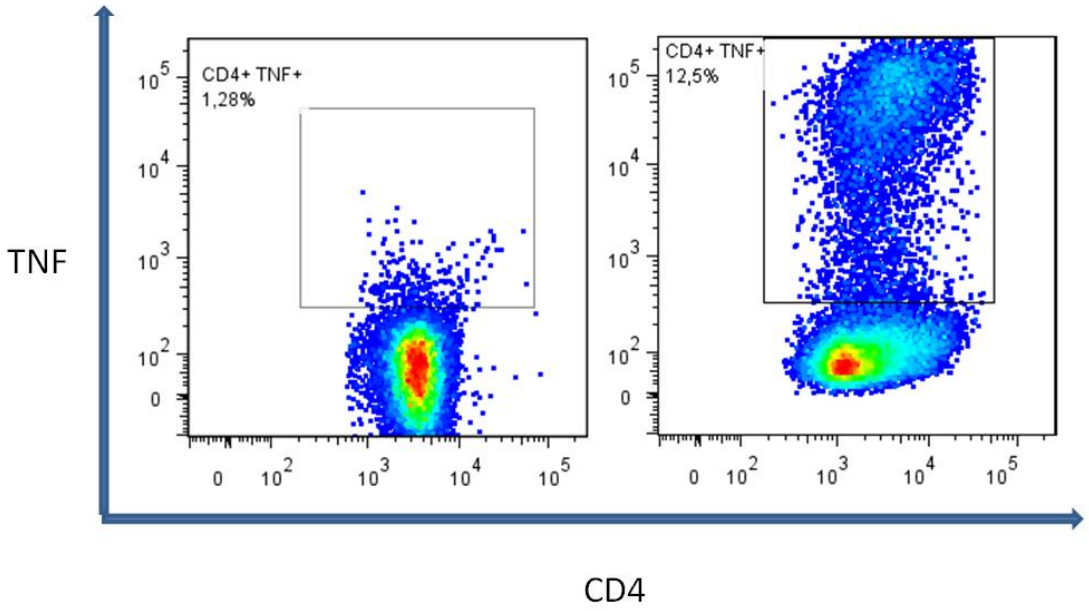


Figure 4.13: Expansion of mycobacteria-specific CD4+ T cells. Effector cytokine production is represented by percentage of CD4+ T cells producing TNF (y axis). Left: PBMCs were stimulated overnight using heat-killed *M.avium*, and then stained extracellularly for identification markers and intracellular cytokine production. Right: PBMCs were cultured for 10 days in the presence of heat-killed *M.avium*. On day 10 they were co-cultured with autologous MDMs that were later stimulated with heat-killed *M.avium*. After co-culturing overnight, cells were stained extracellularly for identification markers and intracellular cytokine production. Expansion and analysis of expanded cells were performed by Marit Bakke.

4.3 Mycobacteria-specific effector cytokine analysis by qPCR

For the purpose to confirm cytokine production findings from flow-cytometry and to extend cytokine analysis from T cells only to all PBMCs in the stimulated wells some of the donor samples were stimulated for quantitative PCR (qPCR) and ELISA (4.4) analysis as further methods for cytokine analysis. qPCR allows analysis of the intracellular cytokine mRNA levels from lysed cells after stimulation and thus the total cytokine production from all cells in the well can be quantified. There is, however, a major disadvantage. As all cells in the well are lysed, it is not possible to distinguish which cell type produced the mRNA detected. In addition to T cells, APCs are present in the lysate and may produce cytokine mRNA.

PBMCs were stimulated overnight (3.2.3), and as no protein transport inhibitor was added, produced effector cytokines were secreted into the supernatant. After 16 hours of stimulation, the supernatant with the produced cytokines was removed (and used for cytokine detection with ELISA as a third method to analyze mycobacteria-specific cytokine production, see 4.4),

and the cell suspension was lysed, and mRNA prepared (see section 3.5). Probes used targeted three cytokines; IL-1β, TNF and IFN- β.

Results from a healthy donor are presented in figure 4.14. For probes for IFN- β, only heat-killed *M.avium* yielded a significant increase in RQ value, but for TNF and IL-1β, there was an increase in RQ value for all three mycobacterial antigens above the unstimulated calibrator. RQ values are largest for the cytokine IL-1β. Heat-killed *M.avium* is the stimulating mycobacterial agent among the three that yields the best results (i.e. highest RQ value) for all three probe pairs.

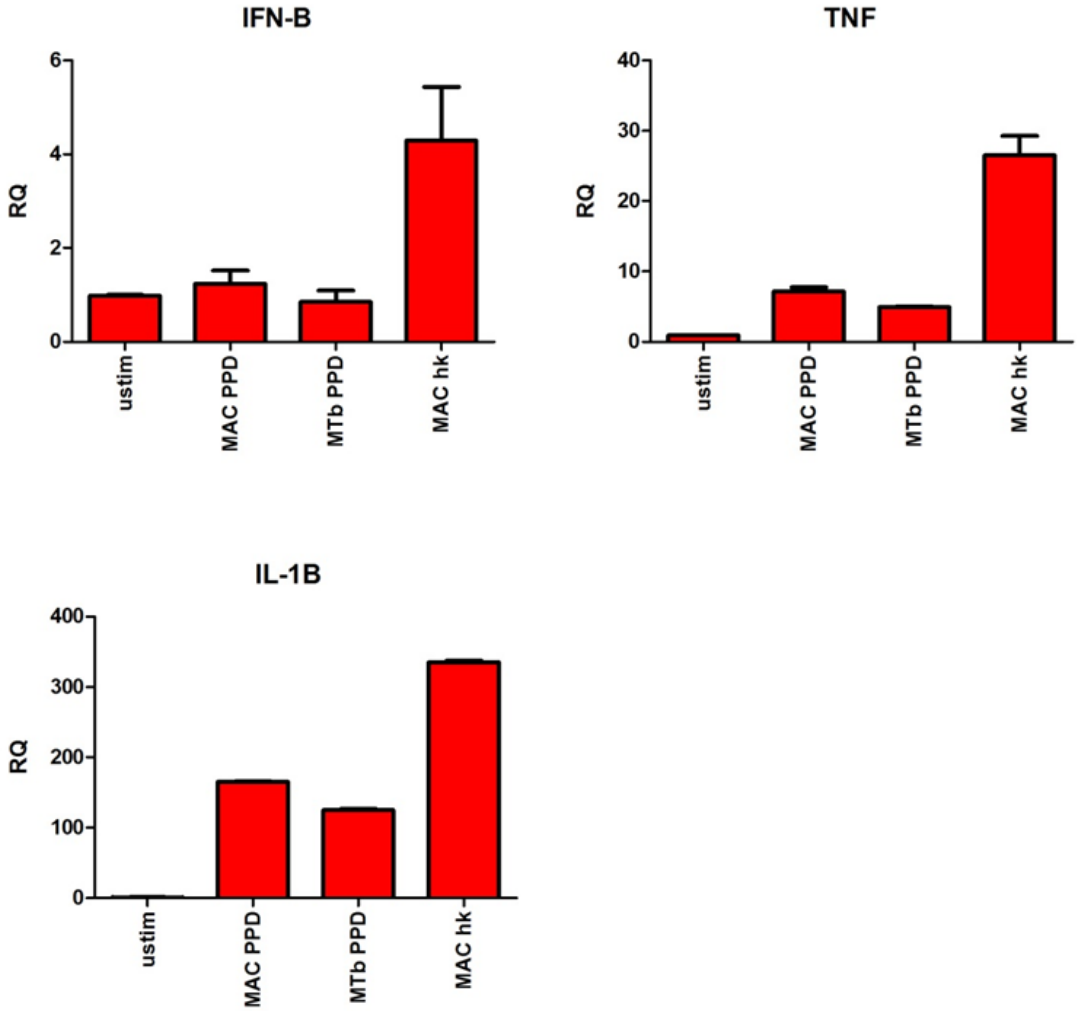


Figure 4.14: Cytokine production in a healthy donor in response to *M.avium* PPD, *M.tuberculosis* PPD as well as heat-killed *M.avium* represented by analysis using quantitative PCR and probes for the three proinflammatory cytokines IFN-β, TNF and IL-1β. Cytokine production is indicated by the RQ (relative quantification) value and unstimulated control is used as a calibrator, and given the RQ value of 1. As endogenous control, the housekeeping gene GAPDH was used.

The same procedure was performed on one of the HIV patients to see if cytokine mRNA levels deviated from the healthy donor. Unfortunately, it was found that this sample produced no cytokines above background during flow cytometric analysis after overnight stimulation with mycobacterial antigens (*M.avium* PPD, *M.tuberculosis* PPD and heat-killed *M.avium*), see 4.2.1.3. qPCR was performed nevertheless, and it was possible to obtain results using probes for the same cytokine mRNA as with the healthy donor; IFN-β, TNF and IL-1β. In figure 4.15, results are shown for one of the cytokines, IL-1β. The figure compares the results from the healthy control and the HIV patient. It is evident that also the HIV patient produced IL-1β mRNA (as RQ values are larger than the calibrator with value 1) but the RQ values are far from as high for the HIV patient as it was for the healthy control, ranging only from 20 to 30 for the HIV patient.

Lysate from additional HIV patients were stored in -80 °C, making the future analysis of mycobacteria-specific cytokine production by PBMCs using qPCR possible.

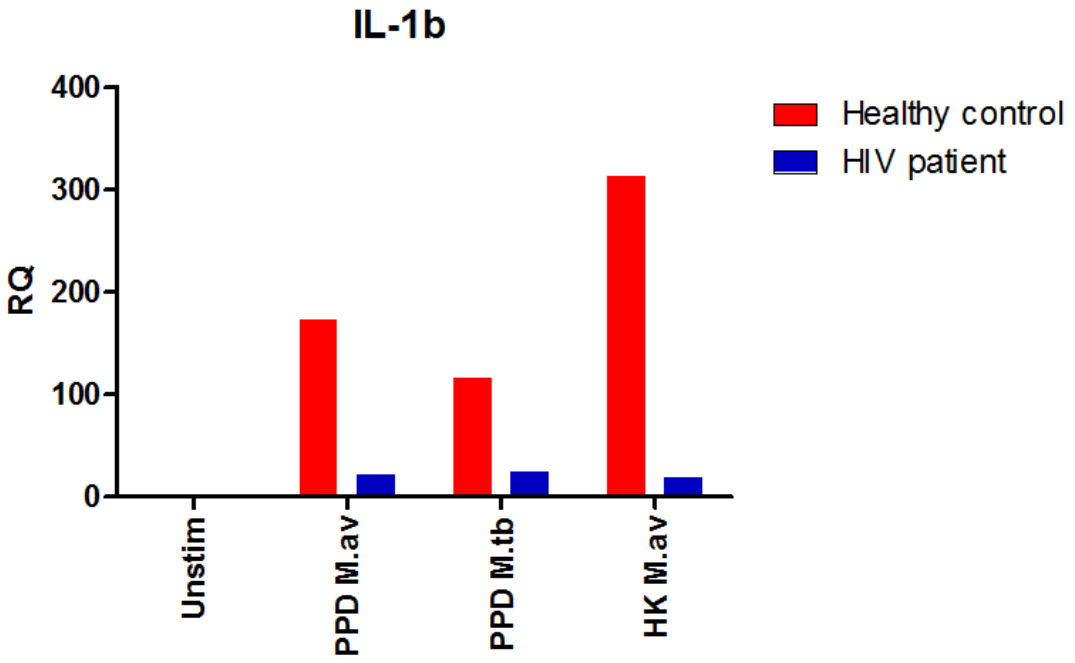


Figure 4.15 Cytokine production in a HIV patient compared to a healthy control in response to *M.avium* PPD, *M.tuberculosis* PPD as well as heat-killed *M.avium* represented by analysis using quantitative PCR and probes for the proinflammatory cytokine IL-1β. Cytokine production is indicated by the RQ (relative quantification) value and unstimulated control is used as a calibrator, and given the RQ value of 1. As endogenous control, the housekeeping gene GAPDH was used.

4.4 ELISA

The third method used to analyze mycobacteria-specific cytokine production, was ELISA (Enzyme-Linked ImmunoSorbent Assay). Supernatants from stimulation as described for qPCR sample acquisition (section 4.3) were analyzed for cytokine production. In this experiment, TNF was targeted, using the Human TNF-α DuoSet kit provided by R&D Systems (refer section 3.6). This method of cytokine production analysis can be said to show the same disadvantage as for the qPCR; the lack of ability to distinguish from which cell type the cytokines were produced.

ELISA was performed using only one healthy donor. Supernatants from stimulated HIV patient samples were stored in 80 °C for future analysis.

Results from the healthy donor is presented in figure 4.16. The results show the presence of TNF in the culture supernatants as healthy PBMCs are stimulated overnight using the mycobacterial antigens *M.avium* PPD and heat-killed *M.avium*.

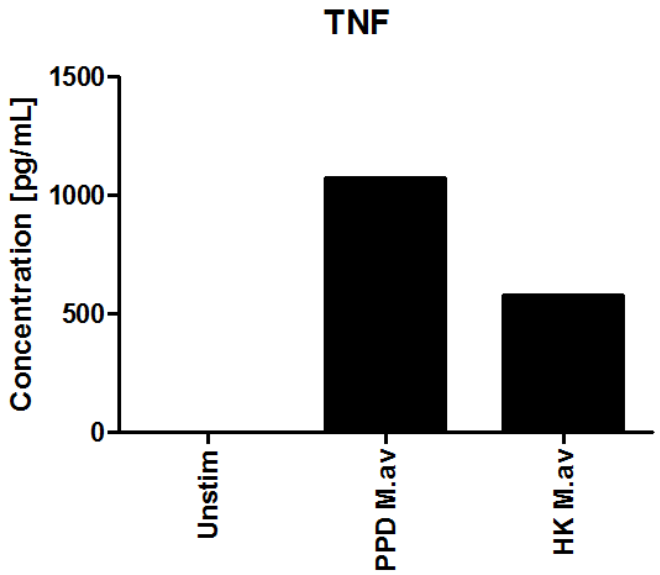


Figure 4.16: Total mycobacteria-specific production of TNF in supernatants from healthy PBMCs stimulated overnight using *M.avium* PPD and heat-killed *M.avium*. Amount of TNF is represented by the concentration in pg/mL.

5 Discussion

An estimated number of 1.4 million people will die from tuberculosis every year (1), and the problem of co-infection with HIV presents a huge threat in Sub-Saharan areas. BCG, the only vaccine available for protection against tuberculosis, has lately been declared ineffective against adult, pulmonary tuberculosis (172).

Immune functions against mycobacteria seem to be impaired in HIV patients even after onset of antiretroviral therapy (130) and at relatively normal CD4+ T cell counts (173). The reason for this increased susceptibility of HIV patients to mycobacterial infections is still unclear and thus this project aimed at investigating the immune functions towards *M.tuberculosis* and *M.avium* in humans infected with HIV and compare to healthy controls. Decreased polyfunctionality of CD4 T cells (95)(106)(133)-(139) and skewed T cell differentiation (106)(112)(133)(141)-(147) are believed to contribute to the accelerated deterioration of immunological functions, and these were central topics for the work. A better understanding of the immunological processes involved is also crucial for drug development.

CD4 T cells are depleted in HIV patients during infection. At the same time, CD4+ Th1 cells are potent producers of IFN-γ, a key cytokine in controlling tuberculosis infection. Their absence may therefore in part explain why infection with *M.tuberculosis* causes such a serious threat for HIV patients, and the main focus of this study was directed towards this immune cell population. The study of CD4 T cells is commonly done by using technologies of flow cytometry, and a main part of this work was the establishment of a multicolor flow cytometric assay for simultaneous study of CD4 T cell polyfunctionality and memory differentiation. Both healthy controls and HIV patients were enrolled in the study by the donation of heparinized blood.

The effect of cryopreservation on T cell effector function was examined by the storage of healthy PBMCs at sub-zero temperatures for up to three months and the subsequent analysis of viability and cytokine production. Two methods of storage were used; -80 °C and liquid nitrogen. Viability was found not to be much affected by cryopreservation, although there were signs of a small decrease in viability post stimulation for cells kept in -80 °C. The

viability seems therefore not to be compromised during cryopreservation, making the analysis of surface markers possible even after cryopreservation of PBMCs in regular freeze containers holding -80 °C. Cytokine production, however, seems to be best analyzed from fresh cells, as frozen cells were not able to completely retain their effector function after thawing. Cryopreservation pauses all intracellular activity, and cells seem not to fully recover after this, proposing the cryopreservation is not able to pause all activity, or exposes cells to irreversible stress. Storage at low temperatures (here: liquid nitrogen) is better for cells, as a larger proportion of cells stored in liquid nitrogen are able to produce cytokines than cells stored in -80 °C. The results from these experiments led us to the recommendation that analysis of CD4+ T cell effector cytokines seems to be best performed from fresh cells. Cells stored for up to three months in liquid nitrogen can be used if absolutely necessary and yields results that are comparable to fresh cells, but storage in -80 °C is not sufficient for analysis of cytokine production. However, both storage methods are recommended for surface marker identification, as viability is retained after thawing by careful consideration of freezing and thawing protocols. It is not inevitable to think that customizing of protocols may affect the effector functions after thawing. For example it could be necessary to distribute protein transport inhibitor differently to cells that have been thawed than fresh cells, if frozen cells takes longer to recover and cytokine production therefore is slower. There are examples of other studies having used cryopreserved PBMCs for intracellular staining of effector cytokines as well (98)(174), reporting high viability and effector function. Cryopreservation of cells may be necessary if sampled for example from developing countries lacking technologies for analysis, and seems to be a widely accepted method for the purpose of investigating cytokine production. The advantages of being able to analyze several samples simultaneously, and including relevant controls, with the same settings, reduce variability between experiments. This may even overweight the disadvantage of reduced total cytokine production.

Distribution of protein transport inhibitor to the cells needs to be adjusted to the aim of the experiment and the protocol should remain unchanged within different studies. Altering the time point of adding protein transport inhibitor alters the resulting cytokine profile of the cells. After stimulation and activation of T cells, not all responsive genes are transcribed simultaneously (83). Genes transcribed are categorized as immediate, early or late, depending on the cell's needs. Cytokines are mainly transcribed in the early phase, but there are also individual differences. TNF was found to be the earliest detectable cytokine in supernatant

from stimulated PBMCs, but it decreased rapidly and to an almost undetectable level when IFN-γ peaks (unpublished results from ELISA assays in our research group). When adding the protein transport inhibitor after 16 hours and for four hours (as was chosen as the routine protocol for this project), there is a risk that the production of some cytokines might be missed, if they peak early after stimulation and decrease rapidly. Tests comparing addition of inhibitor the next day for just four hours with addition of the inhibitor already one hour post stimulation and kept with the cells overnight showed a loss of TNF production when inhibitor is added the next day, but an increase in production of IFN-γ. IL-2 remains unchanged for the two different methods. When adding the protein transport inhibitor already one hour after stimulation and for the whole 16 hours, it would be expected that all cytokines produced in that time period accumulates in the cell and are present in the result. However, this seems not to be the case, although we found in some samples huge fluorescence values for TNF when secretion inhibitor was added early.

The protein transport inhibitor mix contains brefeldin A, a substance that blocks the transport of secretory proteins from ER (175), leading to the accumulation of cytokines in the ER compartment. The other substance included in the mix, monensin, also have a role in inhibiting protein transport. We propose that these compounds exert a toxic damage to the cells, interfering with their normal function, and that the mix should be distributed to the cells for as short time as possible, while still achieving sufficient amounts of cytokines. The time frame for how long cytokine secretion from specific T cells should be inhibited, needs to be individually tailored for each study, and keeping to the protocol is an absolute necessity throughout a study to minimize variability between experiments and allowing comparison of data acquired from different experiments. For our study, we decided to use overnight stimulation and then add protein transport inhibitor for another four hours. This resembles other studies also leaving the protein transport inhibitor with cells for around five hours (133), however, initial stimulation with antigens is often shorter than 16 hours, for example seven hours. As our study was based on fresh patient samples requiring PBMC isolation, this would be impracticable, and overnight stimulation yielded adequate results. There are, however, also examples of incubation with protein transport inhibitor for 16 hours (95)(174).

For choice of cell number per well and fluorochrome amount, compromises must often be made. In order to be able to test as many different conditions as possible from a limited volume of donor sample, it is desirable to minimize the number of cells used for flow

cytometric assays, but still obtain adequate results. Sampling of at least 50 000 cells is required for analysis of cytokine production, due to a limited proportion of mycobacteria-specific T cells that was usually found to be less than 1 % of analyzed cells. For HIV samples, this number would preferably be increased to 100 000. Repeated washing steps during staining of cells also leads to loss of cells. It was found from the experiments in this study that from stimulation of half a million PBMCs in one well of a 96-well plate it was, after harvesting, washing and staining procedures, possible to acquire approximately 100 000 events in the flow cytometer. As there was no significant increase in signal from half a million to one million cells per well, half a million cells was found to be an adequate number for PBMC stimulation and usually 50 000 cells analyzed in the flow cytometer. As we usually received approximately 20 mL of blood, resulting in 15-30 • 10^6 isolated PBMCs, we could thus stimulate 10 samples (5 million cells necessary) plus some controls as well as freeze down two tubes (4-7.5 • 10^6 cells each) with donor PBMCs for repeat analysis.

To determine the concentration of fluorochromes to add for each sample, manufacturer's provided guidelines is often a good starting point. For example BioLegend adjust their concentrations to a 5 µl «test size», and «The test size products are pre-titrated for optimal staining of 1 million cells in 100 µl volume»(176). Ideally, amounts should be tested («antibody titer») for each individual study. It was desirable to see how much this recommended amount could be lowered without the subsequent loss of signal, and different concentrations of dye for intracellular cytokine staining was tested. It became apparent that volume should not be decreased too much, as that would lead to a false low signal, as minimal amounts of fluorescent dye fail to stain all cells. A concentration was chosen that produced sufficient results for out purpose, however, this is below that recommended by the manufacturer, and needs perhaps be increased. However, this needs to be validated using proper negative controls and deciding on a proper signal-to-noise ratio.

Storage of flow tubes was found possible for at least up to five day without any obvious change in fluorescence signal. Manufacturers claim even the unstable tandem dyes are stable for up to three days. Despite this, samples were always analyzed on the same day as the intracellular staining procedure.

The analysis of T cell effector functions and memory differentiation in healthy controls versus HIV patients took use of the optimized methods for isolation of PBMCs, stimulation and

extracellular and intracellular staining of surface markers and effector cytokines. Variability in anti-mycobacterial T cell response among healthy donors was found to be quite large and greatly influenced the results. This also makes generalization of effector cytokine response among healthy individuals complicated. This donor variation may originate from previous exposure to mycobacterial antigens, via vaccines or bacteria in the environment (177). The BCG vaccine was a part of the Norwegian Childhood Vaccine Program up until 2009, when it was taken off the list due to a low prevalence of disease and the believed inefficiency of the vaccine (178). The vaccine should provide long-term immunization by stimulating immune cells to differentiate into memory cells that will recognize mycobacterial antigens upon second encounter. We were able to retrieve one blood sample from a non-BCG vaccinated healthy donor, originating from a country where BCG-vaccination is not standard during childhood. It was found that this BCG naïve donor had a small, but existing, population of CD4+ T cells which produced effector cytokines in response to stimulation with mycobacterial antigen. The percentage of specific CD4+ T cells was below the mean for the rest of the healthy donors, indicating that the BCG vaccine does play a role in evoking the immune system and establishing a pool of memory cells. The degree of polyfunctionality for the non-vaccinated donor was found to be a bit below the mean for the rest of the healthy donors, and combined with the low overall frequency of specific cells, this means very few cells produced all three cytokines. Polyfunctionality of CD4+ T cell responses may be a prerequisite for protective immunity against tuberculosis (92)-(97). The variability between the rest of the healthy donors is not easy explainable, but probably points to a general feature of the population. This could perhaps be due to environmental exposure or individual differences in immune response.

A central step during tuberculosis infection is the activation of T cells recognizing mycobacterial antigens, resulting in production of CD4+ Th1 cytokines IFN-γ and TNF that are of great importance in elimination of intracellular pathogens. Mice defective in IFN-γ succumb rapidly to infection with MTB (81)(80). Genetic susceptibility studies in humans have further revealed that defects in for example IFN-γ signaling (179) increase the risk for disseminated nontuberculous mycobacteria (NTM) diseases (described in (180)). It also seems like there is some kind of kinetics involved in cytokine production from T cells, where TNF is secreted early after stimulation and IFN-γ a bit later. Highest amount of IFN-γ are detected when protein transport inhibitor is added after overnight stimulation and for another four

hours. Response towards *M.avium* resembles that of *M.tuberculosis*, due to great similarities between the two species, they share 75 % of the same proteins (181).

Response in our study towards mycobacterial antigens were characterized by a considerable production of IFN-γ after overnight stimulation. This trend was seen for both healthy donors and one of the HIV patients, making IFN-γ the predominant cytokine produced in single producers for most of the donors. No considerable difference in production of IFN-γ was found in the response towards *M.tuberculosis* and *M.avium*, both healthy individuals and HIV patients responded to antigens from both agents. This trend can be further investigated as more participants are enrolled in the study. Infection with *M.avium* is a complication among patients with severe AIDS disease, and it is possible that response towards *M.avium* differs from that towards *M.tuberculosis*, even though the bacteria share great similarities.

TNF is a proinflammatory cytokine produced by activated macrophages and T cells. The cytokine plays a role for granuloma development and containment of infection with *M.tuberculosis* and *M.avium* (182)(86)(183)(184), although the importance of TNF in mouse *M.avium* infections is not fully clarified (185)(186). Recently our group found surprisingly low levels low levels of TNF in *M.avium* -infected mouse tissue (Haug *et al*. 2013 submitted). For our study, it was found that TNF levels were usually present to a less degree than IFN-γ, but a significant proportion of the single producers secreted TNF. For one of the healthy donors and one HIV patient, TNF was the predominant cytokine produced in response to heat-killed *M.avium*. TNF seems to peak early after stimulation, and the chosen window for cytokine secretion may not be optimal for accumulation of TNF in mycobacteria-specific CD4+ T cells. For the one non-BCG vaccinated healthy donor, the production of TNF in single producers seemed to be somewhat higher compared to the production of IFN-γ. Significant amounts of TNF were also found in healthy controls during ELISA and PCR analysis. For PCR, the response was largest towards heat-killed *M.avium*, however, the response was largest towards *M.avium* PPD in ELISA. These methods are not cell specific, and may, in addition to production by T cells, also reflect the ability of for example macrophages to produce TNF in response to stimulation. TNF has received great interest in HIV patients, but its role and expression are debated. TNF regulation seems to be impaired in HIV patients (described in (187)).

As already mentioned, IFN-γ produced by T cells are important for macrophage activation, and TNF is important for granuloma maintenance in tuberculosis infection. There has recently also been focus on cells that are also capable of IL-2 production, as IL-2 is important for T cell proliferation and self-renewal. T cells simultaneously producing several of these cytokines are called polyfunctional T cells. Polyfunctionality, or the quality, of CD4+ T cells receives increased interest, and the simultaneous production of IFN-γ, TNF and IL-2 has been shown to correlate with long-term protective immunity against *M.tuberculosis* (97), as well as vaccine-induced protection against *L.major* (188). However, it was recently suggested that this polyfunctionality alone is not sufficient for long-term immunity (189).

As HIV patients are, even at relatively normal T cell counts, more susceptible to mycobacterial infections than healthy individuals, there might be differences in the polyfunctionality. Although we could in this study test only T cell responses towards mycobacterial antigens from a very low number of patients, cytokine production for HIV patients seems to be characterized by a low degree of polyfunctionality. The low number of study participants, and problems with low CD4 T cell count and/or low proportions of mycobacteria-specific T cells makes it difficult to draw definite conclusions, but from the preliminary results obtained, there seems to be a marked decrease in polyfunctionality of mycobacteria-specific CD4+ T cell responses i HIV patients. Most samples showed almost a total monofunctionality towards the different antigens (ranging from 70 to 92 % single producers and 0 to 20 % triple producers for the three different mycobacterial antigens). This in contrast to the healthy individuals, where polyfunctionality was always present to some degree (triple producers ranging from 4 to 14 % for the three different mycobacterial antigens). These results correspond to other studies (138)(133), where polyfunctionality was also found to be impaired during HIV infection.

A generally low total mycobacteria-specific CD4+ T cell response (in % of CD4+ T cells) was found among some HIV positive donors compared to the mean of the healthy donors. This correlates to other recent findings (133). However, as all recruited HIV patients followed therapy at the time of blood sampling, results are contrary to studies where anti-mycobacterial CD4 T cell frequencies were found to be restored by HAART (134)(135). Interestingly, the highest proportion of mycobacteria-specific T cells (in % of CD4+ T cells) was found to belong to the HIV patient with the lowest number of CD4 T cells. Similar results have been reported by others as well (137).

In summary, our preliminary results indicate that HIV patients might have an impaired polyfunctionality of their mycobacteria-specific T cell population. As a consequence, this correlation may impact their increased susceptibility towards tuberculosis, even though their CD4 T cell population is restored by treatment with HAART.

CD4+ T cells are depleted in HIV patients, but the population is restored during treatment with HAART. This may impact the status of the CD4+ memory T cell population, as memory T cells may be depleted and replaced with naïve T cells. As a consequence, HIV patients may experience a difference in their memory T cell composition as compared to healthy individuals. The memory population is very heterogeneous and hard to characterize, as a variety of surface markers are up- and downregulated during memory differentiation. A set of markers were chosen after literature studies, and their expression was studied in HIV patients and healthy controls. The chosen markers were, as follows: CD27, CD28, CD45RO, CD57, HLA-DR, PD-1 and CCR7. Identification markers CD3, CD4 and CD8, as well as cytokine production (IFN-γ, TNF and IL-2) were also included for the study of memory markers. Unfortunately, CD28 often yielded unreliable results, and were excluded from further analysis. One analysis was even performed on the CD8+ T cell memory population, and it is possible to look further into this population at a later stage.

In this study, memory differentiation among healthy donors was shown to shift between the whole CD4 population and the specific cells. CCR7 showed a decrease in expression in the specific cells, indicating that these cells are of a more differentiated effector memory phenotype, these cells are described as T_{EM} (58). Cytokine production has been found in other studies to increase with the loss of CCR7 expression (119)(102), which is in agreement with the results mentioned. CD27 should be expressed on all naïve T cells and most memory cells, but the expression has been described to be lost during memory differentiation (107). In our experiments, for the healthy donors, CD27 expression clearly decreased in activated and specific cells compared to naïve CD4 T cells. This means the specific cells have undergone further differentiation. CD27- cells have been associated with high effector functions in CD8 T cells (59), a phenomenon also described by other studies (119).

The splicing of CD45RA to form CD45RO upon antigen encountering is a well-known feature characterizing the majority of memory T cell subsets (103)(104). Among healthy

donors, all mycobacteria-specific CD4+ T cells were found to express CD45RO, whereas only 40 % of the total CD4+ T cells expressed CD45RO with the rest of the cells probably being naïve cells. Expression of CD45RO may, however, be lost at late stages of CD4 T cell differentiation. Expression of CD45RA is then regained, forming the so-called T_{EMRA} subpopulation of T_{EM} (59)(60).

Expression of PD-1 was in our characterization of healthy donors found to be higher in specific cells than in the CD4 population as a whole. This is consistent with findings of PD-1 being present on effector memory cells rather than central memory cells and naïve T cells (113). Expression of HLA-DR was found almost absent from the CD4 T cell population as a whole, but is, consistent with other findings (114) (115) present on a larger proportion of the activated or specific CD4 T cells in healthy donors.

While most naïve T cells are CD27+CCR7+, expression of CD27 as well as CCR7 is gradually lost as CD4 T cells mature from naïve into cytokine-producing memory cells. For the total CD4+ T cell population in healthy donors, we found the majority of cells to be of a CD27+CCR7+ phenotype, although a significant proportion was CD27+CCR7-, and some were CD27-CCR7-. Looking only at the mycobacteria-specific effector CD4+ T cells we found CD27 and CCR7 differentially expressed with CD27-CCR7- as the predominant. This is consistent with the description of an effector memory T cell phenotype where CD27 expression may be both present and absent, and the CD27- effector memory cell population are associated with a great effector function and advanced differentiation (107)(119)(58). Some of the mycobacteria-specific effector CD4+ T cells were CD27+CCR7-, and a few CD27+CCR7+.

We did not fint cells of the CD27-CCR7+ phenotype for any of the two CD4+ T cell populations, indicating CCR7 expression is lost before CD27 expression. Some cells, both in the total as well as mycobacteria-specific T cell population of healthy donors were found to be CD27+CCR7-.

When comparing the expression of the memory markers on the total CD4+ T cell population in healthy individuals and HIV patients, we did not see obvious variations for most of the markers. The marked decrease of CD27+ T cells, as reported (147), was not seen, and the expression of CD45RO and CCR7 were also similar for the two groups. HLA-DR and PD-1,

however, seem to be more frequently expressed on CD4+ T cells of HIV patients. It has been observed by others (190) that expression and replication of HIV is higher in HLA-DR positive than HLA-DR negative T cells, and that expression of HLA-DR correlates with higher expression of HIV. This could mean these cells are infected with HIV.

The PD-1 pathway induces replicative senescence and inhibits cytokine production from T cells. Blocking of this pathway restores these characteristics in CD8+ T cells (145). The reduced capacity to produce cytokines is not necessarily due to the expression of PD-1 itself (146). It was reported by other studies that HIV-specific CD8+T cells show increased expression of PD-1, and this may be independent of their maturational status (146). The expression of PD-1 on CD4 T+ cells is also correlated with viral load and negatively correlates with CD4+ T cell count (112), which may explain the increase in PD-1 expression that we found in our study on total CD4+ T cells of HIV patients.

Studies have shown that untreated HIV patients have a higher proportion of CD4+CD57+ than seronegative individuals (144), indicating that many cells are unable to divide, and that therapy did not normalize these conditions. The same study indicated that CD57 expression was predominantly found in the CCR7- T cell population and mainly among the CD4 T cells producing IFN-γ alone (144). Our findings show that CD57 is more expressed in healthy donors than in HIV patients, and thus seem not in accordance with Palmer and colleagues. It can generally be said that expression is low in both groups. It should be noted that CD57 was not identified for all study participants, as the fluorescence channel was mainly used for identification of CCR7. Additional samples are required to draw conclusions, but this could mean that our patient cohort show a normal expression of CD57, and that the CD4+ T cells are able to divide as normal.

Comparison of total and mycobacteria-specific CD4+ T cells from HIV patients regarding CD4+ T cell memory status is difficult due to the small number of analyzed donors for specific T cells from patients so far. Analysis of more donor samples are needed before conclusions can be drawn. In addition, the fairly low number of specific cells made the analysis complicated, and results seem to differ a whole lot from the healthy controls. For HIV it has been said that increased memory differentiation among the CD8+ T cell population is associated with decreased polyfunctionality (143). Others again claim that HIV-infection does not alter the T cell memory phenotype (133).

As for the healthy donors, we found for the HIV patients, total CD4 T cells to be mainly of a CD27+CCDR7+ phenotype. Our preliminary findings seem to point towards some differences between healthy and HIV patients when taking the specific CD4 T cell population into consideration. Results indicate that the majority of the specific T cells are CD27+CCR7+, the rest being almost equally distributed between CD27+CCR7-, CD27-CCR7+ and CD27-CCR7-. This means a lot of the specific cells show a phenotype indicating an early differentiated stage compared to the healthy controls. As earlier mentioned, loss of CCR7 expression is associated with an increase in cytokine production (119)(102). Among the HIV patients, around 40 % of the specific cells seem to experience the CCR7- phenotype, the percentage is more than 90 % in the healthy individuals. This may indicate that the memory status of mycobacteria-specific CD4+ T cells in HIV patients is different from healthy controls with a higher proportion of CCR7+ cells. This might contribute to the lower proportion of cytokine-producing mycobacteria-specific cells in (some) HIV patients. In addition, this finding supports the view of specific memory CD4 T cells of HIV patients being of an early differentiated stage, as represented by the intact expression of CCR7 in the majority of the cells. As effector memory cells (predominant in the healthy individuals) lack the CCR7 marker, most memory T cells in our HIV patient cohort seem to be of another memory T cell population than T_{EM}, which contradicts earlier reports (106). From these results, specific memory CD4 T cells from HIV patients seem to be of an central memory phenotype, T_{CM}. This subset is characterized by little cytokine production, but is able to differentiate further to effector cells (58).

Detection of intracellular cytokine mRNA expression using quantitative real-time PCR yields results showing that PBMCs stimulated overnight with mycobacterial antigens does produce proinflammatory cytokines. This method of analyzing cytokine production is not cell-specific. That means the whole PBMC population is analyzed for cytokine production, not just the T cells. PBMCs are a large group of cells that in addition to T cells also include for example B cells, DCs and monocytes/macrophages. This means real-time qPCR is not the best method for a project like this, where the T cells are in focus. Still, it provides an image of the overall PBMC status, and thus gives and idea of the total cytokine production. The different cytokines are also induced by different signaling pathways. IFN-β may be induced by TLR4 and the subsequent activation of transcription factor interferon regulatory factor (IRF) 3 and 7 (191). TNF is an inflammatory cytokine that mainly signals via TLRs and transcription factor

NF-κB (192), whereas IL-1β is secreted in response to activation of inflammasomes (reviewed in (99)). For all three sets of probes (IFN-β, IL-1β and TNF) the negative, unstimulated control shows little response, whereas heat-killed *M.avium* shows the greatest response towards all three mycobacterial antigens.

In the qPCR experiments, an increase in signal from stimulation with heat-killed *M.avium* compared to purified protein derivatives (PPD) from *M.avium* and *M.tuberculosis* was seen. This can perhaps be explained by the fact that PPD consists only of isolated proteins from bacteria. Heat-killed bacteria are complete organisms, just inactivated, meaning they perhaps are better at mounting an immune response as they better resemble the real situation of infection. However, the differences in cytokine mRNA production from total PBMCs between PPD and heat-killed mycobacteria seems not to affect T cell activation, as no significant difference was seen between the two stimulating agents in flow cytometric analysis.

ELISA targeting TNF was used as a third method for analysis of total mycobacteria-specific cytokine production from PBMCs. This assay was performed using only one healthy donor. However, supernatants from overnight stimulated PBMCs using mycobacterial antigens were stored in freeze containers for future analysis. ELISA results also show a production of proinflammatory cytokines from PBMCs stimulated with mycobacterial antigens overnight. This method shares a disadvantage with PCR, it is not possible to know from which cells the cytokines originated.

One of the most important limitations of this study was the relatively small number of HIV patients. Due to a late approval of the project by the regional ethics committee, the process delayed, and before the end of the study, it was possible to recruit only seven HIV patients. The pool of HIV positive patients in Trondheim approaches 120, and patients are not often in for controls. To increase the number of samples, it is possible establish a cooperation between research groups, in Norway and even across national borders.

Also, only 2 % of T cells are found in the blood stream (54). Although both major types of memory T cells are supposedly present in the blood (reviewed in (59)), effector memory T cells express surface markers making them home to infected tissues. Responses in T cells of the lung, where mycobacteria enter the body, may not resemble the responses found in peripheral blood.

6 Conclusion and future perspectives

In this study, methods for investigation of anti-mycobacterial CD4+ T cell effector functions and memory T cell composition in HIV infected individuals were established and applied. Individuals with underlying HIV infection are more susceptible to mycobacterial infections than healthy individuals, even after the onset of therapy. We hypothesized that this is due to impaired CD4+ T cell quality and skewed CD4+ T cell memory differentiation. To investigate these matters, we recruited HIV patients from the indoor clinic at St. Olavs Hospital, as well as healthy controls from the institute staff members. Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated using mycobacterial antigens (PPD from M.tuberculosis and M.avium, as well as heat-killed M.avium), and the mycobacteria-specific CD4+ T cell cytokine response and memory differentiation were studied. Flow cytometry was the main method used, but cytokine production was confirmed using qPCR and ELISA.

Before characterization of mycobacteria-specific CD4+ T cell immune responses in HIV patients, we established a protocol to ensure best possible conditions for simultaneous analysis of CD4+ T cell cytokine production and memory differentiation. The effect of cryopreservation on cytokine production was assessed, and it was found that cryopreservation for PBMCs for up to three months in liquid nitrogen yields results that are comparable to those from fresh cells. However, total percentage of mycobacteria-specific CD4+ T cells decreased as cells were stored at sub-zero temperatures, and we concluded that use of fresh cells should be considered. Cryopreservation may be useful if necessary technologies are limited, or to reduce variability between experiments by analyzing a set of samples simultaneously, including relevant controls.

We established a multicolor flow cytometric assay where we simultaneously could analyze CD4 T cell identification markers (CD3, CD4 and CD8), intracellular cytokine production (IFN-γ, TNF and IL-2) and memory markers (CD27, CD28, CD45RO, CD57, HLA-DR, PD-1 and CCR7). 11 fluorochromes were analyzed at the same time. For each donor sample, 10 wells were stimulated (plus controls), half the set was stained for the analysis of CD4+ T cell cytokine production, while the other half was stained for the analysis of CD4+ T cell differentiation status. Relevant controls were always included to be able to distinguish

between negative and positive populations during flow cytometric assay, and for the evaluation of signal-to-noise ratio. Fluorochromes were carefully chosen and brighter colors were designated the least abundant populations (cytokines, but also memory markers).

Response from mycobacteria-specific CD4+ T cells to overnight stimulation with antigens from M.tuberculosis and M.avium was detected in both healthy donors and HIV patients. This response manifested by the identification of effector cytokines IFN-γ, TNF and IL-2 during flow cytometric assays. The frequency of total CD4+ T cell responses to overnight stimulation with mycobacterial antigens was low, usually below 1 %. Polyfunctionality of CD4+ T cells is believed to be central in mounting a potent immune response towards mycobacteria, and it has been proposed that the decrease in CD4+ T cell polyfunctionality among HIV patients may contribute to their impaired immune response. We were able to analyze the mycobacteria-specific CD4+T cell cytokine production from two HIV patients only, but when comparing their results with the healthy individuals, our preliminary findings point to a lower polyfunctionality among the mycobacteria-specific CD4+ T cell population. This may be important in explaining the increased susceptibility of HIV patients to mycobacterial infections, and may confirm that the increased risk is not restored by antiretroviral therapy. These preliminary results should be verified by enrolling more HIV patients to the study, as great donor variability was discovered among the healthy controls, which may also be the case among HIV patients.

In addition to decreased polyfunctionality, skewed CD4+ T cell memory composition in HIV patients may influence the response towards mycobacteria. When considering the total CD4+ T cell population and comparing healthy controls to HIV patients, we did not find large differences for most of the memory markers. However, HLA-DR and PD-1 seem to be more frequently expressed in HIV patients than in healthy individuals. Interestingly, the mycobacteria-specific CD4+ T cells of HIV patients seem to be of an earlier differentiated phenotype, as indicated by a large proportion of CD27+CCR7+ cells. Among the healthy individuals, few cells are CD27+CCR7+, the predominant phenotype is CD27-CCR7-, indicating cells are late differentiated. Cytokine production is believed to peak as CCR7 expression is lost, in the effector memory cell population. Long-term immunity potential, however, may be best preserved as CCR7 expression is intact. These preliminary results suggest there are significant differences in CD4+ T cell memory population composition between healthy individuals and HIV patients. This needs to be further investigated, as it was

possible to analyze the mycobacteria-specific CD4+ T cell memory phenotype from very few HIV patients.

In summary, our preliminary findings point to central differences between healthy controls and HIV patients in mycobacteria-specific CD4+ T cell effector cytokine production and polyfunctionality, as well as CD4+ T cell memory phenotype. Results from this pilot study should be further validated with more HIV patients.

The results from our study might after completion contribute to a better understanding of abnormalities in the CD4+ T cell responses (polyfunctionality and memory subset composition) of HIV patients. Increased knowledge of the T cell subsets necessary for successful anti-mycobacterial immunity is important to develop new and more effective vaccine strategies. Vaccines against tuberculosis are urgently needed, as tuberculosis is a global problem, and multidrug-resistant strains are on the rise.

The results already obtained can be used as indications for further research. As additional HIV patients are recruited to the study, it would be possible to group the patients according to their disease progression. Healthy controls may be compared to HIV seropositive individuals before onset of therapy, and patients already receiving therapy. Their responses towards stimulation with mycobacterial antigens may be compared to investigate how susceptibility to tuberculosis changes during disease progression. This may also be correlated to CD4 T cell count and even viral load. If possible, it would be interesting to do follow-up analysis on the same patient, preferably before and after onset of antiretroviral therapy. In Norway, however, therapy is initiated quite soon after HIV is diagnosed and without waiting for CD4+ T cells to decrease under a certain limit, which means HIV patients before onset of therapy are hard to find. To increase the patient cohort, collaboration with other national and international research groups is an option. This is a possible option as we here showed that CD4+ T cell functions can be analyzed from cryopreserved samples, thus allowing to ship isolated PBMCs on dry ice. Long-term nonprogressors (or elite controllers) could also be enrolled in the study. These are HIV positive individuals who control the disease without treatment with antiretroviral therapy.

A promising finding was the ability to generate an expanded population of mycobacteria-specific T cells from the isolated PBMCs from one of the HIV patients. T cells stimulated

with mycobacterial antigens were cultured for approximately 10 days, and autologous MDMs were generated simultaneously. *In vitro*-generated macrophages that can be co-cultured with antigen-specific CD4+ T cells opens for a wide variety of manipulations. Macrophages may be modified, using for example siRNA knock-down of proteins, and mycobacteria can also be modified. It should also be noted that shortly, there will be facilities available for handling live M.tuberculosis at the institute, which also increases the possibilities for more realistic analysis of immune responses during *in vitro* co-infection with HIV and tuberculosis.

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Appendix I: Isolation of PBMCs

Material: Heparinized venous blood from healthy donor or HIV positive patient.

All solutions should be room temperature.

In a 50 mL tube, add around 15 mL blood. Add PBS up to 30 mL.

In another 50 mL tube, add 15,0 mL of Lymphoprep. Use a 25 mL pipette and gently add the blood/PBS mix to the Lymphoprep so that it forms a layer on top of the blank solution.
Spin tubes at 1800 rpm for 20 minutes at room temperature (brake 7^3).

The white blood cells now form a ring between the Lymphoprep and the serum. Remove this ring of WBCs with a 10 mL pipette and add to a new 50 mL tube. Remove about 10 mL of fluid, be careful not to include too much Lymphoprep, as it is toxic to the cells. Spin at 2000 rpm for 10 minutes at room temperature.

Washing step 1: Gently remove the supernatant and discard it. Leave some fluid in the tube to avoid losing cells. Resuspend the cells in the remaining liquid and add HANKS 2% FCS up to 25 mL. Spin at 800 rpm for 8 minutes at room temperature.

Washing step 2: Gently remove the supernatant and discard it. Leave some fluid in the tube to avoid losing cells. Resuspend the cells in the remaining liquid and add HANKS 2% FCS up to 25 mL. Spin at 1400 rpm for 6 minutes at room temperature.

Gently remove the supernatant and discard it. Leave some fluid in the tube to avoid losing cells. Resuspend the cells in the remaining liquid and add RPMI medium (+Glu+HEPES) 10% A+ up to 5 mL.
Count the cells using Countess: Mix 10 uL Trypan blue and 10 uL sample, and add to a count slide. Follow instructions on the machine.

Appendix 2: Protocols for HIV study

Biobanking of PBMCs

Freezing cells

Prepare freezing medium (80% FCS 20% DMSO) and add 0,5 mL to cryo tubes (size 1,2 mL).

Transfer PBMCs to a 15 mL tube. Dilute to 5 mL using PBS 2% FCS. Spin at 1400 rpm for 6 minutes. Discard supernatant. Use a 1000 uL pipette to remove as much of the supernatant as possible, but be sure not to throw away any cells.

Carefully resuspend the cells in pure FCS and transfer 0,5 mL to each cryo tube.

Cryo tubes are then placed in Mr Frosty and Mr Frosty is placed in -80 °C. Cells going to liquid nitrogen are removed after 24-72 hours.

Thawing PBMCs

Cells should be thawed as quickly as possible after removal from freezer/nitrogen.

Prepare medium (RPMI 10% FCS) and heat it to in 37 °C.

Loosen the cap of the tubes a bit and thaw by placing in water bath (37 °C) or under hot running water. As soon as they are completely thawed, move cells to 5 mLwarm medium and wash the cryo tubes once.

Spin cells at 1300 rpm for 5 minutes. Discard the supernatant and resuspend carefully in remaining liquid by flicking the tubes.

Add medium (RPMI+Glu+HEPES+10% A+) to 1 mL and count the cells.

Dilute the cells and proceed with stimulation

Stimulation of mycobacteria-specific T cells

Dilute the cells (thawed or fresh) to a working concentration of 2,5 x 10^6 cells/mL in T cell medium (RPMI +Glu+HEPES+10% A+)

Add costimulatory signal (anti-CD28 and anti-CD49d).

Pipette 0,5 x 10^6 cells/mL (equals 200 uL) in a 96 well round-bottom plate.

Stimulate the cells:

- Unstimulated
- *M.avium* PPD 10 ug/mL
- *M.tuberculosis* PPD 10 ug/mL

- Heat-killed *M.avium* (MOI 1:1 = 0,5 x 10^6 bacterias per well, calculate from OD measured)

- 1:10 diluted eBioscience 500x cell stimulation cocktail, 4 uL

Incubate overnight (16 h) at 37 °C.
Last four hours: Add 1:10 diluted eBioscience 500x protein transport inhibitor cocktail, 4 uL.

Extracellular staining

Transfer cells to flow tubes containing 500 uL flow wash buffer (PBS 2% FCS), and wash tubes once (1500 rpm, 6 minutes). Discard supernatant.
Pool the surface antibodies and add them to the cells (1 uL of each antibody).
Vortex gently. Incubate 15 minutes, dark and on ice.

Live/dead stain (eBioscience fixable viability dye eFluor 780)

Add 0,5 mL PBS to each well. Wash cells at 1500 rpm for 6 minutes and discard the supernatant. Repeat this process once.
Add 0,5 mL PBS to each well, and then add 0,5 uL live/dead stain. Vortex gently, and incubate for 30 minutes, dark and on ice.
Add 0,5 mL wash buffer, and spin at 1500 rpm for 6 minutes.

Discard supernatant. Resuspend cells in 0,5 mL freshly prepared PBS 2% PFA, and incubate 20 minutes in room temperature and in the dark.
Spin cells at 1500 rpm for 6 minutes, and discard supernatant. Resuspend in 0,5 mL wash buffer and spin at 1500 rpm for 6 minutes.
Discard supernatant, add 0,5 mL wash buffer, and proceed with intracellular staining or leave at 4 °C and dark overnight.

Intracellular staining

Spin cells at 1500 rpm for 6 minutes and discard supernatant.
Resuspend cells in 0,5 mL permeabilization buffer (PBS 1% FCS 0,5% saponin). Incubate for 5 minutes in room temperature before spinning at 1500 rpm for 6 minutes. Discard supernatant. Repeat this procedure once.
Pool the intracellular antibodies and add them to the cells (1 uL of each antibody).
Vortex gently. Incubate 30 minutes, dark and in room temperature.
Add 0,5 mL of the regular wash buffer and wash at 1500 rpm for 6 minutes. Discard

supernatant. Repeat this procedure once.

Resuspend cells in 2-3 drops wash buffer before running flow cytometry.

Lysate for PCR and supernatant for ELISA

Stimulation is performed the same way as for flow cytometry, except they are seeded in 48 well plates (0,5 mL – 1,25 x 10^6 cells).

After 16 hours, spin down the whole plate at 1400 rpm for 6 minutes.

Supernatant

Remove the supernatant, be careful not to touch the cell pellet with the pipette tip (remove around 450 uL). Put supernatant in a new 48 well plate and freeze in -80 °C. This supernatant can be analyzed using ELISA.

Cells

Add 350 uL lysis buffer to each well (lysis buffer = RCL with mercapoethanol). This should be done quickly after removal of supernatant, as cells may dry out. Pipette up and down a couple of times.

Let work for 2-3 minutes.

Resuspend thoroughly 6-7 times and remove all of the liquid to a new 48 well plate. Freeze in -80 °C. Thaw for RNA isolation and eventually PCR.

T-cells and macrophages for expansion experiment

Making an expanded population of mycobacteria-specific T-cells

Day zero: Dilute PBMCs to a working concentration of 3,5 x 10^6 cells/mL in RPMI 10% A+. Seed cells in flat bottom 24 well plates, 1 mL per well. Stimulate T-cells using heat-killed M.avium, MOI 5:1. Incubate in 37 °C .

At day two/three: Add IL-2 to the T cells. Units: 20

Split cells and give them fresh medium if they reach confluence over 80%.

Making autologous monocyte-derived macrophages (MDM)

Day zero: Dilute PBMCs to a working concentration of 2 x 10^6 cells/mL in RPMI 10% A+. Seed cells in flat bottom 96 well plates, 100 uL per well. Incubate cells in 37 °C for 1 hour to have the monocytes adhere to the plate.

Remove medium and wash cells three times using warm HANKS. Avoid cells drying out. Add RPMI 30% A+ to the cells and incubate in 37 °C for 6-8 days.

Keep an eye on the cells and add more medium if nutrients become scarce.

Adding T-cells to macrophages

Day 6-8: Wash T-cells (HANKS 2% FCS) and count them using Countess.

Stimulate macrophages using the preferred agents.

Add T cells to macrophages in the flat bottom 96 well plate. Use 0,5 x 10^6 cells/well. Co-culture for 16 hours, add protein secretion inhibitor, and proceed with extracellular staining, intracellular staining, and flow cytometry.

Appendix 3: Staining panel

Staining for cytokine effector functions

EPITOPE	FLUOROCHROME
CD3	BV785
CD4	BV711
CD8	Alexa 700
Live/dead	eFluor780
IFN-γ	PE
TNF	BV421
IL-2	APC
CD45RO	FITC
CD28	PE/Cy7
CD27	BV510
PD-1	BV605

Staining for identification of cell surface markers

EPITOPE	FLUOROCHROME
CD3	BV785
CD4	BV711
CD8	Alexa 700
Live/dead	eFluor780
IFN-γ / TNF / Il-2	PE
CD45RO	FITC
CD28	PE/Cy7
CD27	BV510
PD-1	BV605
HLA-DR	APC
CCR7 or CD57	BV421 or eFluor450