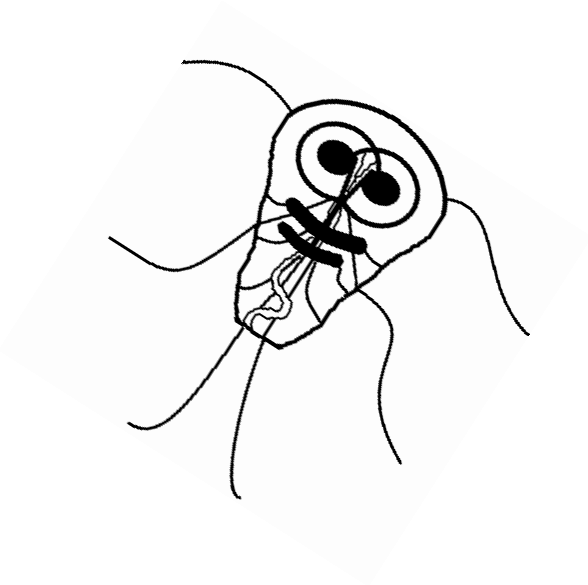
Developing a flow cytometric method to characterize human T cell responses against the enteric protozoan parasite *Giardia lamblia*

Master thesis in Pharmacy

Christina Skår Saghaug



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Bergen, May 2014

Christina Skår Saghaug

Confessions of a student during master(ful) times

- In times of trouble, the lab was daily double.
Motivation I needed and strength as well
to guide me through the analysis of a cell.
Day light; a seldom sight
I had to be finished with the thesis
with all of my strength and all of my might.

The help I have gotten from friends and fellow mates
have opened my mind to the researching gates.

Without all the help and guidance, I'd be lost.
All the time I have stolen, it can be an exhaust.
Guilty as charged to this I will plea
I hope I have gotten enough vitamin D.

Thank you for all the help you provided
a main supervisor like you, Kurt, I feel prided.
Always aware and always kind,
you're a positive contribution to human kind

The lab techniques are not easy to handle
Without your laugher and genius mind,
Steinar, It'd be a scandal.

The writing of a thesis requires time,
and a lot of hindrances have to be climbed.
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Maturation year they say about masters
but without any help and support it'd be disastrous.
Independence one should truly show as well
and bad motivation one have to dispel.

My family always supported with love
Without caring and embrace I'd feel like a lost dove
My mom with supporting love and belief
My dad with calming words of relief
My sister saying there would be no grief

Friends so dear and close to my heart,
Never you disappoint or will fall apart.
Alise, your quick mind and loyalty to me
always reassuring and make my days problem free
Sara and Anne, your laughter and joy.
The three musketeers nothing can destroy.
Beate, the tea breaks and discussions we had
hugely appreciated and you make me glad.
Aasta, your enthusiastic attitude,
making me happy I give you my gratitude.
Aslaug, the wisest girl that I know,
I thank you for your company and times you made me grow.
Lisbeth, your smile and down to earth attitude
I thank you for company and I give you my gratitude

Fellow students in crime
I thank you for company and genius words;
Oddgeir, Linda, Jack, Matej, Mari, Steffen, Torunn and Jan Fredrik,
Without you all,
loneliness would have increased with at least two-thirds

A master can be classified as the worst of times, yet the best of times.
The *Giardia* study is finally (almost) done, and I have no more words that rhymes. -

By Christina Skår Saghaug

Abbreviations

ADH	Arginine dehydrolase pathway
ADI	Arginine deiminase
Ag group	Recruited individuals exposed to *Giardia*, termed recent giardiasis
APC	Antigen presenting cell
CD	Cluster of differentiation
CD14/CD19	Surface markers expressed on respectively monocytes and B cells
CD197 or CCR7	A chemokine receptor expressed on central memory and naïve cells
CD25	IL-2 receptor present on activated cells
CD26	T cell recall activation marker
$CD3^+$ T cell	T cell receptor required for activation of the cell
$CD4^+$ T cell	A lymphocyte also known as helper T cell
CD45RA	Marker for naïve or non-activated cells
CD45RO	Marker of memory or activated cells
$CD8^+$ T cell	A lymphocyte also known as cytotoxic/suppressor T cell
CV	Coefficient of variation
DC	Dendritic cells
DMSO	Dimethyl Sulphoxide
FAB	Fluorochrome-conjugated antibody
FMO	Fluorescence minus one
HLA-DR	Human leukocyte antigen complex that presents antigens
IC	Inomomycin calcium salt
IFN-γ	Interferon gamma, pro-inflammatory cytokine
Ig	Immunoglobulin
IL-10	Interleukin associated with anti-inflammatory responses/regulatory cytokine
IL-17A	Interleukin associated with autoimmune diseases, pro-inflammatory cytokine
IL-4	Interleukin associated with differentiations of naïve T cells to T_h2
IL-2	Growth factor for T cells
LPS	Lipopolysaccharide
LR group	Recruited individuals with low risk of ever having *Giardia*, termed low risk control
MED	X-vivo medium used for cell culturing X-vivo medium used for cell culturing
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NA	Not applicable
NO	Nitric oxide
NOS	Nitric oxide synthase
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Dulbecco's Phosphate Buffered Saline
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PPD	Purified protein derivate
PW	Perm wash for intracellular staining
RCF	Relative Centrifugal Force
RPM	Rounds per minute
RT	Room temperature
SD	Standard deviation
SEB	Staphylococcal enterotoxin B
SSA	Soluble proteins from *Giardia lamblia* assemblage A
SSB	Soluble proteins from *Giardia lamblia* assemblage B
TCR	T cell receptor
T_h	Helper T cell

TNF-α	Tumor necrosis factor alpha, pro-inflammatory cytokine
T_{reg}	Regulatory T cell
VSPs	Variant-specific surface proteins
WHO	World's health organization

Glossary of Giardia assemblages

GS	*Giardia lamblia* Assemblage B
WB	*Giardia lamblia* Assemblage A

Abstract

Giardia lamblia is an enteric protozoan parasite, which causes infection in humans worldwide. The impact of the infection varies from asymptomatic carriers to severe disease such as malabsorption syndrome. Evidence for acquired immunity against *Giardia* infection has been found in previous studies. $CD4^+$ T cell responses have been detected in humans, but data regarding cytokine producing profiles of these T cells is limited.

This study aimed to develop a flow cytometric method to investigate *Giardia*-specific $CD4^+$ T cell responses in individuals with recent giardiasis. Early cytokine profiles in addition to later surface markers and proliferation were combined to explore *Giardia*-specific $CD4^+$ T cell immune responses by flow cytometry.

In the development of the flow cytometric assay, fluorochrome-conjugated antibodies were titrated and different clones tested, detector voltages on the flow cytometer were adjusted, CellTrace proliferation dye labeling method and concentration was optimized, spectral overlap was minimized, compensation matrices were acquired and different fixation and permeabilization reagents were tested.

To explore the function of the assay, cultures of peripheral blood mononuclear cells (PBMCs) from a group of individuals with recent giardiasis were stimulated with *Giardia* assemblage A and B sonicated soluble proteins (SSA and SSB), and responses were compared to responses in PBMCs from a group of low risk healthy controls. Early cytokine profiles in addition to later surface markers and proliferation were compared between these groups to explore *Giardia*-specific $CD4^+$ T cell immune responses by flow cytometry. The first assay (day one assay) investigated cytokine expression of TNA-α, IFN-γ, IL-17A, IL-10 and IL-4 in effector memory $CD4^+$ T cells ($CD197^-CD45RA^-$) after 24 hours of stimulation with *Giardia* soluble proteins and controls antigens. The other assay (day six assay) investigated proliferation by CellTrace dye dilution and activation markers HLA-DR, CD45RO, CD25 and CD26 after six days of stimulation.

The results were analyzed in FlowJo, and statistical analysis was done using SPSS. Cytokine responses were stronger in the *Giardia* exposed group when stimulated with SSA and SSB, but only IL-17A production was found to be significantly elevated in this group. Two participants with current, on-going, giardiasis had markedly elevated production of all cytokines, except IL-4, in response to SSA and SSB, but not to control antigens.

In the day six assay CD45RO and HLA-DR positive cell percentages were found to be

significantly different between the groups when stimulated with SSA. A positive correlation between SSA and SSB induced effector memory $CD4^+$ T cell cytokine production, as well as proliferation responses was found, indicating considerable cross-reaction between these two assemblages.

In conclusion, we find that the developed assay performed well and can be used to assess *Giardia*-specific immunity, but it has some shortcomings. Although the assay showed generally higher responses in the giardiasis exposed group for most of the outcomes, only IL-17A production, and $HLADR^+CD45RO^+$ activation turned out to be statistically significant. Future studies using purified recombinant *Giardia* proteins as antigens may improve this. Some of the low risk controls may also be cross-reacting or unknowingly been previously exposed to *Giardia*. A novel finding in this study is that T_h17 $CD4^+$ T cells may play an important role in the immunity against *Giardia* in humans.

Table of contents

1. Introduction

1.1 Immune system of humans

1.1.1 The innate and adaptive immune system of humans

The immune system is an advanced interplay between many different cells in order to protect an individual against infective agents and harmful components [1]. These agents can for instance be invading microbes and other potential harmful microbe parts such as toxins (usually proteins and polysaccharides) or chemicals. A collective term for these agents is antigens.

Antigens are classified as non-self-molecules and are capable of triggering an immune response. Sometimes the immune system starts recognizing its own body components as antigens, and a state called autoimmunity can be initiated.

The immune system can be divided into a two-part system, where one is activated as an initial rapid response and gives warnings for the other, which requires longer time to get activated, but has a remarkable capacity of generating immunological memory. These two systems can accordingly be named the innate immune system and the adaptive one.

Figure 1.1 displays the components and activation time of the innate and the adaptive immune responses.

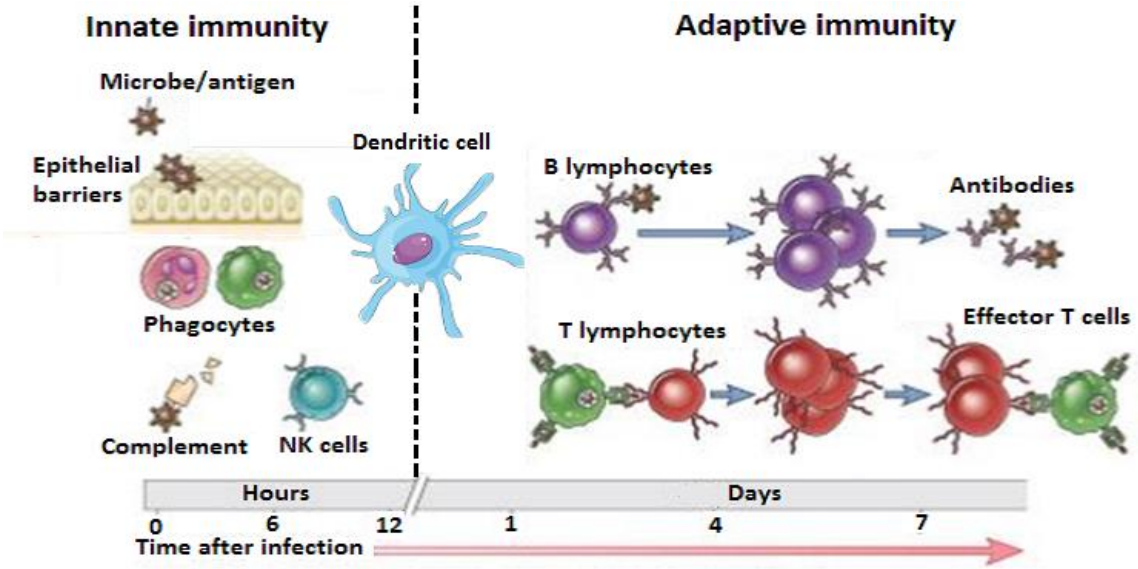


Figure 1.1: Components of the innate and adaptive immune systems and activation time.
The innate immune system is activated within hours of intrusion. The adaptive immune system takes longer time to be activated, but is more specific. Figure is produced using Servier Medical Art and adapted from [1].

Cells in the immune system can express specific receptor/ligands on their cell surface known as cluster of differentiation (CD). CD expressed on the surface of a cell can be used to distinguish between different cell types, and may be used for phenotyping [1].

1.1.1.1 The innate immune system

The innate immune system is congenital and is the initial response against an infection. It is not specific against certain types of antigens, because it is present before an infection takes place. The antigens recognized by the innate immune system, are defined in the germ-line of humans, and is predetermined before a human encounters antigens in the environment after birth. The innate immune system will therefore react the same way against all potential antigens, and no memory responses will be generated [1].

Parts making up the innate immune system are: different barriers, where both physical and chemical, (including the skin, and mucosal surfaces), antimicrobial substrates produced by different cells, phagocytic cells and also a cell type called natural killer cell (NK cell). The phagocytic cells compromise of neutrophils, mast cells, macrophages and monocytes. Dendritic cells and macrophages, are called antigen-presenting cells (APC), and can bind and ingest intruding microbes and mitigate, reducing the antigen load in the host.

Proteins circulating in the blood are also a part of the innate immune system, where proteins of the complement system (C1-C9) and other inflammatory proteins are vital. The complement system comprises of serum and cell surface proteins, which can react with one another or cooperate with other immune components, in eradication of antigens. An activation of the complement system leads to a cascade of happenings, leading to a proteolytic cleavage of the protein C3, creating the products C3a and C3b [2]. This will again trigger cleavage of C5 to C5a and C5b. C3a mediates inflammation, favoring phagocyte recruitment and the C5a work as a chemoattractant (entice phagocytes to migrate to the infection site). C3b coat surfaces of microbes and work opsonic (enhance phagocytosis). C3b can also bind with C6, C7, C8 and C9, making a membrane attack complex and lyse/penetrate antigenic cell membranes by making pores [2].

Other inflammatory proteins important for the innate immune system, are called C-reactive protein, serum amyloid A protein, proteinase inhibitors and coagulation proteins [2].

A type of highly specialized phagocytic cells are termed dendritic cells (DC). Dendritic cells (DC) possess important cellular elongations, called dendrites, and collect antigens constantly form i.e. lumens of mucosal surfaces. DCs can recognize pathogens by receptors for pathogen-associated molecular patterns (PAMPs) [1, 3]. PAMPs include nucleic acids that only

microbes expresses, such as double stranded RNA, carbohydrates such as lipopolysaccharides (LPS) and glycoproteins such as mannose-rich oligosaccharides [1].

After DC antigen uptake, DCs mature and can migrate to peripheral lymph nodes in order to start antigen specific immunity by interaction with lymphocytes of the adaptive immune system. The DC processes the sampled antigen into smaller peptide fragments and display them on a receptor called major histocompatibility complex (MCH). Two subclasses of MHC exist, and are termed class I, and class II. Class I MHC is found on every nucleated cell in the body and is recognized by $CD8^+$ T cells. Class II MHC is displayed on APCs and is recognized by $CD4^+$ T cells. This is described more in detail later. The display of antigen fragments on the DCs MHC class II receptor makes them into APCs. Through this receptor, DCs can present antigens to, and activate, cells of the adaptive immune system. They are therefore know as a bridge between the two immune systems [2].

1.1.1.2 The adaptive immune system

The adaptive immune system (or acquired immunity) on the other hand is stimulated by foreign substances and, when activated can confer specific protection against a specific infectious agent. It is established only after encountering foreign substance and needs longer time to be effective, compared to the innate and initial immune response. The adaptive immunity consists of different cells making up an advanced interaction. The main cells involved are the lymphocytes and the products they secrete against an infectious agent [1]. The adaptive immune system is capable of differentiating between very similar microbes and react towards them in different ways. Existence of memory cells makes an individual capable of eliciting a faster and stronger immune response towards previously encountered pathogens. Memory immune responses and antigen specificity are characteristics of the adaptive immune system. The specific memory responses can exists for several years after an antigen exposure, and give protective immunity [1].

The adaptive immune responses can be divided into two different pathways, where one is called humoral immunity, produced by B cells, and the other one is called cell-mediated immunity, and involves T cells.

1.1.1.3 Humoral and cellular immunity

Both humoral and cellular immunity is based on recognition of specific antigens or a part of it. The parts which they can recognize are called antigenic determinants or epitopes. An individual has a diverse repertoire of about 10^7-10^9 different lymphocyte clones, which means that the

lymphocytes can distinguish between an enormous amount of antigens. When a lymphocyte finds its epitope and becomes activated, it can undergo clonal expansion (proliferation), where many clones of this lymphocyte can help in the removal of an antigen.

The cells responsible for the humoral immunity are called B lymphocytes, or B cells and most of these expresses the surface marker CD19. The maturation of B cells starts in the bone marrow, but before they are fully matured, they go into the circulation and thereafter travel to the peripheral lymphoid organs and here they can become fully matured. B cells have a membrane-bound antibody receptor which they use to recognize antigens. A differentiated B cell, called a plasma cell, produces proteins called antibodies or immunoglobulins (Ig) which are secreted into blood and on mucosal surfaces. The secreted antibodies' major responsibilities are to recognize antigens and microbial secreted toxins, thereafter render them harmless and make them available for removal by several other cells or mechanisms (including the phagocytes and the complement system) [1].

Igs are highly specific proteins capable of distinguishing between different antigens. Igs can be separated into different classes called IgA, IgD, IgE, IgM and IgG. Polysaccharides and lipids stimulate naïve B cells into plasma cells producing Igs known as the IgM class, followed by a weak IgG response. This is recognized as a primary response. If the same infectious agents are met again, a secondary response can be triggered and is faster and stronger than the primary one. IgG is the dominant Ig in a secondary response [1].

The cellular immune responses are carried out by a cell type called T lymphocytes (T cells). T cells will mature completely in the thymus before they travel with the blood circulation and then reside in peripheral lymphoid tissues.T cells express a T cell receptor (TCR). A part of this receptor is called CD3 and can be used to identifythese cells. The TCR can be divided into αβ-TCR and γδ-TCR, where αβ-TCR is the most common one for T cells, and the γδ-TCR T cells usually lack CD4 and CD8 surface receptors (CD4 and CD8 are discussed later) and can mostly not recognize peptides displayed by the MHC on APCs [1]. The γδ-TCR T cells are mostly found in the intestine functioning as intraepithelial lymphocytes [4]. They make up around 3-5 % of T cells in peripheral blood [5].

T cells can only recognize epitopes having peptide structures and they have to be displayed on MHC expressed by APCs. Professional APCs include dendritic cells, macrophages and B cells, and they express MHC class II. Macrophages do not normally express MHC class II, but this receptor can be up-regulated during an immune response [1]. The most efficient APCs are the DCs, which can collect antigens entering the body by endocytosis, transport them to lymphoid organs and thereafter present them to naïve T cells

($CD45RA^+/CD197^+$) by interaction of the MHC and the TCR. CD45RA is a high molecular weight surface receptor expressed on naïve T cells. This receptor will not react rapidly against recall antigens. During an activation, the expression of CD45RA will be lost, and the cell will instead express the low molecular weight structure, known as CD45RO. The CD45RO receptor reacts rapidly against recall antigens [1, 6].

A co-receptor (CD28) in addition to the TCR is needed for a naïve T cell to become activated. Activated T cells can thus turn into a memory cell, either a central memory cell ($CD45RO^+/CD197^+$) or an effector memory cell ($CD45RO^+/CD197^-$) [7]. The CD197 molecule is an important chemokine receptor for cytokines produced by lymphoid tissue and CD197 is known as a homing receptor. This receptor favors migration toward lymph nodes, where T cells can be activated into effector memory cells, provided they encounter the specific antigen for their receptor presented on MHC on an APC. The CD197 receptor will be lost during activation, and T cells will be able to migrate to the site of infection and produce cytokines as their effector function [1, 8].

Recently activated cells will express CD25 (an autocrine receptor for the cytokine IL-2)[1]. Activated T cells can also be positive for a proteolytic enzyme, known as CD26, shown to be up-regulated during activation. CD26 can also be termed a recall antigen marker [9, 10]. HLA-DR is also a surface marker associated with activation occurring in later phases [10, 11]. Another activation marker for T cells is CD69, which is up-regulated in the early phases of activation [12].

The T cells can be divided into two major subsets where one is termed a helper T cell (T_h cell), and another one is termed cytotoxic T cells ($CD8^+$). Some T cells are also functioning to inhibit or regulate immune responses and are termed regulatory T cell (T_{reg}) [1].

Helper T cells ($CD4^+$) recognize peptides in MHC class II displayed on DCs and become activated. After activation $CD4^+$ T cells are responsible for secreting proteins called cytokines. Cytokines are messenger molecules that can exhibit different functions and can stimulate the T cell themselves to undergo proliferation, differentiation and stimulates surrounding cells.

T_h cells can activate B cells into producing antibodies belonging to IgG, IgA or IgE class. The antibodies produced by plasma cells with help have better affinities for the respective antigens. T_h therefore needed to initiate good B cell responses towards protein antigens. To induce B cell activation mediated by T cells, an already activated T cell have to recognize peptides displayed by the B cell's MCH class II.

IgG is important for eradication of an antigen as macrophages express a receptor for this antibody. When this antibody is bound to the antigen, it thereby increases the phagocytosis.

A cytokine called interleukin-2 (IL-2) is produced by T cells and acts as a growth factor for the T cell itself and for nearby cells. T cells can also activate other cells such as macrophages (in conjunction with a cytokine called Interferon-γ) and other leukocytes [1].

Cytotoxic T cells express CD8, a membrane receptor recognizing class I MHC molecules found on all types of cells. These T cells become activated when a cell is displaying peptides on its MHC class I. Cytotoxic T cells monitor and may kill any host cells infected with virus or bacteria (making them go into apoptosis). The $CD8^+$ cell mediated killing is crucial, as antibodies cannot reach the inside of infected cells.

A $CD8^+$ mediated apoptosis is dependent on cytotoxic granules inside the cell. These granules contain perforin and express a surface marker known as CD107a. Theses granules will degranulate when $CD8^+$ cells exert their function. CD107a will then be expressed on the surface of the cell and can be used as a marker of cytotoxicity. Perforin can also be used as a marker of cytotoxicity as this protein gets exposed during the triggered apoptosis of an infected cell, where it creates pores [8, 13].

The lymphocytes and their function of immune responses are displayed in Figure 1.2.

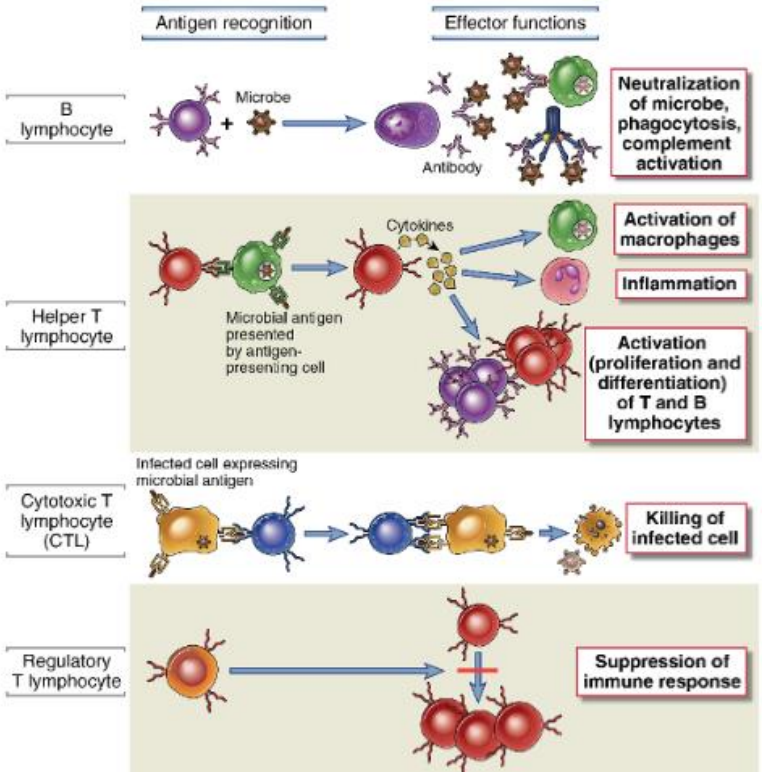


Figure 1.2 Antigen recognition and effector functions of B cells and different subsets of T cells.

The activation of adaptive immune responses and their function of the cells. Figure is adapted from [1].

1.1.1.4 Polarization of naïve CD4+ T cells

Naïve $CD4^+$ T cells can differentiate into different subsets, depending on the type of microbe, tissue and signaling molecules in their environment [14, 15]. The subsets of which naïve $CD4^+$ T helper cells (T_h) can polarize into include T_h1, T_h2, T_h9 and T_h17. Naïve $CD4^+$ T cells can also differentiate into a regulatory cell known as $T_{reg\ [1]}$. The cytokines important for differentiation of a naïve $CD4^+$ T cell, and the main cytokines the differentiated effector cell produce are displayed in Figure 1.3.

The T_h1 subsets mainly produce pro-inflammatory cytokine crucial for the eradication of microbes residing inside cells. The cytokines most specific for this subset of helper cell are IFN-γ, TNF-α and IL-2. IFN-γ and TNF-α are important for cellular immunity, especially towards intracellular microbes. These cytokines also stimulate phagocytes during an infection, improving their phagocytic properties [1, 4, 16, 17].

The T_h2 subset works antagonistically to the T_h1 subsets, and can stop or regulate responses mediated by T_h1. This subset is important for immunity against helminthic parasites and allergens. Typical cytokines produced by T_h2 cells include IL-4, IL-5, IL-13 and triggers IgE production from B cells. A regulatory cytokine known as IL-10 is also a characteristic T_h2 cytokine [1, 16, 17].

The T_h9 subsets can be associated with T_h2 subset, as IL-4 is required for T_h9 development. The polarization of T_h9 subsets are thought to occur both for naïve $CD4^+$ T cells, and for already polarized T_h subsets (Figure 1.3). The characteristic cytokine for T_h9 is IL-9 [14].

The T_h17 subset has IL-17 as their classical cytokine. This subset of T_h cannot occur if INF-γ or IL-4 is produced [1]. Important properties for T_h17 cells are to mediate pro-inflammatory responses against extracellular microbes has been shown to cause tissue damage connected to autoimmune diseases [1, 4].

T_{regs} are essential for inhibiting or limiting immune responses and interleukin-10 (IL-10) is a regulatory cytokine produced by these cells [1].

1.1.1.5 Important cytokines relevant for polarization

The messenger molecules, cytokines, can show pleiotropism and redundancy. Pleiotropism means that one cytokine can function on different kinds of cells, giving one cytokine the chance of carrying out different biological effects. Redundancy means that different cytokines can exert the same biological function and hence give the same responses. Combinations of cytokines can produce synergetic effects. Antagonism can also happen where one cytokine is inhibiting

an activation of another [1].

Some of the cytokines are important in mediating pro-inflammatory, anti-inflammatory or regulative reactions. Many of these cytokines are produced by $CD4^+$ T cells and play vital roles in immune responses [1, 16].

Tumor Necrosis Factor alpha (TNF-α) is the main cytokine for acute inflammation and can cause systemic symptoms associated with infections. This cytokine can be termed pro-inflammatory as it is an important cytokine in recruitment of various phagocytotic cells such as neutrophils and monocytes to site of infection, and for activation these. TNF-α also makes endothelial surfaces permeable to phagocytes by inducing the endothelium to express adhesion molecules that facilitate phagocytotic migration to the site of infection [1].

Interferon-γ, IFN-γ, is the major cytokine for macrophage activation and is important for cellular immunity against intracellular microbes. IFN-γ activates macrophages, influence differentiation of naïve $CD4^+$ T cells to T_h1 and promotes Ig switching in B cells. It is a pro-inflammatory cytokine, and is a mediator in turning naïve $CD4^+$ T_h into a T_h1 response and turning antibodies secreted by plasma cells into IgG type [1].

Interleukin-4, IL-4, is the main promoter for IgE antibodies secreted by plasma cells, and also an important mediator for the polarization of naïve $CD4^+$ T_h into a T_h2. The T_h2 response is essential for mast cell/eosinophil-mediated reactions. IL-4 can be classified as a T_h2 characteristic cytokine [1].

Interleukin-17A, IL-17A, is a pro-inflammatory cytokine important for protection against bacterial infections. IL-17A is produced by a subtype of T_h cells called T_h17. The T_h17 cells require Transforming Growth Factor-β (TGF-β), IL-23 and innate cytokines such as IL-6 in order to develop. IL-17A has a speculative role for being a promoter of destructive properties of autoimmune diseases in mice and also in inflammatory bowel disease in humans [1].

Interleukin-10, IL-10, is an important cytokine for inhibition of cell-mediated immunity. Due to the regulative properties of immune responses, it is characterized as a regulatory cytokine. It is likewise an inhibitor of macrophages. IL-10 is also known to inhibit INF-γ production and can therefore down-regulate a pro-inflammatory response [1].

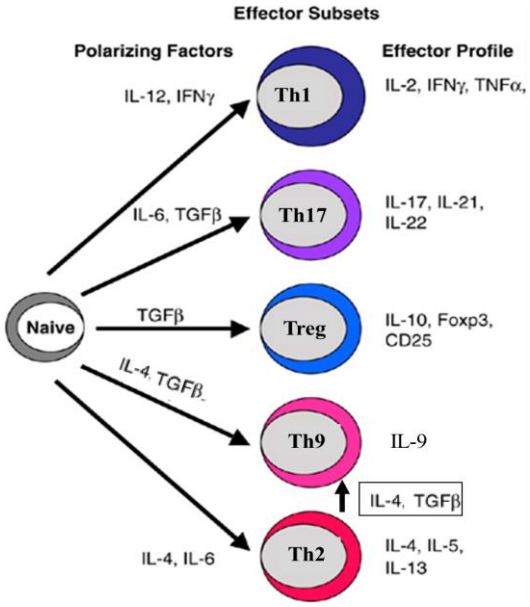


Figure 1.3: Differentiation of naïve CD4$^+$ T cells and their signature cytokines.
Figure adapted from [15]

1.2 Immunity in the intestine and the epithelial barrier

Many organs are covered by mucosal surfaces including the gastrointestinal system, the respiratory tract, the reproductive and the urinary tract. These surfaces play an important role in protection against the environment [18].

The epithelium covering the small intestine is important for nutritional and hydration status in humans, as it absorbs both nutrients and is a regulator for water and electrolytes. To maximize the absorption capacity, enterocytes with microvilli make up an apical brush border with a huge surface area (size of a tennis court). Intracellular junctions and tight junctions between the epithelial cells are important for a working barrier function [18]. These junctions are made up of different proteins, including cytoskeletal F-actin and α-actin [19].

Several different microorganisms flourish in the small intestine and make up a micro flora largely favorable for the human host. Other factors important for absorption, digestion and health include gastric acid, digestive enzymes, bile salts and peristalsis (involuntary constriction and relaxation of the muscles) and CD8$^+$ intraepithelial T lymphocytes [18].

The barrier covering the small intestine is renewed as often as every 4-5 days by undifferentiated, proliferating progenitor cells in the crypts, while the villi are replaced with non-proliferating cells, which are not specialized. Stem cells at the base of the crypts is the source of three cells types that reside in the villus. These cells are called absorptive enterocytes, enteroendocrine cells and goblet cells. The stem cells can also differentiate into a cell located at the base of the crypts that is called Paneth cells [18]. A demonstration of the renewal of some of these cells can be seen in Figure 1.4.

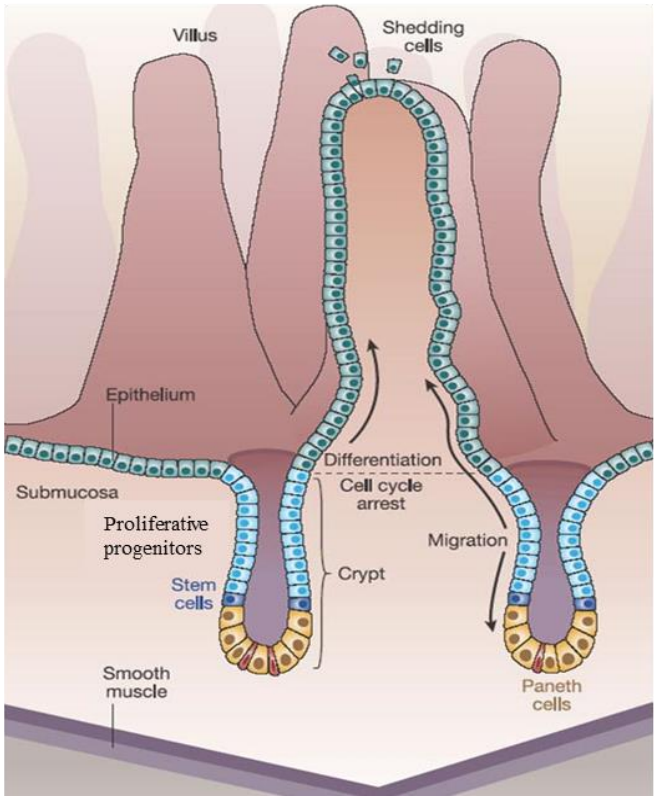


Figure 1.4: Renewing of the cells covering the epithelial barrier in the small intestine.
The renewal of villus, paneth cells and epithelium originated form the crypts of the small intestine. The figure is adapted from [20].

1.2.1 Secretory substrates protecting the small intestine

Goblet cells are responsible for production of mucin glycoproteins, making up a protective inner and outer mucus layer. Paneth cells produce α-defensins which are peptides with antimicrobial properties [18]. The epithelium secretes lactoferrin, lysozyme, peroxidase, Nitric oxide [21], reactive oxygen species (ROS) [19], cathelicidin and α- and β-defensins [18].

Nitric oxide (NO) is produced enzymatically by NO synthase (NOS) and requires the protein arginine. The NO has antimicrobial properties and can act towards both bacteria and parasites [22]. Many pathogens infecting the intestine absorb and utilize free arginine from the surrounding milieu in hosts. Several different pathogens been suggested to compete for free arginine including *Mycobacterium*, *Giardia*, *Trypanosoma*, *Helicobacter*, *Schistosoma* and *several Salmonella* types. This strive for free arginine makes it a competition between the host cells and the pathogens [3, 23, 24].

Right underneath the epithelial barrier, a region called lamina propria is found. Cells mediating immune responses can also be found here and include lymphocytes, macrophages, dendritic cells and stromal cells [18]. Plasma cells are producing protective antibodies, mast

cells are a cell type important for the immunity against helminthic infection and also for part of allergic reactions, producing histamine [25].

Antibodies have a specific way of getting into the intestinal lumen in order for them to perform their functions against pathogens. The polymeric Ig receptor is needed in the transport of IgA and IgM from the lamina propria to the lumen. IgA is the dominant Ig on mucosal surfaces. Transport of IgG happens via the neonatal Fc receptor, and can go both directions (from lumen to lamina propria and vice versa). IgE is often made in response to parasitic infections and can be transported via CD23(FCεRll) [18].

1.2.2 Recognition of pathogens in the small intestine

Pathogens residing in the intestinal lumen need to be recognized by host cells in order to elicit a defense mechanism in the form of adaptive immune responses. There are many ways for this immune response to happen, but three pathways are of special importance. One of the pathways involve M cells (specialized epithelial cells), lymphoepithelial structures found in Peyer's patches(a region in the intestine resembling lymphoid tissue) in addition to isolated lymphoid follicles. The M cells can take up antigens from the lumen of the small intestine and forward them to APCs, without being one themselves. Another pathway important for stimulating immune responses is mediated by transcytosis (transportion of macromolecules from the surroundings and into the interior of a cell), where Ig bound to antigens make up complexes which can be presented to and captured by professional APCs (dendritic cells) found in lamina propria. After activation, these APCs can migrate to other lymphoid structures in the body, interact with lymphocytes and thus activate a systemic adaptive immune response. The last pathway is where dendritic cells can be located in the submucosa in close proximity to the epithelium. Dendritic cells can extend their dendrites between epithelial cells in order to collect different pathogens from the intestinal lumen [18].

1.3 The gut infective parasite Giardia lamblia

1.3.1 A brief historical perspective of the gut parasite Giardia lamblia

Giardia lamblia (synonyms*: Giardia duodenalis*, *Giardia intestinalis*) is a gastrointestinal protozoan parasite known to infect different hosts, including humans [26]. It was the first protozoan parasite, infectious to humans, to be discovered by the Dutchman Antonie van Leeuwenhoek in 1681 [27]. Vilem Lambl redescribed *Giardia* later, in 1859, and published self-made drawings of the protozoan [28].

The parasite was isolated and described in human fecal samples, but still physicians disagreed about whether or not the parasite was a pathogen or a commensal, i.e. an innocent organism benefiting from host interaction with humans. Research and clinical reports from 1915 and onwards showed, however, that *Giardia* was associated with diarrheal disease and therefor a pathogen [28].

Although the timespan since discovery has been long, the epidemiology and nomenclature of this organism can still be considered confusing and the pathology different than was earlier described. Transmission due to contaminated drinking water and infection with this protozoan, causing the disease known as giardiasis, was first proved when well-documented reports came out in the 1960s in the United States of America [27, 28]. Today the leading route of transmission is the fecal-oral route either indirectly through water or food or directly between persons [27, 29].

Giardia lamblia (from now on *Giardia)* can be termed an antediluvian diverging organism [30]. Figure 1.5 graphically shows the evolution of the eukaryotes, and places *Giardia* to the earliest branches of an rRNA-rooted tree, together with other organisms, all termed Archezoa.

Some people have disagreed about whether *Giardia* developed before or after the mitochondrial acquisition, and it has been proposed that *Giardia* has had mitochondria, but has lost them during evolution [31].

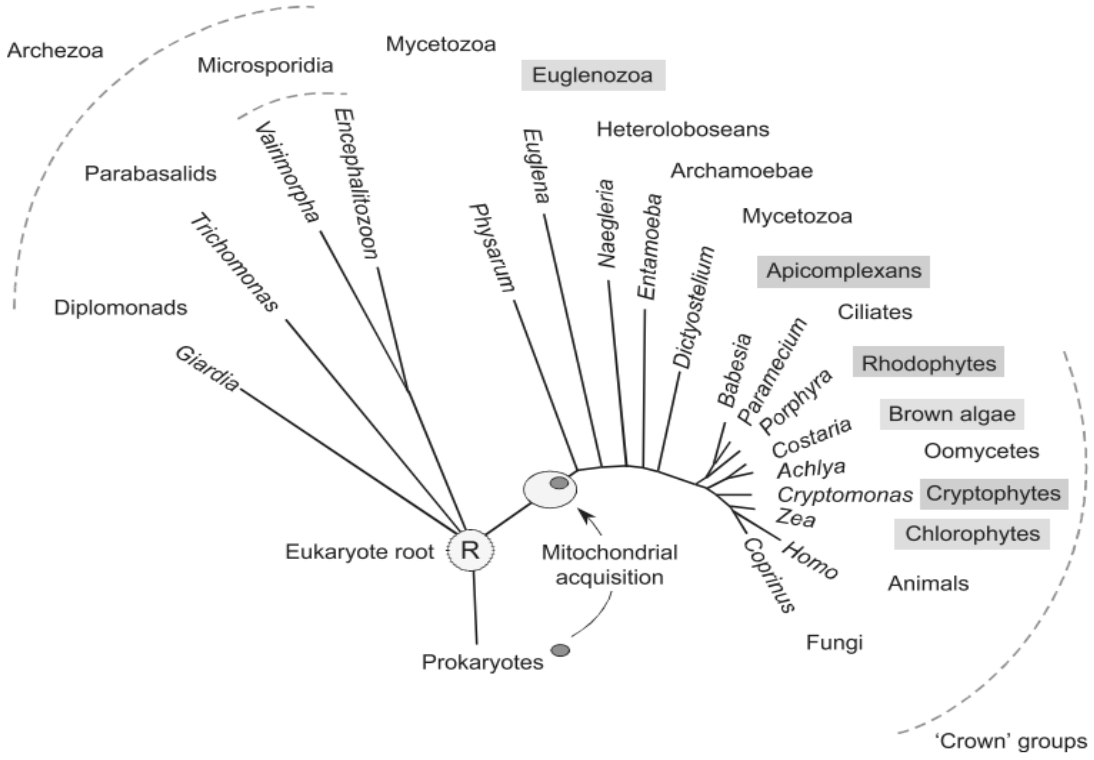


Figure 1.5: A schematic overview of the evolution of eukaryotes, shown by a rooted rRNA-tree.
The branches representing the diplomonands, parabasalids and the microsporidia have a shared root, showing that they branched off before mitochondrial acquisition. The lineages that can be found further to the right of the root are thought to be newer branches, consisting of uni- and multicellular organisms, called the 'Crown' groups [32]. This figure is borrowed from [33].

1.3.2 Taxonomy, nomenclature and genotypes of Giardia

Giardia belongs to the phylum Sarcomastigophora, and to the class Zoomastigophora. The protozoa is classified as a member of the order *diplomonandia*, and is part of the *binuceated* (two nuclei inside its cell) *flagellates* group, known to populate anaerobic or microaerophilic habitats. At the present time, *Giardia is* part of the supergroup Excavata [26, 34].

Giardia cells offer exceptional opportunities for expanding the insight into essential cellular pathways characterized by eukaryotic cells, and for discovering new molecular mechanisms. *Giardia* has a metabolism resembling bacteria, and shows compliant growth in cultures, making it a good 'prototype' in order to examine relic organelles, cellular differentiation and other cellular mechanisms [34].

Eight different genotypes of *Giardia lamblia* have been classified, known as assemblages A-H, and can infect mammals [23, 26]. Assemblage A and B are infective to humans. *Giardia* is divided into different species, based on which host they can infect, and their

morphological appearance identified with light microscope. For instance *Giardia agilis* infects amphibians and *Giardia muris* infects rodents, birds and also reptiles [26].

1.3.3 Epidemiology of giardiasis

Several waterborne pathogens, found in industrialized and in developing countries, cause diarrheal diseases. Annually, there is an estimated 4 billion cases of diarrheal disease worldwide and these result in approximately 2,2 million deaths [35]. Out of the diarrheal diseases, *Giardia* has been estimated to give up to 280 million symptomatic human infections annually. World's health organization (WHO) classified giardiasis as a neglected disease in 2004 [34, 36].

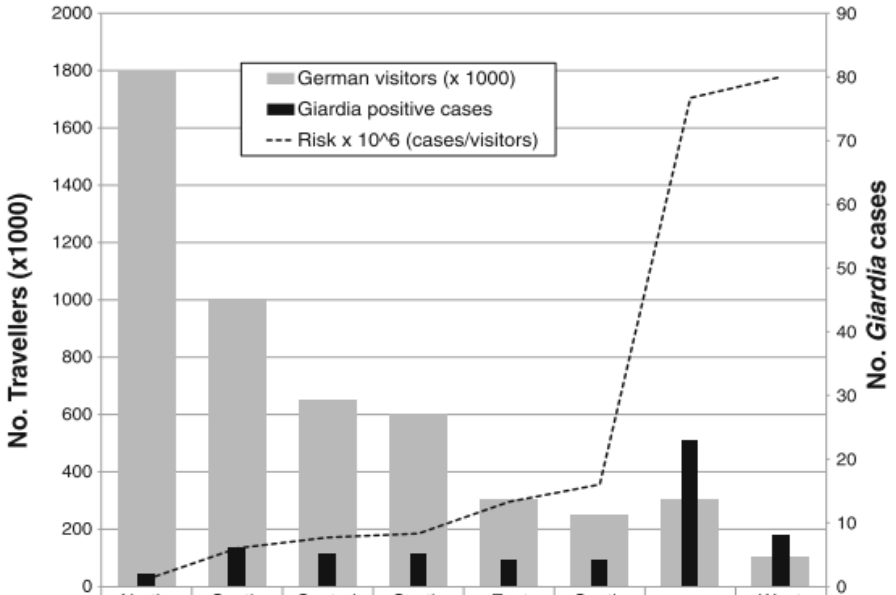
Giardia is a parasite often identified in waterborne outbreaks and is found throughout the world [37]. Prevalence rates of infection in the industrialized world is estimated to be 5 % (3-7 %), and 20 % (4-43 %) in third world countries [19]. Giardiasis is frequently recognized as acute, but can turn into a chronic disease lasting for months or years, both with or without symptoms [38].

Giardia together with *Cryptosporidium* are the two main waterborne infections caused by protozoan parasites producing diarrhea in humans worldwide. These infections can be transmitted by fecal-oral route, water/swimming pools can be contaminated with parasites originating from animals or humans. Infection between family members have been documented and infection can occur during sexual relations involving oro-anal contact [28, 39].

A large outbreak was seen in Bergen, Hordaland County in 2004. A *Giardia* outbreak in this proportion had never been registered in this non-endemic country before. 1300 persons had laboratory confirmed positive *Giardia* stool samples and 2500 people underwent medical treatment due to this outbreak [40].

People infected with *Giardia* in industrialized countries, usually have acquired the disease through travelling in tropical or developing countries where *Giardia* is endemic. Figure 1.6 displays returning German travelers, where those returning from India and West-Africa had higher risk of returning with *Giardia* infection [41].

Giardia outbreaks are common in the USA. A surveillance of *Giardia* positive cases from 2006-2008, demonstrated in Figure 1.7 shows age distribution and numbers of cases. Young children between 1-4 years are most prone to infection, both in the USA and worldwide [37, 42].



	North Africa	South East Asia	Central America	South America	East Africa	South Africa	India	West Africa
German visitors (x 1000)	1800	1000	650	600	300	250	300	100
Giardia positive cases	2	6	5	5	4	4	23	8
Risk x 10^6 (cases/visitors)	1.1	6	7.7	8.3	13.3	16	76.7	80
95% C.I.	0.0-2.7	1.2-10.8	0.9-14.4	1.0-15.6	0.3-26.4	0.3-31.7	45.3-108.0	24.6-135.4

Figure 1.6: *Giardia* infection rates of returning German travelers.

Giardia positive cases among German travelers. The risk of infection per 10^6 cases is highest in the countries India and West-Africa. Figure borrowed from [41].

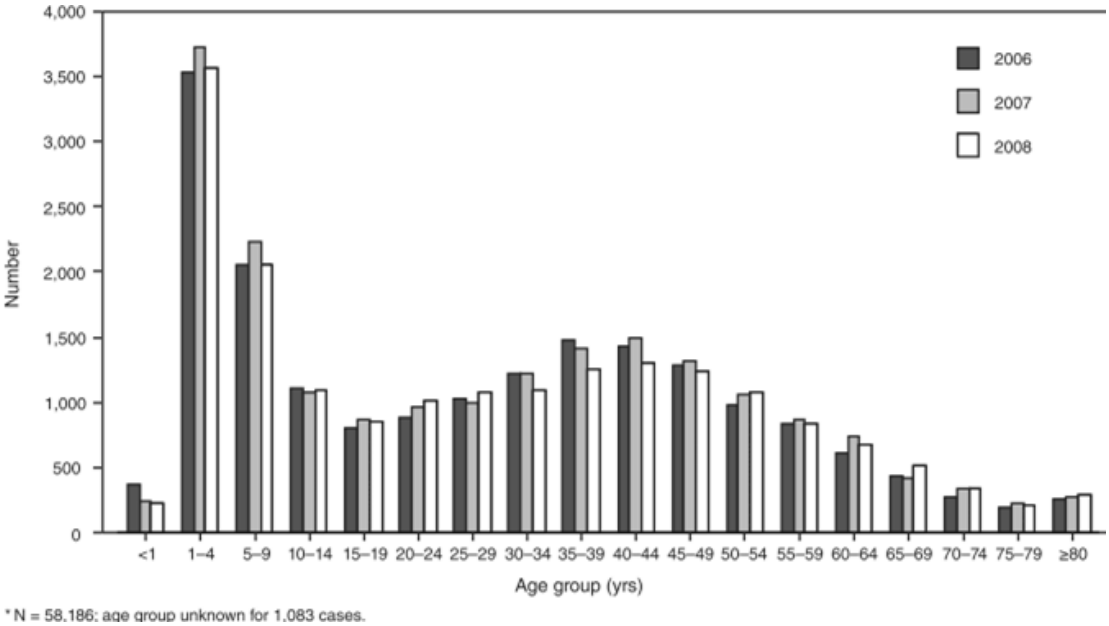


Figure 1.7 Demographics of *Giardia* infection in the United States of America from 2006-2008.

This figure shows that children between the ages 1-4 are most prone to infection. This graph is borrowed from [37].

1.3.4 Clinical manifestations

Giardia infection in humans can on one hand give acute or chronic diarrhea, and at times result in more serious complications such as malabsorption syndrome. On the other hand it can also be an asymptomatic infection where the individual is unaware of the parasite [28]. Even if the infection can be self-limiting or be treated with medicines, it can influence the quality of life to a certain extent [3, 29]. The infectious dose is small, only 10 cysts are needed to establish the infection [34].

Clinical manifestations usually occur after 6-15 days of incubation. When the infection is symptomatic, it can cause watery diarrhea, epigastric pain, nausea and vomiting, which may results in weight loss [3, 29]. The diarrhea caused by *Giardia* can give rise to a severe disease, termed malabsorption syndrome [3]. In some intestinal biopsies from chronically infected individuals, atrophy of the villi has been found by microscopic analysis. Malfunction of the Na^+-glucose uptake and microvillus brush border disruption have additionally been seen [43]. A correlation between symptomatic disease and a dysfunction of the epithelial barrier in the intestine has been suggested [3].

The impact of the infection is often more severe in young children, in malnourished persons, and in individuals incapable of developing an immune response following exposure to *Giardia*. The complications of infection can be macronutrient and micronutrient shortages. Vulnerable children susceptible to infection can due to these nutrient deficiencies suffer failure to thrive resulting in retarded growth and development [28, 29].

Normally, it takes between a few days and up to approximately 6 weeks to eradicate a *Giardia* infection spontaneously without medication [38, 44]. However, giardiasis can be treated with antibiotics of the 5-nitromidazole compounds, metronidazole commonly being the first choice [34, 45]. The different manifestations seen, can be due to different factors, such as *Giardia* genotype, the virulence of the *Giardia* strain, the antigenic variation, how large the ingested dose of cysts was, previous *Giardia* infection, age of the individual, other ongoing infections and the clinical state of the hosts immune system [19, 46-48]. A study with gerbils (an animal that can be infected with both the *Giardia* assemblages infective to humans) showed different durations of infections and probability of re-infection, when alternating re-infection with *Giardia* assemblage A or B. The gerbils previously infected with assemblage A showed resistance to be infected with both of the assemblages, while gerbils previously infected with assemblage B only showed resistance to this assemblage. This indicates that different immune

responses are induced by the two assemblages, and cross-reactivity exists [47].

1.3.5 Diagnosis

Microscopic analysis looking for cysts in stools samples of infected individuals can be used to determine *Giardia* infection in individuals. *Giardia* cysts can be excreted only sporadically, and therefore examination of several stool samples will give better sensitivity. Three separate stool samples collected on different days will increase chances of getting a positive test and thus increase sensitivity to around 90 % [37].

Analysis for presence of *Giardia* can also be done by polymerase chain reaction (PCR). The PCR aims to detect the 18S gene found in *Giardia*, and has shown to have higher specificity and sensitivity than other diagnostic methods [49].

Analysis of trophozoites in duodenal contents can also be used to diagnose *Giardia*, but is a more invasive method and associated with more discomfort for the patient, than for stool sample examination [26].

1.3.6 Treatment of giardiasis

Giardia infections are usually treated with metronidazole as a common first line choice [45]. Metronidazole is the only licensed drug that can be used against giardiasis in Norway. This antibiotic has shown to result in successful eradication of the parasite in 60-90 % of the cases. Several antibiotics may be used to treat *Giardia*, if there are unsatisfactory responses to metronidazole. They are here listed according to empirical preference and range of efficacy effectiveness: Tinidazole (74-100 %), quinacrine (92-100 %), albendazole (24-100 %) and furazolidone (80-100 %). Pregnant women should however use paromycin (55-90 %) [45].

It has been proposed in Norway to use combination therapy, if the first-line antibiotic, metronidazole fails. A second line choice consists of albendazole in combination with metronidazole. Paromomycin can be a third line choice and an option in pregnancy. Quinacrine in combination with metronidazole can be a fourth line choice [45].

1.3.7 Giardia Biology

The trophozoite has a shape, which bulges outwards on the back and bulges inwards in the front, where the adhesive disc (a cytoskeletal organelle [19]) is found. The length of a trophozoite is usually 10-12 µM and is about 5-7 µM wide. One or two structures can be found in the middle of the parasite called the median body, which often have a shape resembling claw

hammers. *Giardia* also have 8 motility organs known as flagella, which consists of four pairs [26]. *Giardia* has been recognized to be deficient of mitochondria [33] and also lacking a normal endoplasmatic reticulum and a Golgi apparatus [30].

Two identical nuclei containing nucleolus are found in the upper part of the trophozoite and can thus look like two eyes. Central- and peripheral mitosomes and peripheral vesicles are organelles found within *Giardia*. Overview of the parasite's two stages, the trophozoite and the cyst, can be seen in Figure 1.8. The ventral adhesive disc, used for attachment, and the flagella are consisting of a type of *Giardia*-specific cytoskeleton proteins termed the giardin family, unique for this parasite, and the tubulin family [26, 34].

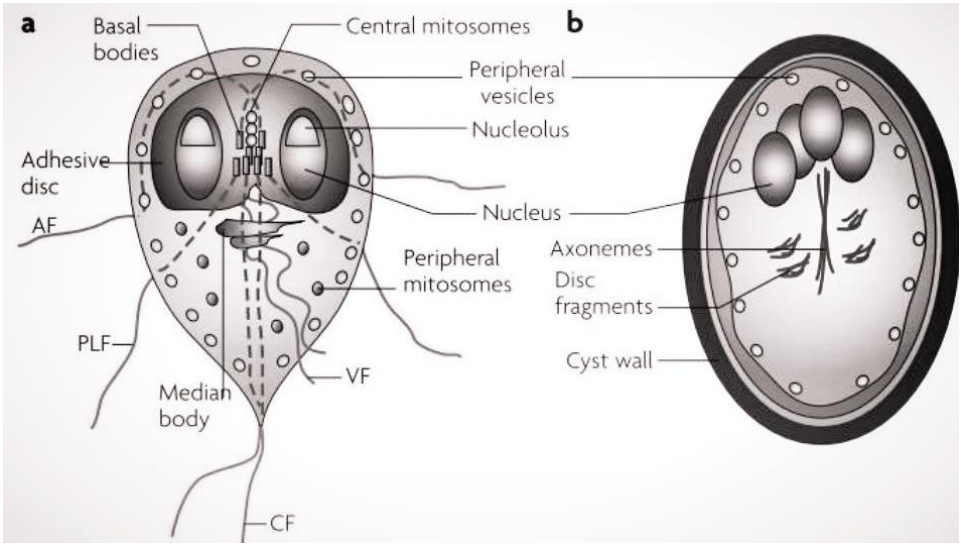


Figure 1.8: The trophozoite state of *Giardia* (left) and the cyst state of *Giardia* (right).
The flagella AF,CF, VF and PLF, seen on the trophozoite are abbreviations for anterior, caudal, posterior-lateral and ventral flagella. Picture borrowed from[34] and colors modified.

The cysts measures 7-10 µM in length and has an oval shape. The cyst wall measures 0.3 µM [26, 28]. When *Giardia* is a cyst, the adhesive disc and the flagella are broken into pieces (axonemes) during encystation and stored as fragments inside of the cyst [19]. The cyst has four nuclei inside the cell.

1.3.7.1 Cell cycle of Giardia

Giardia's natural habitat is in the gastro-intestinal tract system, preferably in the small intestine, where it can adhere to the host's intestinal mucosa in the duodenum and jejunum [19]. The parasite has however been found at more distal sites of the intestine [22].

Two morphologic stages of the parasite exist, namely a binucleated trophozoite stage

and a quadrinucleated cyst stage. The cyst can be termed a non-replicating infectious form and the trophozoite a symptom causing and replicating vegetative form. The cyst can live for many months outside its host, provided that the conditions are cool and moist [26, 34, 48].

The giardial life cycle can be divided into four different steps, where the first one is excystation(I), followed by adherence to brush border villi in the intestinal epitelium (II), trophozoite replication (III) and encystation in order to infect other hosts (IV) [28].

When a cyst has been ingested, it can excyst in the upper part of the small intestine, forming two trophozoites. These disease-causing trophozoites further replicates by binary fission [26]. Figure 1.9 shows the cell cycle of *Giardia*, from an infective cyst to a replicating trophozoite.

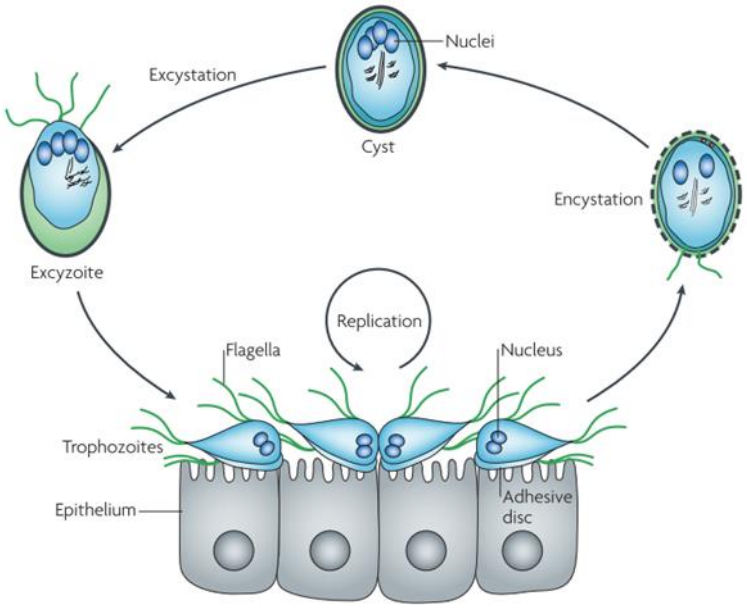


Figure 1.9: **The replication process of *Giardia* lamblia** The cyst, or the non-replicating infective state, is the first and last step in an infection with *Giardia*. The excyzoite is an intermediate before the vegetative, disease causing and replicating cysts are formed. The trophozoite has to undergo encystation before becoming a cyst again and can infect other hosts. Figure is borrowed from [34].

The conversion of a cyst to a trophozoite, excystation, making an excyzoite, is catalyzed by acidic pH (1.3-4 [26]) in the host stomach. Further on the cyst completes its differentiation in the duodenum where the pH is 6.8-7.0. The excystation is finished when the cyst is exposed to excretions from the pancreas. Proliferation/cytokinesis of the trophozoites can at this point (30 minutes after cyst wall disruption [26]) start and the infection is established.

The conversion the other way, encystation, happens in the small and large intestine when the surrounding milieu have low concentrations of cholesterol or high concentrations of bile acid and a basic pH [34, 50]. The infective cysts can then be parted from the host mixed in feces and infect other hosts by the fecal-oral route [51].

1.3.8 Host-parasite interaction

Trophozoites of *Giardia* have been identified to reside in close proximity to the top of enterocytes in the intestine, and also in the small intestinal crypts, where they can attach to the surface of the host cells. *Giardia* can only attach to the host cells, but is not invasive [23, 28]. Figure 1.10 demonstrates a *Giardia* trophozoite attached to human epithelium in the small intestine.

Several *in vitro* experiments have shown that *Giardia* could rearrange some of the proteins binding the enterocytes together with tight junctions. Proteins found to be disrupted *in vitro* are the epithelial cell F-actins and α-actins, impairing the epithelial barrier function [19]. An experiment using a mouse model also showed a cytoskeletal remodeling of the epithelial barrier in the small intestine [52].

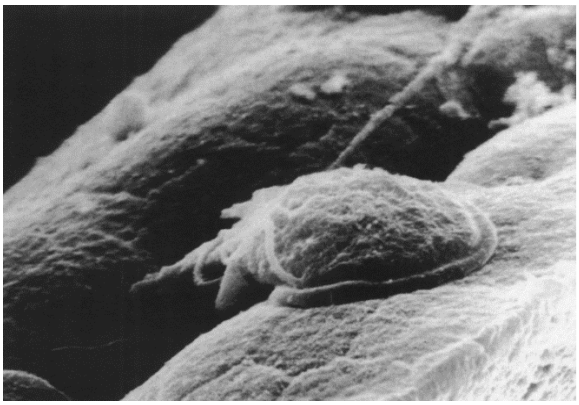


Figure 1.10: Attachment of a *Giardia* trophozoite of the microvillus border on the top of epithelial cells of the human intestine.
Photo borrowed from [26]

Replicating trophozoites have incomplete capacity to biosynthesize fat derived products such as membrane lipids, cholesterol and fatty acids. Accordingly, *Giardia* needs to depend on supplies from the surroundings. Lipids and fatty acids consumed from the host surroundings can be utilized as an energy source and biogenesis of different organelles. Fatty acids and cholesterol have both shown evidence of being important in the involvement and regulation of encystation and cyst differentiation [50].

Giardia use a pathway named the arginine dehydrolase (ADH) pathway to obtain energy. In this pathway arginine deiminase (ADI) has shown to start a progress of an irreversible catabolism, where free arginine from the host is converted to citrulline and NH_4^+ in order to secure energy [3, 51].

The consumption of arginine, has been proposed to affect enterocytes in the intestine by reducing proliferation of stem cells in crypts. In this manner, it can impair homeostasis of the intestinal epithelium and thereby induce villi shortening leading to increased permeability in the intestine and cause diarrhea [23]

1.3.9 Antigenic variation and virulence factors

Giardia is not found to produce any toxins, and the knowledge regarding the main characteristics for defining parasite-host interactions, responsible for causing different outcome for disease is limited [22].

The adhesive disc and flagella are important for the parasite to avoid removal by peristalsis from the host's intestine. The adhesive disc provides the parasite with suction properties, and enables attachment to the epithelial barrier in the intestine of the host. The flagella on the other hand makes *Giardia* capable of moving or "swimming", both in order to get attached to the host cells and to evade peristaltic movements by the intestines [34].

The trophozoite form of *Giardia* is able to switch major surface molecules, making the parasite capable of evading the immune system in the host. The surface molecules are called variant-specific surface protein (VSP), and only one out of around 250 VSP genes are expressed on the surface at any time point. VSPs are bound to the membrane and have an extracellular N-terminal which is variable and a C-terminal which is more conserved [51].

ADI is recognized as a metabolic enzyme, but earlier findings shows that ADI has a capability of citrullinate the VSPs on the surface of *Giardia* cells. This modifies the original VSP biology and will alter the cytotoxic antibody responses from the host [51]. It has been suggested that *Giardia*-mediated VSP shifting is driven by host antibodies, and thus making the parasite evade the immune system of the host [53].

Cysteine proteases have been found in *Giardia* and these have been thought to be a virulence factor as they have shown to be capable of cleaving IgA from the host [26].

The enzyme ADI converts arginine into citrulline as stated above. Earlier findings suggest that ADI is used as a competitor for the free arginine to NOS in the host [51]. The competition can therefore be seen as a virulence factor as it disturbs host nitric oxide synthases from functioning normally, and thereby decrease the secretion of anti-microbial NO [3, 46]

A summary *Giardia* virulence factors can be seen in Table 1.1.

Table 1.1: Overview of *Giardia* virulence factors. The table is adapted from [34].

Virulence factors	Role of the virulence factors
Ventral adhesive disc	Facilitate interaction between parasite and the intestinal endothelium
VSPs on the cell surface	Surface shifting of proteins in order to escape host Immunoglobulins
Flagellar motility	Evading elements in the intestinal lumen and avoid peristaltic movements of the intestine.
Excretion of Arginine Deiminase	Down regulation of host mediated production of Nitric oxide in response to infection, and citurullination of VSPs.
Giardias differentiation from trophozoite to a cyst	A mechanism to protect the parasite from exterior environment and to be able to infect other hosts.
High cysteine protease	Cleaves IgA in order to escape immune responses from host

1.3.10 Cellular and humoral immunity against Giardia

Eradication of, and protection against, *Giardia* is likely to be dependent on both B cell mediated antibody production and T cell mediated immune responses in the host-pathogen interaction [19, 54]. Different barriers and immunity against *Giardia* can be seen in Figure 1.11.

Aquired immunity has been shown to occur as mice challenged with a second *Giardia* infection had far less cysts in feces (98 % less) compared to an initial infection [55].

Individuals who earlier encountered *Giardia* previously are less prone to infection, have been seen in humans who live in endemic areas. The infective rates of giardiasis have been lower than expected for people encountering the parasite often. This indicates that some sort of acquired immunity exists [42, 56]. *Giardia*-specific T cell memory immune responses have been evaluated using flow cytometry and thymidine proliferation assay. A small but pristing $CD4^+$ T cell responses was found to be present even 5 years after individuals were infected with, and successfully treated for *Giardia,* in the Bergen *Giardia* outbreak [10].

Likewise, a waterborne *Giardia* outbreak in 1985 in Creston, Canada showed that people living here were significantly less prone to have a re-infection when a second outbreak

happened again in a five year period. Residents living in this area showed to have higher IgA and IgG specific antibodies towards *Giardia* compared to controls [57].

Most of our knowledge regarding both the cellular and humoral immune responses against *Giardia* is based on studies in mice. $CD4^+$ T cells have been shown to be necessary for immediate responses. Mice without $CD4^+$ T cells cannot control a *Giardia* infection. B cell mediated antibody production did not show to be needed the same way as $CD4^+$ T cells for the regulation of an acute *Giardia* infection [58]. Chronic infection can be caused by decreased or nonexistent CD4 T cells, and gives evidence that these cells are crucial for the murine defense against *Giardia* [58, 59].

An earlier study found that IFN-γ was secreted by human intestinal and blood $CD4^+$ T cells when stimulated with *Giardia* trophozoites. The $CD4^+$ T cells were also found to be proliferating in response to the *Giardia* parasites, suggesting specific proliferation of antigen-specific $CD4^+$ T cells [60].

T cells expressing αβ-TCR has been recognized to be of crucial importance in order to control an infection in mice, whereas T cells expressing γδ-TCR have not shown importance [58].

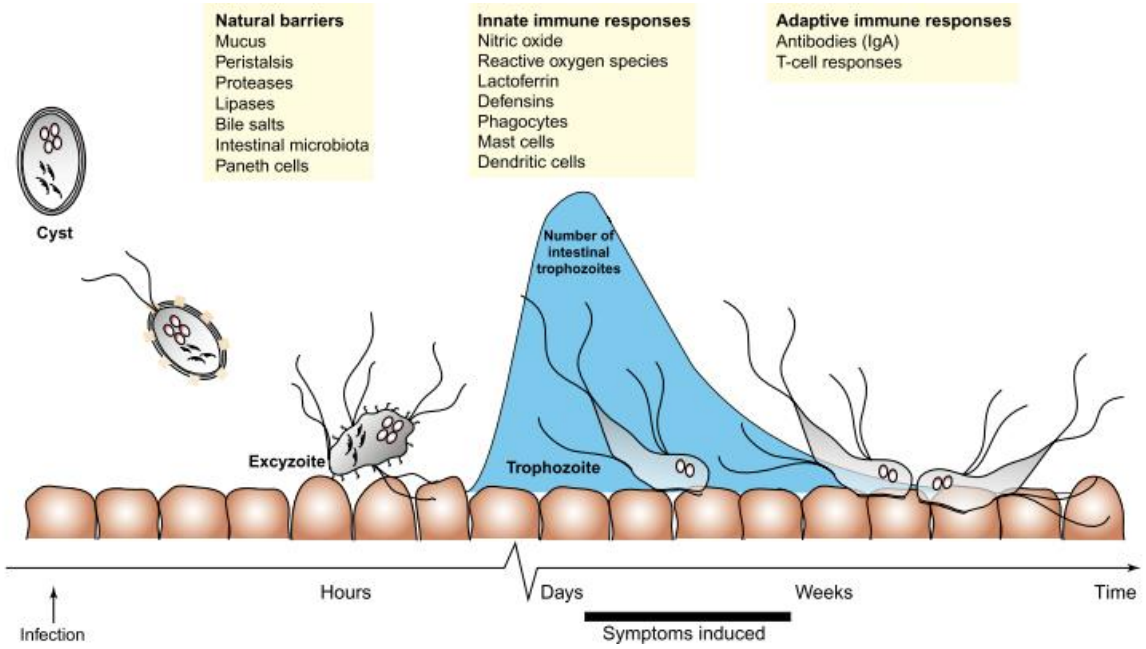


Figure 1.11 *Giardia* infection and immune responses of the host over time.

The timespan of giardiasis varies, but the incubation period is around one week. Natural barriers and the innate immune responses in the intestine are initial protection mechanisms against infection. Adaptive immune responses takes longer time to be established and is more specific. The figure is borrowed from [19].

$CD8^+$ T cells have not shown importance for the immunological control of murine infection, but have been shown to be a contributing factor for intestinal mucosal injury and

increased number of cells in the crypts and villus. Diffuse villus shortening is a result of the injury and makes the intestinal surface area for absorption of micro-and macronutrients smaller, and can thus lead to malabsorption and diarrhea [43].

Studies in mice shown that neither T_h1 nor T_h2 responses are not required for protection against an acute infection of *Giardia* [58]. This finding opens up the possibility that other T_h polarization could be important.

Giardia infections in cattle do often become of chronic character, and they can have cysts in their stools up to at least 112 days after being infected. It has been suggested that the adaptive immune responses in calves are not fully developed, giving fertile conditions for re-infections frequently [53]. *In vitro* stimulation of PBMCs from calves with live *Giardia* trophozoites has shown increased transcription of IL-17 and FoxP3 mRNA in proliferating $CD4^+$ T cells. This could mean that T_{reg} and/or T_h17 responses are important. $CD4^+$ αβ-T cells were shown to be proliferating in response to *Giardia,* supporting other findings that $CD4^+$ T cells play a key role in the protective immunity against *Giardia* infection [53].

Antibodies are shown to inhibit trophozoite growth, and to kill the ones they recognize. Antibodies secreted by plasma cells are probably directed towards VSPs on the surface of *Giardia.* The antibodies can trigger shifting of the VSPs, and make *Giardia* capable of evading the immune system. Trophozoites which express other VSPs than the antibodies can recognize, will evade this immune response and continue their replication in the host [51].

IgA-deficient individuals have shown not be remarkably more prone to infection than healthy individuals are [22]. However, individuals with common variable immunodeficiency experience chronic *Giardia* infection more often and cannot control the infection efficiently. [46, 58]. Deficiencies in B cell mediated production of antibodies when $CD4^+$ T cells are also not working normally is a probable reason for this [58].

1.4 Flow cytometry and principles

1.4.1 Flow cytometery - an introduction to principles and functions

Flow cytometry is a remarkable method for phenotyping and characterize cells [61]. Flow cytometers are multiparametric instruments, as they can record multiple information each cell is carrying simultaneously. By using flow cytometry a homogenous and small population can be filtered out from a larger heterogeneous population.

Using flow cytometry for cell analysis is based on the light-scattering properties (also known as fluorescence emission) that cells exhibit. Cytometry uses basic laws of physics such as electronics, optics and fluidics in order to function. Size, granularity, and if the cells are

stained with a fluorescent antibody/dye, gives the opportunity for flow cytometry to distinguish between different cell types. Figure 1.12 shows how a sample is analyzed in a flow cytometer.

When the flow cytomtric laser has sent its beam on the cells, light will be scattered around. Light scattered in low angles (0.5-10 °) gives information regarding cell size and it is termed forward-scattered light (FSC). Whereas light scattered in large angles (90 °) gives information regarding cell granularity and is termed side-scattered light (SSC).

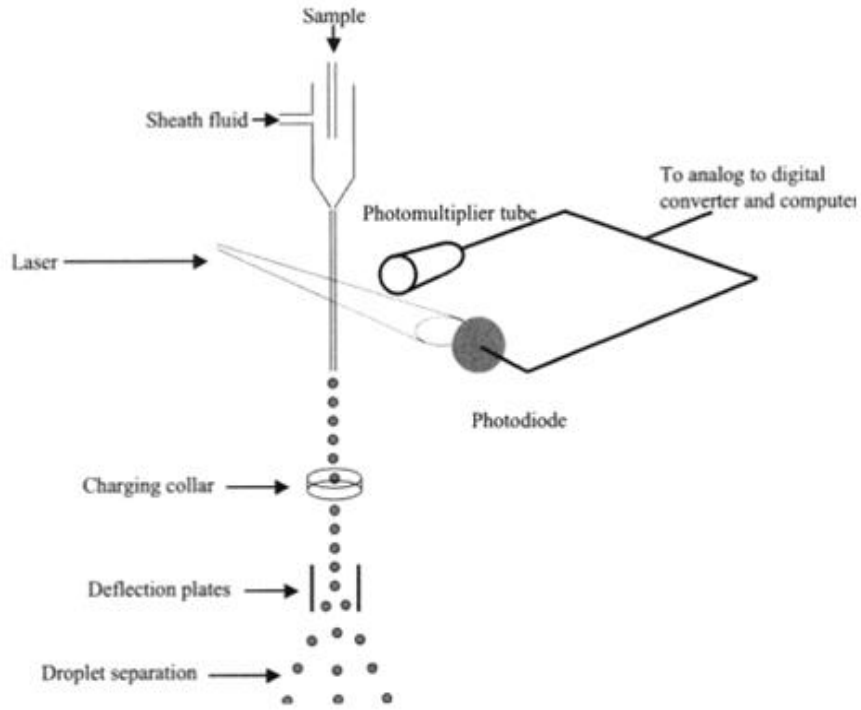


Figure 1.13: **Compartments of a flow cytometer**.

The analysis works by injecting a sample with cells into its system by using sheat fluid. The cells will be under pressure as they go into the system. A laser (many different can exist giving possibility of using different wavelengths) will send a beam out on the cells and they can become excited it carrying a stain or antibody. The light scattering from the cells will thereafter be picked up by a detector and a photomultiplier tube can convert signals and send it to a computer. Here the signals can be transformed to digital data. The signal can then be displayed on a computer screen. Figure is borrowed from [61].

1.4.1.1 Excitation and emission

Fluorescence means that that a substance absorbs light of a wavelength/color and thereby emit the absorbed light in a different wavelength/color. The wavelength for which the substance emits is generally higher. Some components of cells (pyridine- and flavin-containing nucleotides in addition to porphyrins) exhibit autofluoroscence, which mean that they emit absorbed light at a higher wavelength without being stained [61].

The variation between wavelength being absorbed and excited, is called Stoke's shift.

Some energy will also be lost to the surroundings as heat. A small Stoke's shift will mean that the difference between absorption wavelength and emitted wavelength is little, giving small difference between the wavelengths. A large Stoke's shift will on the other hand mean the opposite [62].

The physics behind flow cytometry, can be based on two equations:

Equation 1.1: $\lambda \times \nu = c$

Where λ = the wavelength in meters, ν = frequency in cycles/s and c = the speed of light defined as 3×10^8 m/s.

Equation 1.2: $E = h \times \nu$

Where E = energy in joules for one quantum of radiation and has a frequency of ν (cyces/s), h = Planck's constant ($6{,}626 \times 10^{-34}$ J/s and ν =frequency in cycles/s.

Equation 1.1 demonstrates the relationship between frequency and wavelength. Visible light exists between the wavelength of 400 nm to 700 nm [61].

Equation 1.2 demonstrates the energy which is related to the frequency of radiation [61].

Atoms in a molecule can exist in different energy states, where ground state does not require additional energy to be achieved. Atoms can be excited out of ground state and into a higher state level provided that the correct amount of energy is absorbed [61] . Figure 1.13 demonstrates how an atom can absorb energy and further emit energy of a different wavelength.

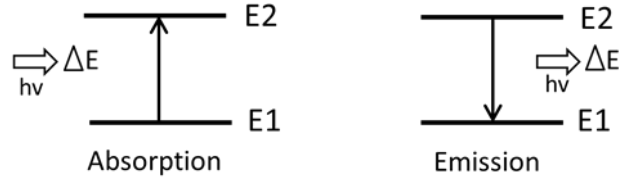


Figure 1.13. Absorption of energy to excite an electron and the emission of light at another wavelength. Energy which is supplied from the laser, hv, excites a valence electron from a lower energy state (E1) to a higher energy state (E2). Emission of the absorbed energy, will follow when the valence electron is dropped from a high energy state (E2), to a lower energy state (E1) and will subsequently release energy, hv.
Figure adapted from [61] and redrawn by Christina Skår Saghaug.

1.4.2 Spectral overlapping and importance of compensation

A fluorescent molecule, or a fluorochrome, bound to a cell will be excited when a laser hits it. A specific fluorochrome has a specific wavelength where the emission will be strongest. Still, different fluorochromes tend to have emission ranges falling into the same or share parts of a wavelength range. When this happens to two or more fluorochromes, it is termed "spectral overlap". A cell can be stained with multiple fluorochromes, and spectral overlap is not possible to avoid, as the more colors used - the more spillover [61].

In order to remove or improve spillover, compensation can be used. Compensation makes it possible to subtract spectral overlap fractions and thus making the signals seen more reliable. A compensation matrix can be made by singly staining beads separately with each of all the fluorochromes used in an assay. A flow cytometric analysis program can be used to make a compensation matrix, and by adding this to the cell analysis, the spectral overlap can be adjusted and subtracted.

1.4.3 Fluorochromes

In order to investigate surface receptors on cells, cytokines, viability and proliferation, a fluorocrome needs to be connected to the target in the cells. A fluorochrome can be conjugated to an antibody, and if the cell expresses the antigen (i.e. receptor or cytokine) for this antibody, it will bind. When a target in a cell cell is bound to a fluorochrome-conjugated antibody (FAB), the fluorochrome will be excited during flow cytometric analysis. The signal seen for fluorochrome stained cells will be at another wavelength than unstained cells [61] .

Fluorochromes are often distinguished into brightness levels according to how bright their fluorescence signal is. This is important to consider when making multicolor panels with a mixture of highly expressed and poorly expressed target. The brightest colors should be used for the poorly expressed targets. Cross-reactive binding of a fluorochrome can also occur, resulting in a higher non-specific background signal in the true negative cells [61].

1.4.3.1 Titrations

When a cell has been stained by a FAB adhering to it, it will be excited by a laser when analyzed in the flow cytometer. The signal from the stained cells can however overlap with the signal from the unstained cells.

It is therefore necessary to maximize the specific-to-nonspecific binding, termed as signal-to-noise ratio, meaning the optimal concentration of a fluorochrome. In order to find the optimal concentration for a fluorochrome, it should be titrated. By testing a dilution row of the FAB the most appropriate concentration of the FAB can be found, good separation between the unstained and the stained cell populations can be calculated using mean fluorescence intensity (MFI). The MFI for the stained and unstained cells can be found. The MFI of the positive cells are divided on the MFI of the unstained cells, and the concentration giving the highest ratio, gives the best separation [61].

1.4.3.2 Fluoroscence minus one

Fluoroscence minus one, or FMO, is a useful tool for the recognition of spectral overlap. When many different fluorochromes are used, the corresponding overlap between the emitting wavelengths will also increase.

If a FMO control is made for a fluorochrome used in an experiment, leakage from other fluorochromes in the assay and autofluoresence into its detection channel, can be acknowledged [63]. FMOs can also be helpful for setting gates determining the threshold for positive and negative cells.

1.4.3.3 Live/Dead discrimination

Dead cells can be a problem when using flow cytometry. Dead cells go through several changes, where the cell membrane will become permeable. Dead cells have been shown to bind FABs non-specifically. In analysis looking for rare events, dead cells can contribute considerably to false positives, and exclusion of these cells is important [64].

Many different methods exists to monitor viability, and measuring membrane permeability gives a good indication of the percentage of dead cells in a population. Such a staining dye can for instance react with DNA, and will only bind to dying or dead cells due to failing membrane integrety. Figure 1.14 illustrates how a staining dye, LIVE/DEAD viability dye (Life technologies) binds to dead cells, but not to live cells.

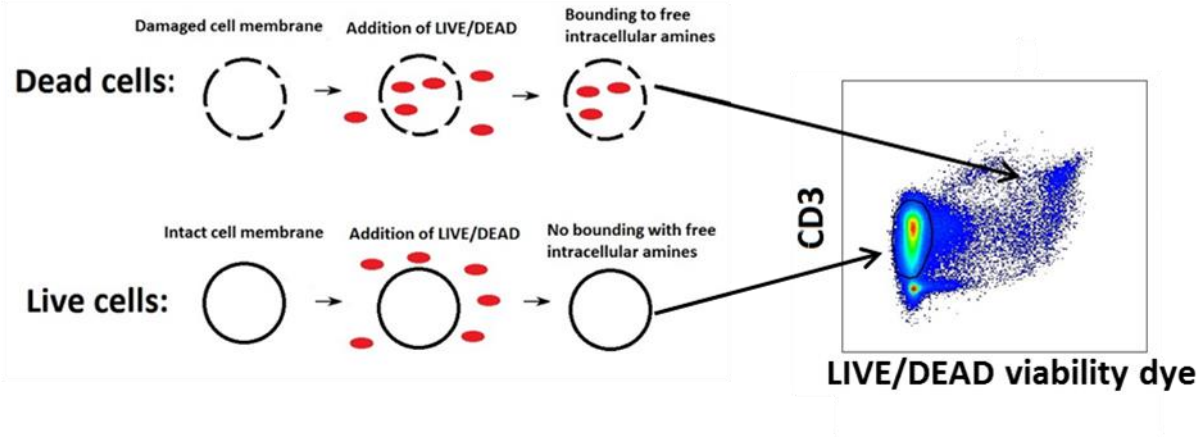


Figure 1.14: Demonstration of how a viability dye works.

Dead cells with damaged cell membrane will be stained with the LIVE/DEAD dye, and can be separated from the live cells, which will not be stained using flow cytometry. Figure is inspired by [64].

1.4.3.4 Proliferation monitoring by dye dilution

Cells that recognize or get activated by an antigen can undergo clonal expansion, proliferation [1]. Proliferation can be tracked by flow cytometry, where several dyes are available. One

proliferation dye, known as Carboxyfluorescein di-acetate succinimidyl ester (CFDA) diffuses into the cells and are cleaved by intracellular esters forming carboxyfluorescein succinimidyl ester (CFSE). The CFSE bind irreversibly to intracellular amines and if the cell is proliferating, daughter cells will contain half the concentration of the dye. The corresponding signal in a flow cytometer for daughter cells, will be of a lower intensity. Generations of the cells can thus be counted according to how many peaks are shifted towards zero in the specter [61, 65].

Another proliferation dye PKH, is lipophilic and will bind non-covalently to the cell membrane and become partitioned in the membrane. The concentration of the dye, will be halved for daughter cells when this dye is used as well [65].

Figure 1.15 demonstrates how proliferation can be tracked when staining with a dye (CellTrace), which diffuses into the cells and has similar properties as CFSE, but has a different fluorescent specter. The cell generations can be counted, and the percentages of proliferation can be calculated.

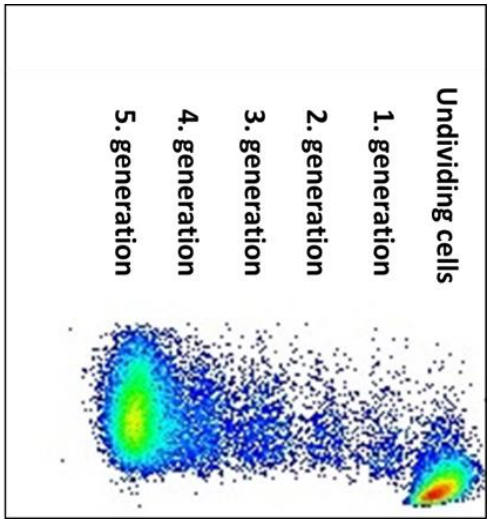


Figure 1.16 Proliferation tracking using flow cytometry.

A flow cytometric plot of undividing cells and dividing cells. The boxes represent the generations. Drawn by Christina Skår Saghaug.

1.4.4 Staining with fluorochromes

1.4.4.1 Surface staining

Fluorochrome-conjugated antibodies (FAB) made for surface receptors can be used to assess the main cell populations, (also called lineage) such as CD3, CD4, CD8 and CD19. Surface staining is also possible for many activation markers, which are up-regulated and expressed on activated cells. Surface staining makes it possible to isolate the cells of interest, and further look at i.e. activation and which proteins these cells produce. Lineage surface markers are often highly expressed, and therefore dim fluorochrome markers can be used for staining these.

1.4.4.2 Intracellular staining

Cells can also be stained intracellularly by using FABs. Cytokines produced inside a cell are often of particular interest, since their production is important for immune responses. The problem with cytokines is that after production they are released to the surrounding mileu. The Golgi apparatus is an organelle responsible for the production of proteins, such as cytokines. When staining intracellular components, it is crucial to add a protein transport blocker to "lock" the cytokines inside the Golgi apparatus, in order to be able to stain them [61, 63].

Since the cytokines are located inside the cell, the fluorochrome-conjugated antibodies need to get into the cell as well. This can be done by first fixating the cell (i.e. crosslinking of macromolecules and prevention from decay), followed by permeabilization where small pores are made in the cell membrane. In this way antibodies directed to cytokines can get inside the cells and bind to them.

1.4.5 Building a multicolor panel

If many fluorochromes are used together making up a multicolor panel, several considerations have to be taken into account, before the results can be trusted.

The optimal concentration for the desired fluorochromes have to be found by titration in order to separate the stained cells from the unstained cell population. The voltages used in the set-up will also be important, as these should be adjusted to avoid spectral overlap into other channels, and the unstained cell population should be distributed around zero in a flow cytometric plot.

Compensation have to be done to adjust spectral overlap, and if more color are used, more spectral overlap will occur. Compensation is therefore crucial in multicolor panels.

Different clones of FABs are available, binding to different epitopes of their cellular target. Clones for the same target molecule have different characteristics regarding non-specific binding, affinity to their target and compatibility with staining procedures and reagents. Some clones can be damaged during fixation and permeabilization and it is crucial to test their functions if used in an assay that requires intracellular staining. Non-specific binding can happen with fluorochrome and give rise to false positives. FMO controls should therefore be included in a flow cytometric assay to see how the spectral overlap manifest in the channel where one fluorochrome is missing. Non-specific binding of rare event or markers of activation can also occur in an unstimulated cell population. The clone used for the fluorochrome should then be investigated, and several ones should be tested if in doubt of non-specificity.

If a target for a antibody is a rare event (weakly expressed), it is important that the

conjugated fluorochrome have a relatively bright staining index. Weaker fluorochromes can be used for targets being highly expressed, such as lineage markers.

The stimulation time for a cell population can also be important for multicolor flow cytometry. The targets of interest will have to be up-regulated on the cells in order to stain them. One should therefore provide an optimal stimulation period.

1.5 Background for the present study

1.5.1Why do research on Giardia lamblia?

Giardia lamblia is an intestinal protozoan parasite giving rise to infection both in humans and animals worldwide. The clinical picture of *Giardia* varies from asymptomatic carriers to severe disease such as malabsorption syndrome. Even if the infection itself normally not result in serious disease and death, it can be a contributing cause to health problems.

Specific immune responses gained by *Giardia*, has generally been studied in mice, and acquired immunity have been seen in these models [55, 66]. The specific responses of T cells in humans have however not been well characterized. There are reasons to believe that various host responses against the parasite is both important for how the symptoms manifest and if the disease turns into chronic state or give rise to more serious complications such as malabsorption syndrome.

Human peripheral blood mononuclear cells (PBMCs) give an opportunity to investigate T cell responses in humans. Giardia-specific T cell responses have earlier been found using flow cytometric analysis [10]. These finding suggest that T cell responses should be investigated further and may be used to characterize *Giardia*-specific immunity.

A detailed insight into T cell responses can be investigated by using soluble proteins from *Giardia* trophozoites to stimulate PBMCs. Cytokines produced by $CD4^+$ T cells and $CD4^+$ effector memory T cells in early phases of stimulation combined with information of later proliferation and activation responses expressed by $CD4^+$ T cells, can be investigated using flow cytometry. Correlation between how cytokines expressed by effector memory cells will affect or not affect later activation can give valuable knowledge regarding specific memory immune responses.

A better insight and understanding for the progress and the duration of a *Giardia*-specific immune response in humans is important and needed for development of a protective vaccine. The study is potentially of interest not just for *Giardia*, but also for the host-microbe interaction for a wide range of gut pathogens.

An improved understanding of the progress, and length of *Giardia* specific immunity in human beings will, and approaches as how to assess these, will assist further to understand underlying general mechanisms for how pathogens may or may not elicit symptomatic disease. Advanced medicine regimes in order to get rid of an infection can lead to decreased compliance and lead to resistance, and understanding immune responses is important to avoid this by develop a vaccine [36, 67].

1.5.2 Aims of the study

An earlier flow cytometric study conducted [10], suggested that cellular immunity against *Giardia* could last up to five years. The surface markers CD25, CD26, CD45RO and HLA-DR were examined in this study in addition to the general proliferation on lymphocytes.

This project aimed to characterize *Giardia*-specific memory $CD4^+$ T cell immune responses in recently infected individuals, by looking at early cytokine responses in the general $CD4^+$ T cell population but also in the effector memory $CD4^+$ T cell population, later surface activation in addition to proliferation of $CD4^+$ T cells.

Specific aim 1: To develop two flow cytometric assays capable of measuring Giardia-specific T cell cytokine responses, as well as proliferation and activation markers was performed in this study.

Specific aim 2. To test performance of these assays using a group of giardiasis exposed persons compared to low risk healthy controls.

Specific aim 3. Given that *Giardia* specific immunological responses were seen in the flow cytometric assays, did the findings correlate with one another, and did they support earlier findings?

2. Materials

2.1 Giardia antigen preparation

2.1.1 Origin of Giardia antigens

In October 2013, *Giardia* antigens were sent to the Department of Clinical Science, University of Bergen, Bergen, Norway from the Department of cell and molecular biology, Uppsala University, Uppsala, Sweden. The laboratory work regarding growing trophozoites, harvesting and acquiring of the proteins in the sonicated lysates was done in Uppsala, Sweden.

2.1.2 Harvesting, lysation and sonication of Giardia trophozoites

Giardia assemblage A (WB-C6, ATTC 50803) and B (GS/M, ATTC 50581) trophozoites were grown in separate Diamond- and Keister medium (TYDK medium) supplemented with bile, supporting the methods of Keister [68], at a temperature of 37 °C.

The trophozoites were collected from a 50 mL falcon tube with an 80 % confluence (approximately $5x10^6$ cells) and washed 3 times in cold sterile PBS.

The cells were harvested at 4 °C using centrifugation at 2500 rounds per minute (RPM) for 5 minutes and re-suspended in 5 mL sterile PBS. The re-suspended cells were snap-freezed-thawed in liquid nitrogen twice and sonicated (3 times for 30 seconds at 50 Watts). Membrane and cell debris were removed by centrifugation at 4 °C at 13000 RPM for 15 minutes. The supernatants containing *Giardia* soluble protein fractions were sent on dry ice to the Department of Clinical Science, University of Bergen, Bergen, Norway and stored in at – 70 °C until further investigation.

2.1.3 Concentrations of the Giardia soluble proteins

The protein concentrations were measured in the received *Giardia* soluble proteins solutions. Measurement was done using the DIRECT DETECT™ system (EMD Millipore corporation, Billerica, MA, USA). The *Giardia* protein solutions were then diluted to 50 µg/mL in X-vivo medium and stored at -20°C[1].

These *Giardia* soluble proteins, named SSA for *Giardia* assemblage A and SSB for *Giardia* assemblage B, were later used to stimulate peripheral blood mononuclear cells (PBMC) in order to elicit *Giardia*-specific T cell responses. The concentrations used for PBMC

[1] This work was done by lab technician Steinar Sørnes

stimulation are shown in Table 2.1.

Table 2.1: Concentrations of the two *Giardia* isolates used in the project and final concentrations used in stimulation culture. The measured concentrations of the received solutions of *Giardia* proteins, the concentration of the stock solutions and the final concentration used to stimulate PBMCs. The sonicated supernatant proteins were used to stimulate cells on day one and day six.

***Giardia* Assemblage**	**Mean[2] measured concentration [mg/mL]**	**Stock concentration [µg/mL]**	**Final concentration [µg/mL]**	**Assay**
Assemblage A, WB-C6[3] (**SSA**)	6.091	50	10	Day one and six
Assemblage B, GS/M[3] (**SSB**)	4.429	50	10	Day one and six

2.2 Reagents for positive and negative controls

Reagents used as positive controls were chosen depending on their stimulation capacity. Lipopolysaccharide (LPS) is a powerful macrophage activator, and macrophages can activate T cells [1]. Staphylococcal enterotoxin B (SEB) is classified as a superantigen and can stimulate more T cells than conventional antigens. SEB has a capacity to stimulate naïve $CD4^+$ cells into proliferation [1]. Purified protein derivative (PPD) was used, as this antigen can stimulate T cells of previously vaccinated individuals [1]. Phorbol 12-myristate 13-acetate (PMA) and Ionomycin calcium salt (IC) were used in combination. PMA can diffuse directly through T cell membranes and activate cells without MHC presentation of antigens.
IC is a reagent triggering calcium release and works synergistically with PMA [69].

[2] Mean of two protein concentration measurements
[3] Kindly provided by Staffan Svärd and his group in Uppsala.

2.2.1 Reagents used for positive controls

Positive controls were used in every experiment to ascertain cell responses. Table 2.2 shows the reagents used for positive controls in the project.

Table 2.2: Concentrations of reagents for the PBMCs stimulation and which assay they were used.

Name of antigen reagents	Stock concentration [mg/mL]	Final concentration [µg/mL]	Assay
Lipopolysaccharide (**LPS**), *Salmonella typhimurium* Rouge strains[4]	0.5	1	Day one
Phorbol 12-myristate 13-acetate (**PMA**) Chemical[5]	1	0.02	Day one
Ionomycin calcium salt (**IC**), *Streptomyces conglobatus*[6]	1	0.5	Day one
Purified protein derivate (**PPD**), *Mycobacterium tuberculosis*[7]	1	10	Day one and day six
Staphylococcal enterotoxin B, (**SEB**) *Staphylococcus aureus*[8]	500	0.1	Day six

[4] Sigma Aldrich, product number: L9516, 5mg dissolved in 10 mL sterile NaCl and stored at -20°C in aliquots.
[5] Sigma-Aldrich, product number: P8139, 1 mg, diluted in 1 mL DMSO and stored at -20°C in aliquots.
[6] Sigma-Aldrich. Product number I0634, 1 mg, diluted in 1 mL DMSO and stored at -20°C in aliquots.
[7] Statens Serum Institut, Copenhagen, Merida number: 3704627. 1mg/mL 1 mL test tubes stored at 4-8°C.
[8] Kindly provided by Ida Wergeland, originally from Sigma-Aldrich, concentration 500 µg/mL stored at 4-8°C

2.2.2 Negative control

Table 2.3: **Cells in medium without stimulating agents was used as a negative control in this project**

Name of medium	Application day	Supplier
X-vivo 15 with Gentamicin and Phenol red (**MED**)	Day 1 and day 6	Lonza via BioNordika

2.3 Solutions made or diluted in the laboratory

Table 2.4: Solutions made or diluted for this project

Solution	Ingredients and storage
Dulbecco's Phosphate Buffered Saline (PBS) pH 7.4. Filtered for unsterile usage. Filtered and autoclaved for sterile usage.	8 g NaCl + 1.44 g $Na_2HPO_4 \cdot 2H_2O$ + 0.2 g KCl + 0.2 g KH_2PO_4 and adjusted up to 1 L with Milli-Q water. Stored at 2-8°C
Paraformaldehyde 2 % (w/v) in PBS. (PFA). Filtered before usage.	2 g paraformaldehyde (Sigma-Aldrich P-6148) was added to every 100 mL PBS and heated to 65 °C until dissolved. Fresh solution was made every 2 weeks. Stored at 2-8 °C
Perm/Wash 1:10 dilution (PW). Filtered before usage.	1 mL 10x Perm/Wash (BD Biosciences Franklin Lakes, New Jersey, USA) was added to every 9 mL milli-Q water making a solution of 10 % Perm/Wash. Stored at 4 °C. Throughout the project, a fresh made solution was made for every new experiment.
Brefeldin 5 mg/mL	5 mg Brefeldin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1 mL Dimethyl sulfoxide (DMSO). Stored in aliquots at -20 °C.
Serum 10 % in PBS	550 µL of Pooled human serum, drawn the 17.04.2008 (Infectious laboratory, Haukeland University hospital, Bergen, Norway), was diluted in 4950 µL PBS and stored at 4-8 °C. Fresh solution was made for every new experiment.

2.4 Kits used in the project

Table 2.5: Purchased kits used in this project

Kit name	Supplier	Catalog nr
LIVE/DEAD Fixable Near-IR Dead cell Stain Kit (Dye coupled to APC-H7)	Life Technologies	L10119
Anti-Mouse Ig, κ/Negative Control (FBS) Compensation Particles Set	BD Biosciences	552843
Anti-Rat and Anti-Hamster Ig κ/Negative Control Compensation Particles Set	BD Biosciences	552845
BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit	BD Biosciences	554714
CellTrace™ Violet Cell Proliferation Kit (Dye coupled to the fluorochrome Pacific-Blue)	Life Technologies	C34557

2.5 Equipment for cell harvesting and culturing

Table 2.6 Tubes and plates used for cell harvesting and stimulation

Equipment	Supplier
BD Vacutainer CPT Na-Heparin 8 mL	BD Biosciences
Centrifuge tube 15 mL Polypropylene	Sarstedt
Centrifuge tube 50 mL Polypropylene	Sarstedt
Tissue Culture 96-well Vee bottom (96 V-well plate)	Sarstedt

2.6 Fluorochrome-conjugated antibodies (FABs)

Table 2.7: Overview of the FABs used in this project.

All the antibodies and dyes used in this project were directly coupled to a fluorochrome.

Antibody	Clone	Fluorochrome	Isotype	Concentration	Supplier	Catalog nr.
CD3	UCHT1	Alexa Fluor® 700	M* IgG1, κ	500 µg/mL	Bio Legend	300424
CD8a	RPA-T8	Brilliant Violet 711™	M* IgG1, κ	50 µg/mL	Bio Legend	301044
CD4	L200	PerCP-Cy™5.5	M* IgG1, κ	25 µg/mL	BD Biosciences	552838
CD14	M5E2	APC-H7	M* IgG2a, κ	50 µg/mL	BD Biosciences	561384
CD45RA	Hl100	Brilliant Violet 510™	M* IgG2b, κ	50 µg/mL	Bio Legend	304142
CD45RO	UCHL1	Brilliant Violet 605™	M* IgG2a, κ	100 µg/mL	Bio Legend	304238
CD26	BAgb	PE	M* IgG2a, κ	50 µg/mL	Bio Legend	302706
CD25	M-A251	APC	M* IgG1, κ	1.5 µg/mL	BD Biosciences	555434
HLA-DR	TU36	FITC	M* IgG2b, κ	25 µg/mL	BD Biosciences	555560
IL-17A	BL168	Brilliant Violet 605™	M* IgG1, κ	50 µg/mL	Bio Legend	512325
IL-10	JES3-19F1	PE	R# IgG2a	25 µg/mL	BD Biosciences	559330
IL-4	MP4-25D2	APC	R# IgG1, κ	200 µg/mL	Bio Legend	500812
TNF-α	Mab11	Brilliant Violet 421™	M* IgG1, κ	100 µg/mL	Bio Legend	502932
IFN-γ	B27	FITC	M* IgG1, κ	50 µg/mL	BD Biosciences	552887
CD197	150503	PE-CF594	M* IgG2a	100 µg/mL	BD Biosciences	562381

*M = mouse and #=rat

2.7 Study population

Two groups of people were recruited in this experiment in order to evaluate differences between a Giardia exposed group and a control group. All the individuals in the study had previously received BCG vaccine against tuberculosis.

2.7.1 Giardia exposed group

Fifteen consecutively identified adults with recent (last 26 months) symptomatic chronic or acute giardiasis were eligible for inclusion. The majority of these individuals were returning travelers. The infection was laboratory confirmed by routine light microscopy. Participants in the *Giardia* exposed group were given study IDs starting with Ag.

2.7.2 Low risk healthy controls

Eleven age and sex matched controls with a low risk of ever having had giardiasis were eligible for inclusion. A low risk healthy control was defined as never having travelled to highly endemic areas (low and middle income countries), not drinking contaminated water in Bergen the Autumn 2004, or known previous giardiasis and having no relatives with known giardiasis in the past.

Participants in the low risk healthy control group were given study IDs starting with LR.

2.7.3 Exclusion criteria for both groups

Exclusion criteria for all groups were age below 18 or above 70, known immunosuppression or ongoing treatment with immunosuppressive medication and autoimmune diseases.

2.8 Instruments and incubator

2.8.1 Flow cytometer

BD LSR Fortessa™ Cell Analyzer (BD BioSciences, Franklin lakes, New Jersey, USA) was used to gather fluorescence properties of cells.

2.8.2 Cell counting

MUSE™ Cell Analyzer (Millipore Corporation, Billerica, USA) was used to count cell concentrations and to assess viability of freshly acquired PBMCs before culture stimulation.

2.8.3 Centrifuge

The centrifuge used for both tubes and plates was a Centrifuge 5810 R (Eppendorf, Hamburg, Germany).

2.8.4 Eppendorf centrifuge

The centrifuge used for spinning down aggregates in FAB mixes was a Centrifuge 5417 C (Eppendorf, Hamburg, Germany).

2.8.5 CO2 incubator

For the stimulation of PBMCs with antigens, a CO_2 incubator model MCO-15AC (Sanyo Electric Co., Ltd, Moriguchi, Osaka, Japan) was used.

2.9 Computer software

2.9.1 Word

The project was written and edited using Word 2013 (Microsoft corporation, Redmond, Washington, USA).

2.9.2 Excel

Graphs and histograms were made using Excel 2013 (Microsoft corporation, Redmond, Washington, USA).

2.9.3 Flow cytometer software

For acquiring data from cell samples, BD Facsdiva version 8 (BD Biosciences, Franklin Lakes, New Jersey, USA) was used. The data was collected as FCS-files, and was transferred to other computers for further investigations.

2.9.4 Flow cytometric analysis program

Analysis of FCS-file data from BD Facsdiva was done in FlowJo version X10 (Tree star Inc., Ashland, Oregon,USA).

2.9.5 Statistical analysis program

To assess statistical significance of comparisons between the groups and responses, Mann-Whitney U non-parametric test, linear regression using Pearson's correlation coefficient test, Fisher's exact test and non-parametric Kruskal Wallis test were applied.

The Statistics software used was IBM SPSS 21 (IBM corp, Armonk, New York, USA).

3. Methods

3.1 Recruitment of individuals

3.1.1 Recruitment of healthy volunteers for method development

Staff and students working at the University of Bergen or Haukeland University hospital were asked to donate blood for the purpose of method development and flow cytometric testing. Some of these healthy volunteers agreed to donate blood several times for method testing, and to serve as low risk healthy controls in the study.

3.1.2 Recruitment of participants to the study; healthy low risk controls and giardiasis exposed persons

Two groups were considered to be relevant for testing the assay. Persons exposed to *Giardia*, and persons with low risk of ever having had giardiasis. The exposed group were recruited through e-mail, phone call or both, based on data from the microbiological laboratory at Haukeland University Hospital. Low risk controls were recruited through the research group's network, and among students, hospital and laboratory staff by direct contact.

Both groups had to answer a set of questions in a Case Report Form (CRF), before the participants could be included/excluded in the study. The CRF contained information regarding exclusion criteria, the nature of the *Giardia* infection, giardiasis risk for low risk healthy controls, abdominal symptoms the past 2 weeks, regular medication, in addition to sample related data. The CRF used in this project is attached in Appendix A. An informed consent regarding participation, storage of samples and storage of personal information, had to be filled in and accepted by the participants. The informed consent form is attached in Appendix B.

During the study period of two months, fifteen out of eighteen available *Giardia* exposed individuals accepted to participate in the study. Eleven healthy controls with a low risk for ever having had giardiasis were recruited. One control had to be excluded due to later information about travel to a *Giardia* endemic area. Thus, 10 low risk healthy controls was included in this study.

The participants were older than 20 and younger than 70 and all turned out to be Caucasians (ethnical Norwegian). All of the participants were tested for *Giardia* by PCR.

3.1.3 Investigation of ongoing giardiasis

To determine if participants were *Giardia* infected at the time of the study, a stool sample was collected from all participants and analyzed for presence of *Giardia* by polymerase chain reaction (PCR). The PCR assay was performed by other laboratory staff[9].

The *Giardia* status of participants had to be known to exclude low risk healthy controls and to be able to treat individuals with ongoing infection.

If the PCR test was positive, the individuals were treated with metronidazole 400-500 mg three times a day for 7-10. A second line treatment, albendazole 400 mg in combination with 250 mg metronidazole two times a day for seven days, was used in order to get rid of infection.

3.2 Collection of peripheral blood mononuclear cells (PBMCs)

Human blood contains many different cells and in order to isolate PBMCs, BD Vacutainer® CPT™ Tube with Na^+/heparin (CPT) was used. These tubes contain a polyester gel and a dense solution and cells in human blood can be separated by centrifugation due to differences in density. The human blood would be segregated into different layers with plasma on the top followed by mononuclear cells and platelets. The dense solution and polyester gel separated the granulocytes and red blood cells, which would be at the bottom of the tube after centrifugation. Figure 3.1 demonstrates a CPT tube filled with blood before and after centrifugation.

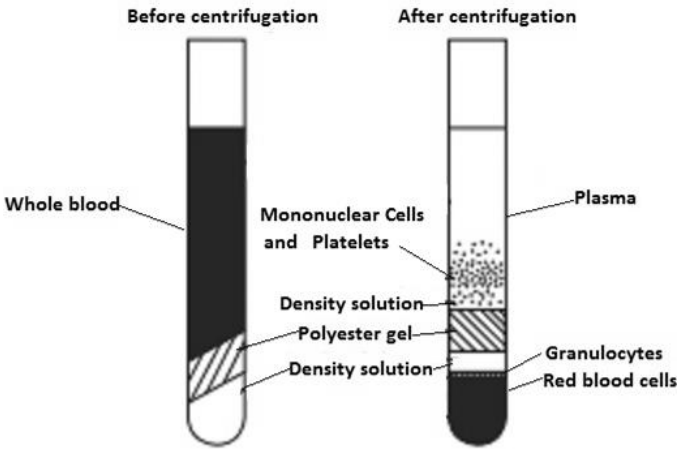


Figure 3.1: CPT tube before and after centrifugation.

Adapted from manufacturers protocol [70].

[9] Done by medical students Martin Kristiansen and Torunn Hjøllo

3.2.1 Procedure of cell harvesting

The procedure of cell harvesting was done in sterile environment using sterile equipment and safety bench. Before blood samples were drawn from the recruited participants or volunteers for method development, one 50 mL size conical centrifuge tube with cap was filled with 20 mL sterile PBS. If cold PBS was used, it had to reach room temperature (RT), approximately 20°C, before the cells were added. One 50 mL centrifuge tube was used for each participant and was marked with study ID.

Blood was collected into 4 CPT tubes per participant using standard venipuncture technique. The CPT tubes had to be inverted 10 times to ensure good mixing of blood and chemicals inside the tube. The CPT tubes were labeled with study number. All the blood sampling was done in the laboratory for infectious diseases.

The time from collection until CPT tube centrifugation did not exceed two hours. The CPT tubes were inverted 10 times immediately prior to centrifugation. Centrifugation of the tubes was done at 1800 Relative Centrifugal Force (RCF) at RT for 20 minutes.

Collection of the PBMCs was done immediately after centrifugation. The plasma layer above the PBMC layer was pipetted away and discarded in a biohazard container. The PBMC layer was then collected using a sterile pipette and tips and transferred into the previously filled 50 mL centrifuge tube with RT sterile PBS. All the PBMCs in the CPT tubes from the same participant were added into the same 50 mL centrifuge tube. The tube was then filled with PBS up to a final volume of 50 mL. The tube was capped and the contents mixed by inverting the tube 5 times.

The cells were then pelleted by centrifugation for 15 minutes at 300 RCF at RT. The supernatant was decanted carefully without disturbing the cell pellet. The cell pellet was re-suspended in the remaining volume in the centrifuge tube, and transferred to a 15 mL conical centrifuge tube using a pipette. To obtain all the cells from the 50 mL centrifuge tube, 1 mL PBS was added, gently rotated, and transferred to the 15 mL centrifuge tube. The 15 mL centrifuge tube was filled to a final volume of 15 mL with PBS. The centrifuge tube was capped and inverted 5 times.

The tube was centrifuged for 10 minutes at 300 RCF at RT and the supernatant was decanted carefully. The cells were re-suspended in the residual volume. X-vivo medium was added to the 15 mL tube to a final volume of 5 mL.

These PBMCs in medium were in the first period of the project used to develop the flow

cytometric method for characterizing human T cell responses against the enteric protozoan parasite *Giardia lamblia*. In the last period of the project, PBMCs obtained by the same protocol were used in the optimized flow cytometric assay to analyze human T cell responses.

3.3 Method development

3.3.1 Titrations of the fluorochrome conjugated antibodies and dyes

To determine the most suitable concentration of the fluorochrome-conjugated antibodies (FABs) and dyes, a dilution series was done. Titrations were done on freshly harvested or stimulated cells. To titrate intracellular antibodies directed towards cytokines, 4 hours stimulation with PMA and IC was sufficient, while the proliferation and surface activation markers were stimulated over a longer period of time (6 days) to ascertain positive cells for these markers.

The washing of cells in tubes was always done by centrifugation at 350 RCF for 5 min. at RT. Cells in plates were washed by centrifugation at 450 RCF for 5 min. at RT and after permeabilization/fixation cells were washed by centrifugation at 600 RCF for 5 min. at 4 °C. The supernatants were discarded by decanting for tubes and flicking for plates.

3.3.1.1 Protocol for titration of CD3, CD4, CD8a, CD14 and LIVE/DEAD

Freshly harvested PBMCs from 3.2.1, in the 15 mL centrifuge tube, were washed 2 times with PBS. A dilution series for each fluorochrome was done using Eppendorf tubes. A 2-folddilution series with final staining concentrations of 5.0 µL, 2.5 µL, 1.25 µL, 0.63 µL and 0.31 µL FAB per 100 µL cell solution was used for CD3, CD4, CD8a and CD14. To the first Eppendorf tube in the dilution series, 90 µL PBS was added and 10 µL of a FAB. The four other Eppendorf tubes in the dilution series were filled with 50 µL PBS. The dilution was done by taking 50 µL from the first tube in the series, mixing it with the next and take 50 µL of this and transferring it to the next tube. The remaining 50 µL from the dilution series was discarded.

The dilution series of LIVE/DEAD was done likewise, but first 50 µL of Dimethyl Sulphoxide (DMSO) was added to the LIVE/DEAD vial and mixed. 1 µL of this dye was added to 999 µL PBS in an Eppendorf tube. 500 µL PBS was added to the other Eppendorf tubes (3 Eppendorf tubes in total).

The cells were spun down by centrifugation and re-suspended in 1150 µL PBS. 50 µL of this cell suspension was added to the wells of a 96-V-wells plate giving columns of cells for each fluorochrome. 50 µL of each of the fluorochromes in the dilution series was added to the

cells. The cells were incubated with the FABs or the dye for 30 min. in the dark at room temperature. The cells were washed two times with PBS. The cells were transferred into marked flow tubes, with a final volume of 300 µL before analysis was done on the flow cytometer the same day.

3.3.1.2 Protocol for titration of TNF-α, IFN-γ, IL-17A, IL-10, IL-4, CD197 and CD45RA

Freshly harvested PBMCs from 3.2.1 with a volume of 5 mL in a 15 mL centrifuge tube was placed in a CO_2-incubator over-night. The next day the cells were stimulated with an antigen.

The stimulation was done by thawing 4 µL frozen PMA and 40 µL IC and dilute them with 9996 µL PBS and 3960 µL PBS, respectively, in 15 mL centrifuge tubes. The two stimulation antigens were added to the cell suspension (280 µL of each), making a final concentration of 0.02 µg/mL of PMA and and 0.5 µg/mL of IC. Brefeldin A was also added (11.2 µL) in the final concentration of 10 µg/mL. The cells were put back in the CO_2 incubator for an incubation time of six hours.

After the incubation time, the cells were washed two times with PBS. The stimulated cells were re-suspended in 1500 µL and 50 µL of this cell suspension was transferred to a 96 V-wells plate making 7 columns of cells, one for each fluorochrome. A 2-fold dilution series was done for CD197 and CD45RA by adding 10 µL of each fluorochrome into 90 µL PBS to the first Eppendorf tube in the dilution series and dilute as stated in 3.1.1.2. Two of the cell columns in the plate were used for the two surface markers CD197 and CD45RA, where 50 µL staining solution from the dilution series was added to the wells. At the same time, all of the cells were stained with the optimal concentration for CD3 followed by an incubation in the dark for 30 minutes at RT. The cells were washed two times by centrifugation. Before the titration of the cytokines could be carried out, the cells had to be permeabilized and fixated. This procedure is stated in 3.6.1.3. The cells were kept on ice further on.

A 2-fold dilution series was used for TNF-α, IFN-γ, IL-17A, IL-10 and IL-4. To the first Eppendorf tube in the dilution series, 80 µL PW was added and 20 µL of a FAB. The dilution series was done the same way as stated in 3.1.1.2.

The cells were incubated with the intracellular FABs for 30 min. in the dark at 4°C. The cells were washed two times with PW and two times with PBS. The cells were re-suspended in PBS and transferred into marked flow tubes, making a final volume of 300 µL. Analysis was done the same day on the flow cytometer.

3.3.1.3 Protocol for titration of CD25, CD26, CD45RO, HLA-DR and CellTrace

Freshly harvested PBMCs from 3.2.1, in the 15 mL centrifuge tube, were used for the titrations. 196 µL cells per well was transferred to a 96 V-wells plate, making a total of 4 columns with 5 rows, suitable for a 2-fold dilution series.

1 mL of cell suspension was left in the centrifuge tube, and was used for the CellTrace titration. This was done by adding an excess of 500 µL medium to the centrifuge tube, mix the contents and divide the suspension into three 15 mL centrifuge tubes, with 500 µL cell suspension in each tube. The CellTrace staining dye had to be mixed before labeling of cells could be done. The mixing was done by adding 20 µL DMSO to a CellTrace vial (from CellTrace™ Violet Cell Proliferation Kit) and 2 µL of this fluorescence dye was mixed with the first cell suspension, 1 µL and 0.5 µL was added to the other ones in the dilution series. The cells were incubated for 10 min in the dark at RT. The cells were washed two times by centrifugation at 350 RCF for 5 min. at RT by filling the tubes with medium. The cells were re-suspended in 196 µL medium and transferred to the same 96 V-wells plate with the previously added cells.

8 µL of SEB (Sigma-Aldrich, concentration 500 µg/mL), was diluted in 72µL PBS and 4 µL of this antigen mix was added to all the wells, making a final concentration of 0.1 µg/mL. The plate was placed in a CO_2-incubator for 6 continuous days.

After the antigen stimulation, the cells were washed two times out of the stimulation medium with PBS. The cells were re-suspended in a total volume of 50 µL.

A dilution series for each fluorochrome was done using Eppendorf tubes. A 2-fold dilution series was used for CD25, CD26, CD45RO and HLA-DR. To the first Eppendorf tube in the dilution series, 90 µL PBS was added and 10 µL of a fluorochrome. The dilution was done the same way as stated in 3.3.1.1. The CellTrace labeled cells were at the same time stained with the optimal concentration of CD3. The cells were incubated for 30 min in the dark at RT. The cells were washed with PBS two times before they were transferred into marker flow tubes, making a final volume of 300 µL and analyzed the same day on the flow cytometer.

3.3.1.4 Finding the optimal concentrations from the titrations

The optimal concentrations used in this assay for a FAB or a dye, were based on the results from titrations. The MFI for a positively stained cell population was found in the flow cytometric analysis program. The MFI for a positive cell concentration was divided by the MFI for a negative population, and would give a ratio. The highest ratio would mean the maximum signal-to-noise ratio, and thus the best separation between the two cell populations.

3.3.2 Compensation and voltage settings

Before compensation was done, different voltages were tested for each of the fluorochromes. The testing was done by checking the spectral overlap into other channels using histograms for all the parameters used, in the flow cytometer software BD Facsdiva. The negative stained beads should lie around zero for the optimal voltage as well.

A compensation matrix was obtained using Anti-Mouse Ig, κ/Negative Control (FBS) Compensation Particles Set and Anti-Rat and Anti-Hamster Ig κ/Negative Control Compensation Particles Set. The different FABs were mostly from mouse, but a few were produced in rat. The relevant type of compensation beads for each fluorochrome were used in order to get a positive staining. CellTrace labeled PBMCs were used when obtaining the compensation matrix where Celltrace was included.

Flow tubes were used when the compensation was carried out and one flow tube was designated to each of the FABs or dyes. The beads from the Compensation Particles set were vortexed and one drop of the positive control and one drop of the negative control was added to every flow tube. For the CellTrace compensation, cells labeled with CellTrace and unstained cells were added to a flow tube.

The beads were washed with PBS two times. The optimal concentration for each of the fluorochromes found in the titrations were used. The beads were incubated 30 minutes in the dark at RT and washed one time and re-suspended in 300 µL PBS.

The compensation beads were run on the flow cytometer the same day as the staining was carried out.

3.3.3 Fluorescence minus one (FMO)

FMOs were done for all the experiments involving recruited individuals to the study. The FMOs were done as a quality control for the gating strategy and also to determine spectral overlap/spill-over from other channels, into the channel where one fluorochrome was missing.

3.3.3.1 FMOs for cytokines

Cytokines are dim markers and few positive cytokines can be seen in a large cell population. It is important to exclude false positives, and FMOs can be used to guide gating. When an intracellular staining was done, FMO staining cocktails where made for each of the cytokine FABs. This was done by adding all the FABs, except the one in question.

3.3.3.2 FMOs for surface markers

When surface activation markers FABs were added to the wells, FMO staining cocktails were added as well. This was done by adding all the surface activation FABs, except the one in question.

3.3.4 Exploring CD8⁺ responses

$CD8^+$ and $CD4^+$responses were investigated by testing two fluorochrome panels where perforin, CD69 and CD107a was included (information in Appendix D).

CD69 and perforin were titrated the same was as CD45RA and CD197, while CD107a was titrated using six days of stimulation where this FAB was added in a dilution series to PBMCs in the last six hours of stimulation and a cytokine transport blocker protein, monensin (Appendix D), was added to the wells during the staining. These FABs were tested the same way as 3.6.

3.5 Cell counting, adjusting cell concentrations and stimulation times

3.5.1 Cell counting

Cell counting was done by diluting 20 µL of cell suspension from freshly harvested cells with 380 µL or 780 µL MUSE™ count and viability kit (EMD Millipore corporation, Billerica, MA, USA) . The dilution factor used was determined by the cell pellet size during harvesting. The counting was carried out using MUSE™ Cell Analyzer according to core facility's instructions.

3.5.2 Cell concentrations

According to the cell count, the PBMC stock solution had to be separated into two tubes with two different concentrations. One concentration was used for the day one assay and another one for the day six assay. In the day one assay, concentrations of $6\text{-}10x10^5$ cells/mL were used. In the day six assay concentration of $2x10^5$ cells/mL were used. The total volumes needed for the assays were calculated with regard to how many wells were used in each setup (duplicates or triplicates and extra wells for FMOs).

3.5.3 Stimulation time and antigens

3.5.3.1 Plates and stimulation time for day one assay

Two 96 V-wells plates, one for the day one assay and one for the day six assay, were prepared with 100 µL of the different antigens. For day one, SSA, SSB, PPD and LPS were put into wells in twice the final concentration needed. 100 µL MED was added to the negative control wells

in addition to the wells designated PMA/IC, since the addition of these antigens was done the next day. 100 µL cell suspension for day one was added to each well with the antigens and controls. The 96 V-wells plate was placed in a CO_2 incubator at 37°C for stimulation for period of 24 hours. The day six cells had to be labeled with CellTrace before incubation with antigens.

Figure 3.2 demonstrates a typical set up for the 96-V wells plates with antigens and negative controls used in day one and day six assays.

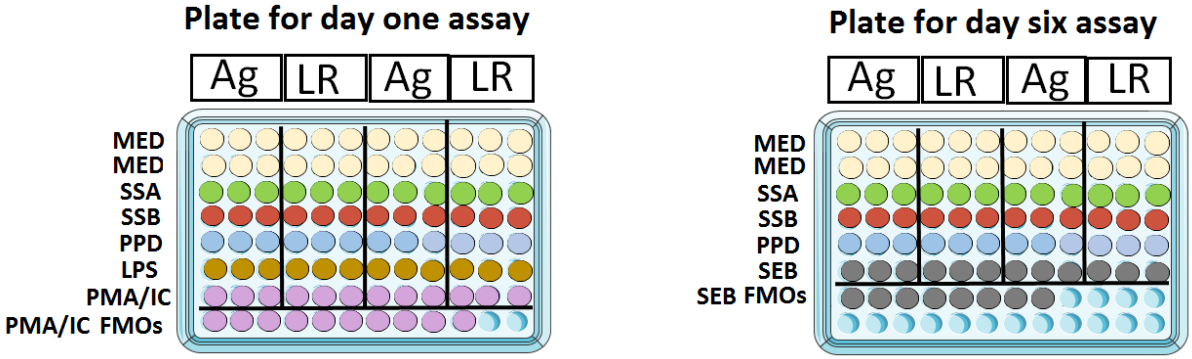


Figure 3.2: Stimulation plates for day one and day six assay.

Antigens used for the stimulation of PBMCs. These plates represents an experiment using 2 individuals from each group (Ag and LR) and triplicates used for each antigen and two triplicates for the negative control, MED.

3.5.3.2 Labeling day six assay PBMCs with CellTrace and stimulation time

The volume of the cells in medium with the concentration of 2 x 10^5 cells/mL was adjusted to 1 mL.

CellTrace was mixed by thawing the two components in the CellTrace™ Violet Cell Proliferation Kit at RT. CellTrace is sensitive for light, and the light was turned off in the safety bench when doing the labeling. The working solution of CellTrace staining dye was made by adding 10 µL of DMSO to the CellTrace vial and vortex it. Labeling was carried out by adding one drop of 100 µL PBS to the inside of the tube, with cells, while the tube was held slantingly. Then 2.1 µL of CellTrace was added to the PBS droplet, the tube was raised to an upright position and immedialtey vortexed, thus rapidly mixing the dye evenly with cells. The cells were then incubated in the dark for 10 minutes at RT. Labeling of CellTrace was inspired by [71].

The tubes were filled up with medium and washed. The supernatant was decanted, the cells re-suspended and the washing was repeated once, and cells were re-suspended in the final volume needed to fill duplicate or triplicate wells in a 96 V-wells plate. For the day six assay,

100 µL SSA, SSB, PPD and SEB had been added to wells in twice the final concentration needed. 100 µL MED was added to the negative control wells (the 96 V-wells plate in shown in Figure 3.2). 100 µL of labeled PBMC suspension was added to each well. The 96 V-wells plate was placed in a CO_2 incubator at 37°C for 6 consecutive days.

3.6 The two different assays looking at Giardia-specific immune responses

Both of these assays were developed combining several other methods [61, 63, 65, 72-76], optimizing and validating results along the way. Figure 3.3 shows a flow chart of the two assay protocols combined.

3.6.1 Day one assay protocol

3.6.1.1 Washing the cells out of stimulation media and viability staining

18 hours into the stimulation for the 96 V-wells plate for day one, the plate was taken out of the CO_2 incubator, placed in a sterile laminal Air Flow bench. 11.2 µL of PMA and 11.2 µL of IC (made as stated in 3.3.1.2) were added to the positive control wells. Brefeldin A was diluted 1:10 and added to all wells in a final concentration of 10 µg/mL, and the plate was put into the CO_2 incubator for another 6 hours, making the total stimulation period 24 hours long.

After the incubation, the cells were pelleted by centrifugation and re-suspended in PBS. The duplicates or triplicates of each participant were gathered into two columns, where one column served as stained cells and one as unstained cells. More cells were transferred into the column representing the stained cells. The cells were washed two times with PBS.

Viability staining was done by using Near Infrared dye (LIVE/DEAD). One vial of the fluorescent reactive dye (Component A) and the vial of anhydrous DMSO (Component B) was brought to RT before the caps were removed. 50 µL of DMSO was added to the vial of reactive dye. The contents were mixed and it was visually confirmed that all had been dissolved. The solution of reactive dye was used within a few hours of preparation. The remaining solution was kept in aliquots in a freezer (-20°C) for a maximum of 2 weeks.

1 µL of LIVE/DEAD was mixed with 999 µL PBS. 30 µL of this solution was added to the wells containing 30 µL cell suspension and mixed. The cells were incubated in the dark at RT for 30 min. The wells were filled up with PBS and washed twice. The cells were placed on ice after the last washing step and the temperature in the centrifuge was adjusted to 4°C.

3.6.1.2 Blocking the Fc Receptors and surface staining

Blocking of Fc Receptors with serum was used in order to reduce nonspecific binding of fluorescent staining. Before staining with FABs the PBMCs were incubated with 50 µL 10 % normal human serum in PBS at 4°C for 15 min. The cells were washed two times out of the serum media, and re-suspended in the residual volume.

A staining cocktail with all the surface marker FABs (CD3, CD4, CD8a, CD14, CD45RA and CD197) was made in an Eppendorf tube using PBS as staining media. All the FABs were added in double concentrations needed according to titrations The mix was spun down in an Eppendorf centrifuge at 14 000 RPM for 1 minute in order to let FAB aggregates fall to the bottom of the tube.

30 µL of the staining mix was added to the wells containing 30 µL of cells. The cells were incubated at 4°C in the dark for 30 minutes. The wells were filled up with PBS and washed two times.

3.6.1.3 Fixation, permeabilization, intracellular staining and analysis

FABs have to get inside the cells to bind to cytokines. Fixation and permeabilization was done prior to intracellular staining was carried out. The fixation in the present study was done by adding 100 µL of Fix/Perm solution to all the wells, mix the cell suspensions, and incubate for 20 minutes at 4 °C. The permeabilization was done by using PW as the washing media after fixation. The wells were filled with PW and washed two times. The cells were kept in PW after fixation and in the consecutive washing after staining, as PW is a reversible permeabilization agent.

The intracellular FABs (TNF-α, IFN-γ, IL-17A, IL-4 and IL-10) were added in double concentrations needed, according to titrations, to an Eppendorf tube containing PW, making a staining cocktail. The tube was spun down in an Eppendorf centrifuge at 14 000 RPM for 1 minute in order to let FAB aggregates fall to the bottom of the tube.

30 µL of intracellular FAB cocktail was added to 30 µL cell suspension and incubated for 30 minutes at 4°C in the dark. FMOs for all the cytokine FABs was done by mixing a FAB cocktail including all FABs except the one to be controlled. After the staining incubation, the wells were filled up with PW and washed two times. Two additional washes were done with PBS before filling the wells up to a final volume of 250 µL. The final volume in each well was based on the requirements of the accessory plate reader during analysis on the flow cytometer done same day.

3.6.2 Day six assay protocol

3.6.2.1 Washing the cells out of stimulation media and viability staining

After six days of incubation, the cells were taken out of the CO_2 incubator and the cells were washed out of the stimulation media two times with PBS. The cells were pelleted by centrifugation and re-suspended in PBS. The duplicates or triplicates of each participant were gathered into two columns, where one column served as stained cells and one as unstained cells. More cells were transferred into the column representing the stained cells. The cells were washed two times with PBS.

Viability staining was the done by using Near Infrared dye (LIVE/DEAD). The previously mixed solution from day one was used on day six. The vial with the dye had to gain RT before it was used. 1 µL of LIVE/DEAD was mixed with 999 µL PBS. 30 µL of this solution was added to the wells containing 30 µL cell suspension and mixed. The cells were incubated in the dark at RT for 30 min. The wells were filled up with PBS and washed twice. The cells were placed on ice after the last washing step and the temperature in the centrifuge was adjusted to 4°C.

3.6.2.2 Blocking the Fc Receptors and surface staining

The blocking of the Fc receptors were done by incubating the cells with 50 µL 10 % normal human serum in PBS at 4°C for 15 min. The cells were washed two times out of the serum media and re-suspended in the residual volume.

The preparation of all the surface marker FABs was done by adding all the staining antibodies (CD3, CD4, CD8a, CD14, CD25, CD26, HLA-DR and CD45RO) in double concentrations needed, according to titrations, to an Eppendorf tube containing PBS, making a staining cocktail. The mix was spun down in an Eppendorf centrifuge at 14 000 RPM for 1 minute in order to let FAB aggregates fall to the bottom of the tube. 30 µL of the staining mix was added to the wells containing 30 µL of cells. FMOs for the CD25, CD25, HLA-DR and CD45RO FABs were stained by a FAB cocktail including all FABs except the one to be controlled. The cells were incubated at 4°C in the dark for 30 minutes. After the staining, the wells were filled up with PBS and washed two times.

3.6.2.3 Fixation with paraformaldehyde and analysis

Fixation was done after the second wash with PBS by adding 100 µL 2 % Paraformaldehyde in PBS (PFA) to 100 µL cell suspension, making a final concentration of 1 % PFA. The suspension was mixed and incubated 30 minutes on ice. 50 µL PBS were added to all the wells to a final

volume of 250 µL. The final volume in each well was based on the requirements of the accessory plate reader during analysis on the flow cytometer done the same day.

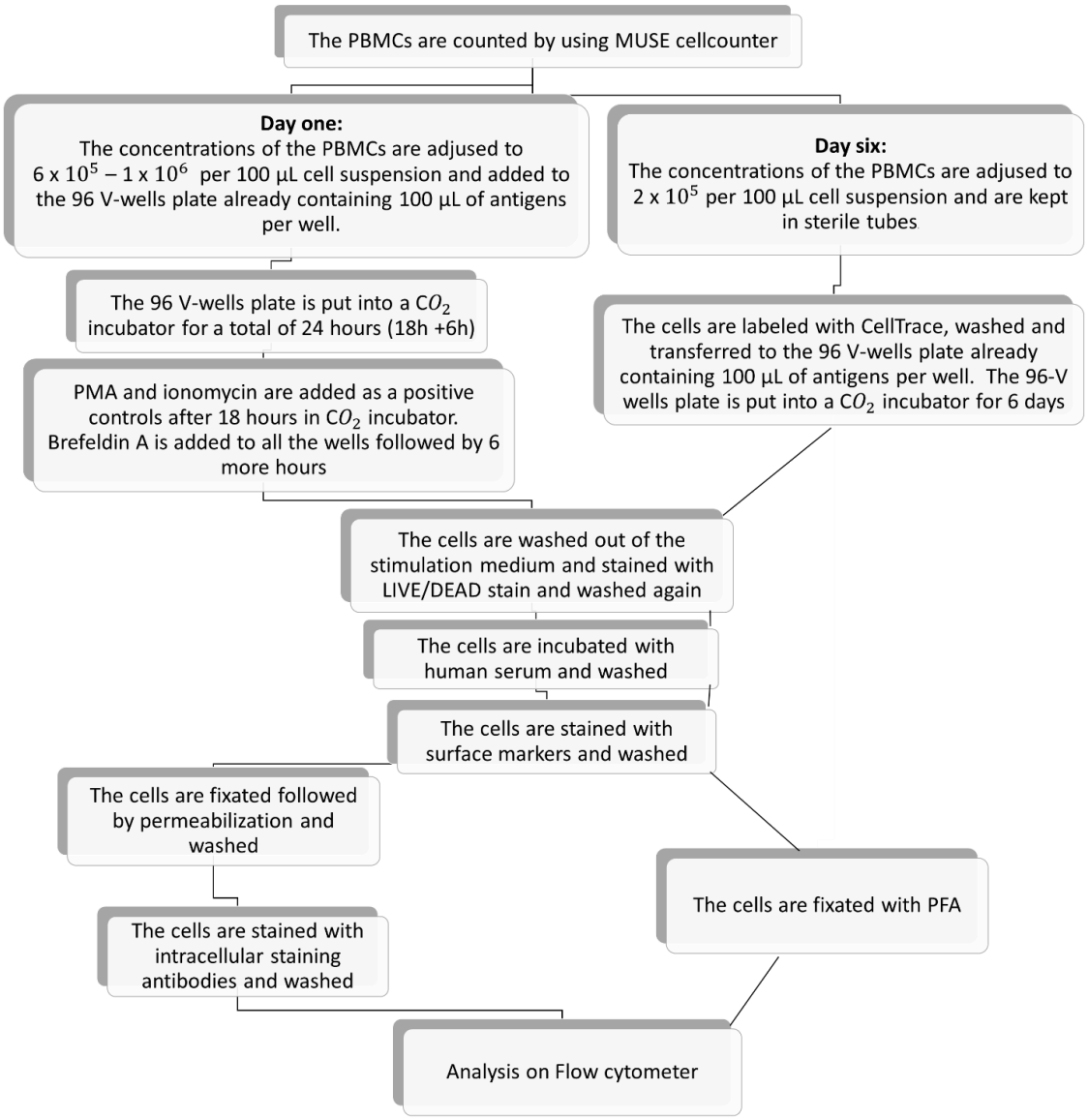


Figure 3.3: Flowchart demonstrating the workflow steps in the day one and the day six flow cytometric assays used in the project. The two different assays have some common protocol steps, but differ due to the investigation of different cell markers.

3.7 Analysis using Flow cytometer BD LSR Fortessa

3.7.1 Setting up flow cytometer

A Cytometer Setup and Tracking (CST) was always done before a flow cytometric analysis took place. The CST was done according to instructions given by the core facility responsible for the flow cytometers. The CST was done as a quality control to assess function and day-to-day variations of the flow cytometer. The delay on the lasers, trigger on the Fluorescence was noted, and flow cytometric analysis could be done if the CST passed.

Before using the plate reader, a washing step with a clean plate filled with Milli-Q water, FACS rinse solution (BD Biosciences) and FACS clean solution (BD Biosciences) was done to avoid remnants and debris in the flow cytometer.

3.8 Flow cytometric analysis using FlowJo

3.8.1 Gating strategy for day one assay

FCS-files from the flow cytometer was transferred to a computer and the compensated parameters were analyzed. The cells were gated using FlowJo analysis program. The gating of cell populations from the day one assay, was done as shown in Figure 3.4.

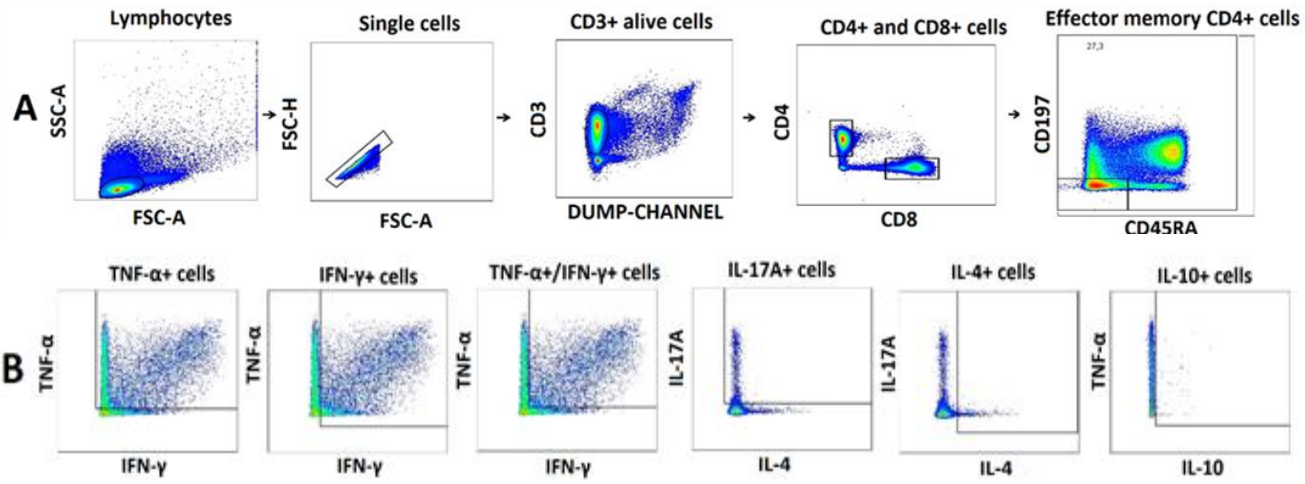


Figure 3.4: Cell gating for day one assay

A: The lymphocytes were gated first using SSC-A vs. FSC-A, followed by single cells gating using FSC-A vs. FSC-H. The $CD3^+$ cells were gated and dead and $CD14^+$ cells were excluded by plotting CD3 vs. LIVE/DEAD. Next the $CD4^+$ cells were found plotting CD4 vs. CD 8. The $CD4^+$ population was further investigated, plotting CD197 vs CD45RA, and the Effector memory $CD4^+$ cells could be found.

B: Cells positive for IFN-γ, TNF-α, IFN-γ and TNF-α, IL-4, IL-17A and IL-10 was gated from the effector memory $CD4^+$ T cells.

3.8.2 Gating strategy for day six assay

The cell gating done for day six assay can be seen in Figure 3.5. The lymphocytes gated on day six were generally more blastic and had larger size compared to day one lymphocytes.

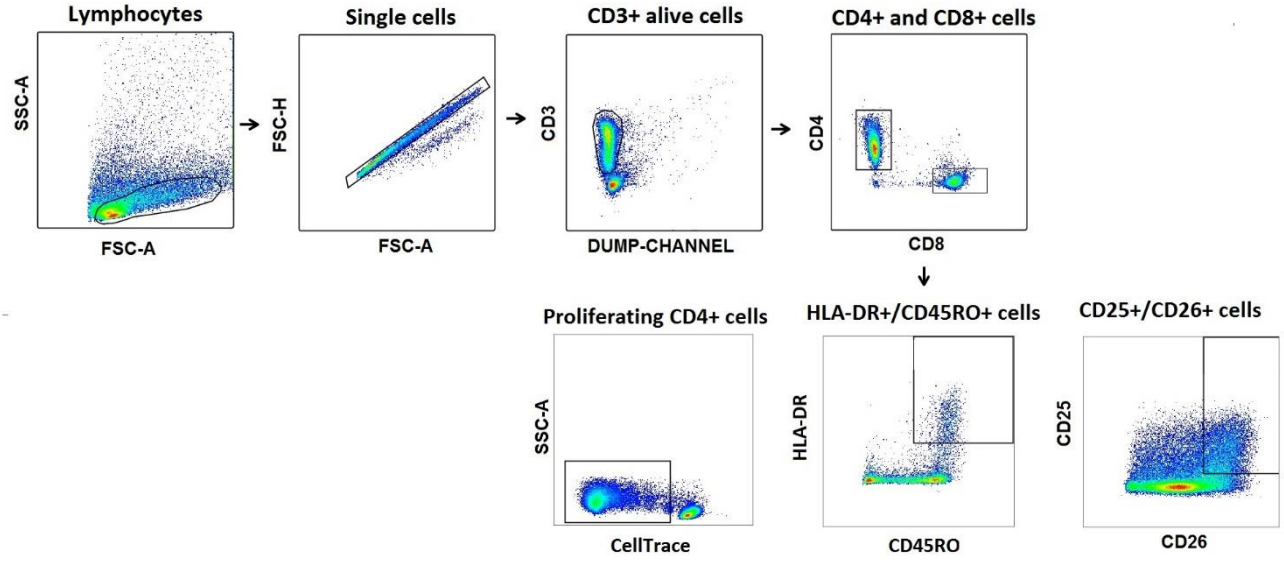


Figure 3.5: Cell gating for day six assay

The lymphocytes were gated using SSC-A vs. FSC-A. Next the single cells were gated using FSC-A vs. FSC-H. The $CD3^+$ cells were found and dead and $CD14^+$ cells were excluded by plotting CD3 vs. LIVE/DEAD. Next the $CD4^+$ cells were found plotting CD4 vs. CD 8. $CD25^+CD26^{++}$ were found plotting $CD4^+$ cells in a CD25 vs. CD26 plot. $HLA\text{-}DR^+CD45RO^+$ were found plotting $CD4^+$ cells in a HLA-DR vs. CD45RO plot. Quadruple positive cell for all the four surface markers were gated, combining the two gates with $CD45RO^+HLA\text{-}DR^+$ cells with $CD25^+CD26^{++}$ cells (not shown in Figure). The proliferating subset of $CD4^+$ cells were found plotting CellTrace vs SSC-A.

3.9 Data analysis

Data analysis was done by transferring flow cytometric data to excel. The percentages of the responses in the day one and day six assays in the various stimulation media were adjusted for the background responses in the negative control, medium. If the percentages were negative in stimulation media, it was corrected to zero. This was done done to ensure that background signals did not contribute to the responses seen for SSA and SSB, and also for the positive controls.

The data was transferred to SPSS version 21 for statistical testing could be done. Differences in cytokine producing cells, in cell activation and proliferation between the *Giardia* exposed group and the low risk healthy control group, were investigated by a 2-tailed Mann-Whitney U test. To investigate demographical differences between the groups, Fisher's exact test was done for categorical variables and non-parametric method with and Kruskal Wallis test

was done for continuous variables. Correlation between responses was done using linear regression with Pearson's correlation coefficient. P-values < 0.05 were considered to be statistically significant.

3.10 Rare events in flow cytometry

Cytokine producing cells are rare events, and it is important to have enough cells in the final analysis to detect these cells with flow cytometry.

If a cell subset i.e. consists of 5 %, or less, the number of cell required to collect can be calculated following equations:

Equation 3.1: $SD = \sqrt{r}$

Where SD = standard deviation and r = positive rare events.

Equation 3.2: $CV = 100/\sqrt{r}$

Where CV = coefficient of variation and r = positive rare events.

These equations can be combined giving:

Equation 3.3: $r = (100/CV)^2$

If a CV value of 5 % is adequate and the cell population of interest comprise 5 % the entire cell population, following equation 3.3, 400 positive rare events have to be collected by flow cytometric analysis. In order to obtain 400 positive events for the cell population of interest, $\frac{400}{5\ \%}$ x 100 % = 8000 events have to be acquired. If a subpopulation comprise 0.1 % of a cell population, 400 000 events have to be collected to give a CV of 5 %. The more rare an event is, the more cells have to be used. This can provide difficulties, because unlimited number of PBMCs cannot be drawn from an individual. A higher CV should therefore be accepted if the number of cells required is unattainable [61].

3.11 Ethical considerations and funding

The pilot study involving human subjects was approved by the Regional Committee for Medical Research Ethics and performed in correspondence to the Declaration of Helsinki. Participation was voluntary and the recruited individuals could withdraw at any time without giving any consent.

Blood sampling used in the study was not associated with critical complications. A small

compensation was given to participants in order to cover the costs of travel and time. The sampling, participant compensation, laboratory reagents and analysis were covered by project grants from the Western Norway Regional Health Authority and the Department of Clinical Science, University of Bergen. If supplementary pathological findings were discovered, a physician would evaluate the finding and refer for a follow-up or medical attention.

4. Results

4.1 Method development

4.1.1 Setting up a flow cytometric multicolor fluorochrome panel

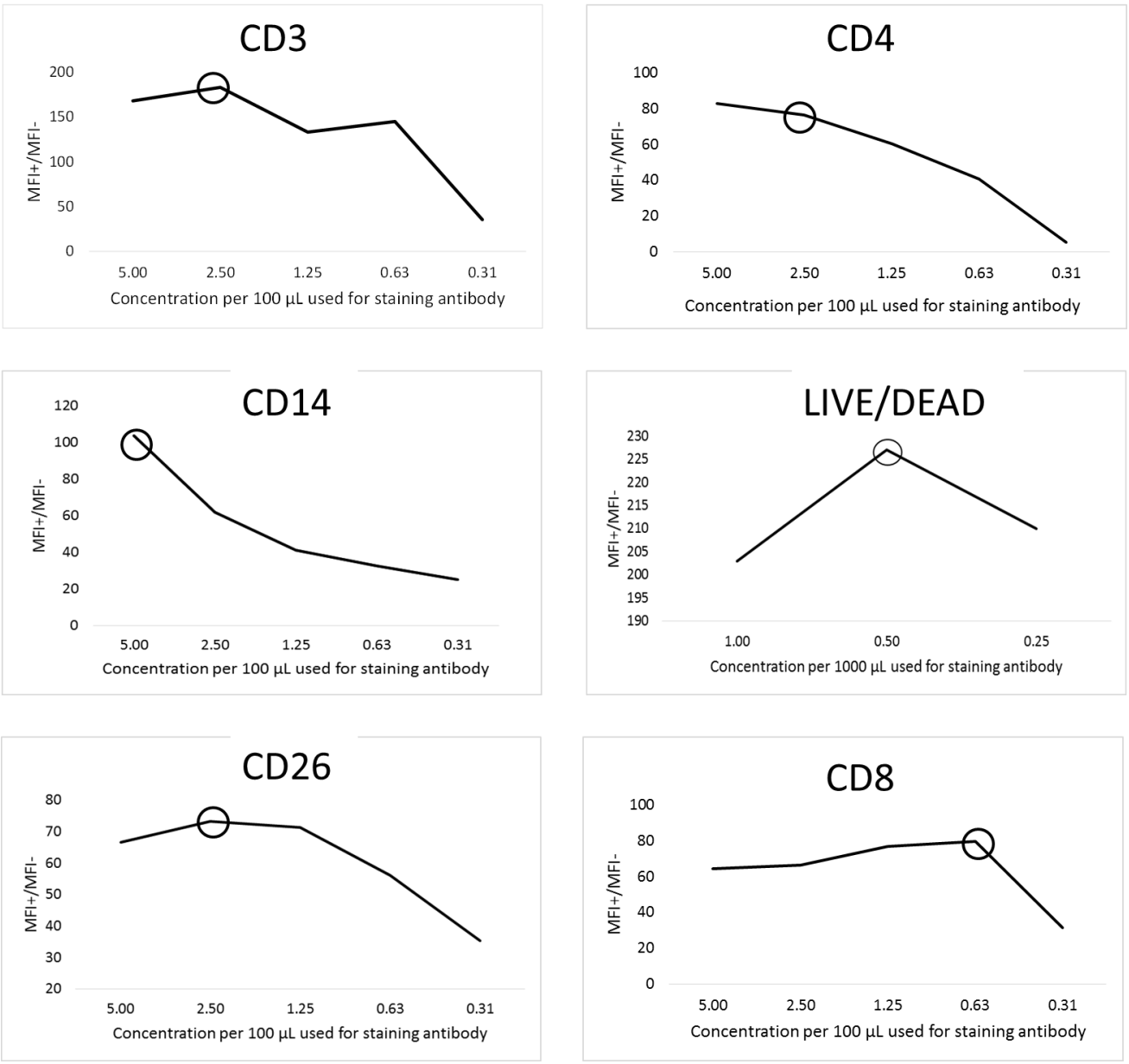
4.1.1.1 Titrations of the fluorochrome-conjugated antibodies or dyes used in the project

All the fluorochrome-coupled antibodies (FABs) or dyes had to be titrated before the optimal concentrations for cell staining could be found. The lowest concentration possible, which at the same time provided a good separation of the positively stained cells from the negative ones, was used.

To find the most suitable concentration of each FAB, results were analyzed in FlowJo The MFI for the positive cell population was found and divided by the negative population. The higher this ratio was the higher separation of the two cell populations.

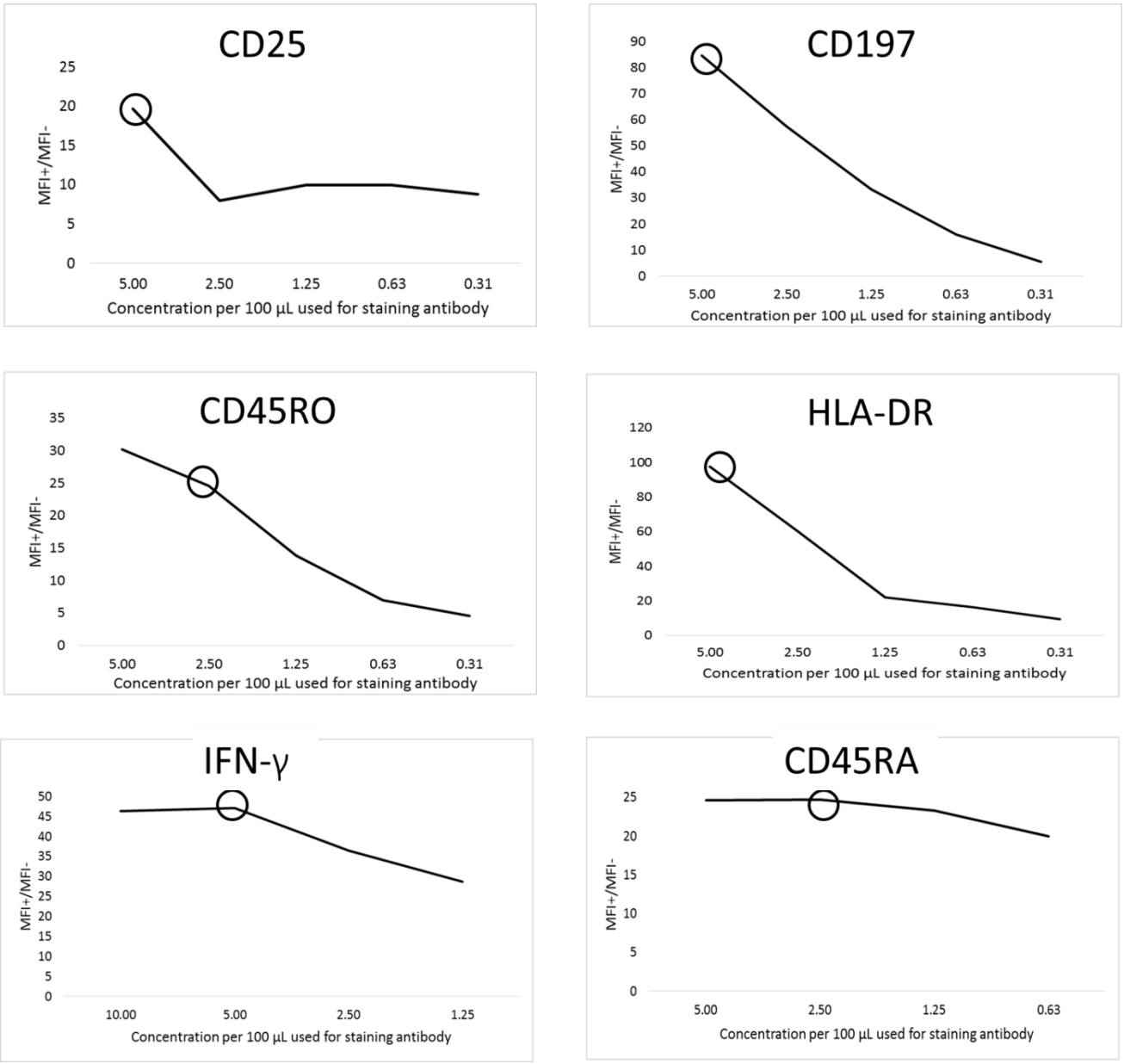
Figure 4.1 A, B and C shows how the separation of the positively stained cells from the negative ones varied, as the concentration got lower. For the cytokine titrations, PMA/IC were used to stimulate the PBMCs, as this antigen has shown to be able to elicit production of all the cytokines investigated in this project. The titrations for the markers CD3, CD4, CD8, CD14 and LIVE/DEAD were done using unstimulated fresh PBMCs. The late activation and memory markers, CD25, CD26, HLA-DR and CD45RO were titrated after six days of stimulation with SEB. CellTrace labeling results are given in section 4.1.4. All of the cells used in the titrations were from healthy volunteers.

The titrations were done for all the antibodies before combinations of different antibodies were tested in the panel setup for development of the flow cytometric method.

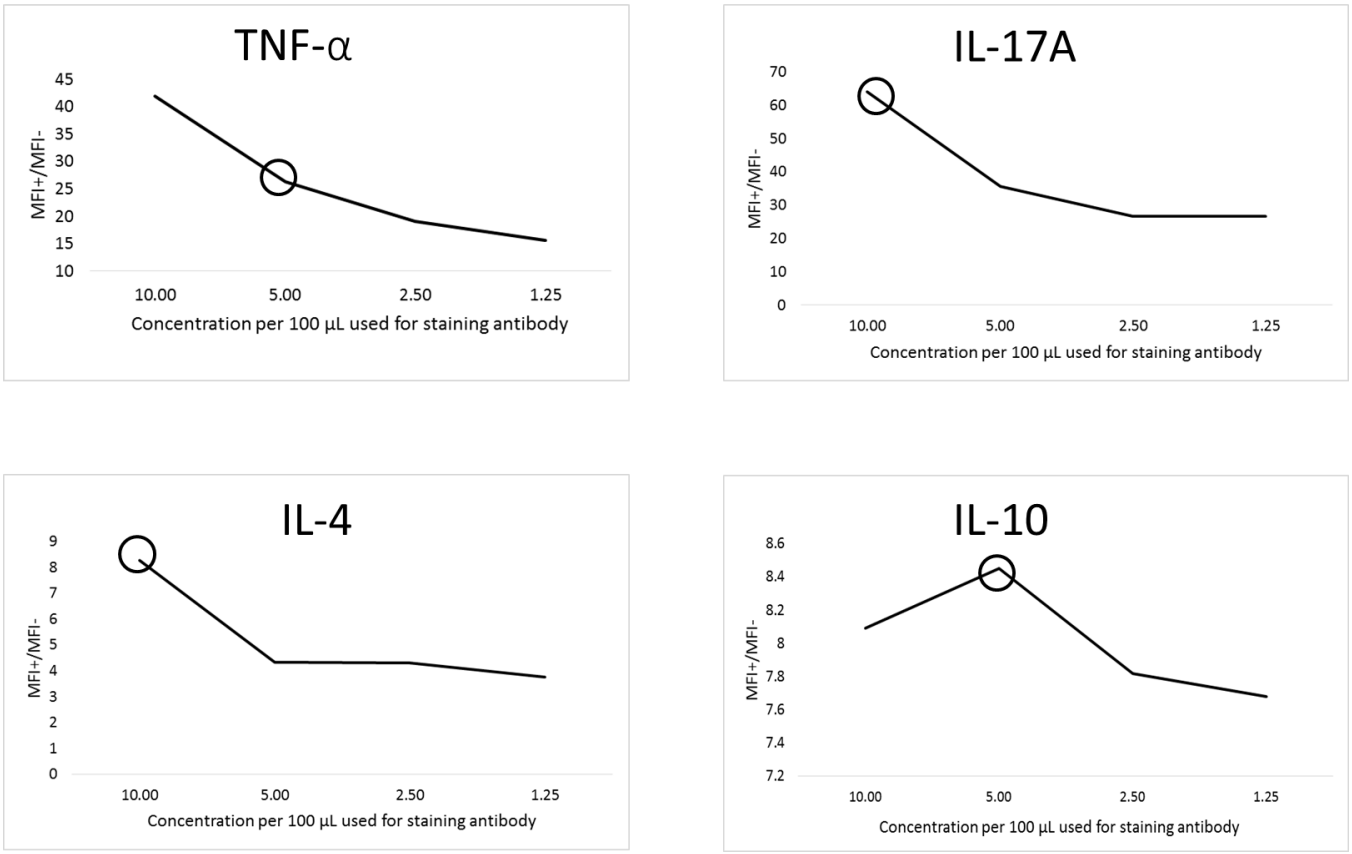


<u>Figure 4.1 A</u> Titration of the fluorochrome-conjugated antibodies and dyes used in this project.

From upper right: titration of CD3, titration of CD4, titration of CD14, titration of LIVE/DEAD, titration of CD26 and titration of CD8. The x-axis represents the concentration of FAB used per 100 µL staining medium. The y-axis represents the mean fluorescence intensity (MFI) of the positive stained cell divided by the MFI of the negative cells.



<u>Figure 4.1 B</u>: Continuation of the titration of the fluorochrome-conjugated antibodies and dyes used in this project. From top right: Titration of CD25, titration of CD197, titration of CD45RO, titration of HLA-DR, titration of IFN-γ and titration of CD45RA. The x-axis represents the concentration of FABused per 100 µL staining medium (per 1000 µL for LIVE/DEAD). The y-axis represents the mean fluorescence intensity (MFI) of the positive stained cell divided by the MFI of the negative cells.



<u>Figure 4.1 C</u>: Continuation of the titration of the fluorochrome-conjugated antibodies and dyes used in this project.

From top right: Titration of TNF-α, titration of IL17A, titration of IL-4 and titration of IL-10. The x-axis represents the concentration of FAB used per 100 µL staining medium. The y-axis represents the mean fluorescence intensity (MFI) of the positive stained cell divided by the MFI of the negative cells.

4.1.1.2 Final voltages used in the project

Voltages of the different channels were adjusted in order to reduce spectral overlapping. A high voltage in one channel can contribute to "bleeding" into another channel. The voltage is also important for the separation of positively stained cell from the negative ones, as the negative stained cells should have low intensities and center between 0 and 1000 in a flow cytometric plot. The optimal voltages used in this project for the day one and the day six assays can be seen in Table 4.1 and Table 4.2. The voltages were found during the compensation and titrations of all the fluorochromes used in the project.

Table 4.1: Voltages used on the flow cytometer and parameters for the day one assay

Following parameters/channels were used in the day one setup characterizing intracellular cytokine profiles of effector memory T cells. A represents area, while H represents height.

Laser	Channel (wavelength)	Parameter/fluorochrome and antibody target	Voltage
-	488 ± 5	FSC-A	200
		FSC-H	200
		SSC-A	240
Violet (407)	450 ± 25	BV421-A – TNF-α	300
	525 ± 25	BV510-A – CD45RA	410
	605 ± 6	BV605-A – IL-17A	400
	710 ± 20	BV711-A – CD8	420
Blue (488)	530 ± 15	FITC-A – IFN-γ	410
	695 ± 20	PerCP-Cy5.5-A – CD4	490
Yellow Green (561)	582 ± 7.5	PE-A – IL-10	450
	610 ± 10	PE CF594-A – CD197/CCR7	460
Red (635)	670 ± 7	APC-A – IL-4	510
	730 ± 22.5	Alexa Fluor 700-A – CD3	440
	780 ± 30	APC-H7-A – CD14/ LIVE/DEAD	460

*APC-H7 was used as a "Dump-channel".

Table 4.2: Voltages used on the flow cytometer and parameters for the day six assay

Following parameters were used in the setup for the late activation, memory, proliferation and lineage markers on T cells. A represents area, while H represents height.

Laser	Channel (wavelength)	Parameter/fluorochrome - antibody/dye	Voltage
-	488 ± 5	FSC-A	200
		FSC-H	200
		SSC-A	240
Violet (407)	450 ± 25	Pacific Blue – CellTrace	300
	605 ± 6	BV605-A – CD45RO	400
	710 ± 20	BV711-A – CD8	420
Blue (488)	530 ± 15	FITC-A – HLA-DR	410

	695 ± 20	PerCP-Cy5.5-A – CD4	490
Yellow green (561)	582 ± 7.5	PE-A – CD26	450
Red (635)	670 ± 7	APC-A – CD25	510
	730 ± 22.5	Alexa Fluor 700-A – CD3	440
	780 ± 30	APC-H7-A – CD14/ LIVE/DEAD*	460

*APC-H7 was used as a "Dump-channel".

The voltages seen in Table 4.1 and 4.2 were adjusted and optimized according to one another. Voltages should be high enough to ensure that the unstained cells can be found around zero in a flow cytometric plot, while not too high for the positively stained cells to avoid spectral overlap. The compensation matrices for day one and day six assay were used as guidance during the development of the method. If the percentage of spectral overlaps were high (over 40 %) in the compensation matrix, the voltages were adjusted. Antibodies conjugated to APC-H7 were defined as a "dump channel", so that dead cells and monocytes stained with APC-H7, could be excluded from the analysis.

4.1.1.3 Compensation matrices

Compensation was done by using Compensation Particle set beads (Mouse or Rat IgG, depending on the isotype for a specific antibody) for all of the FABs or dyes, except for CellTrace. Live and fresh cells had to be used for obtaining a relevant fluorescence intensity signal for CellTrace on the flow cytometer. The compensation matrices for the project can be seen in Figure 4.2.

Day one compensation matrix

Show All	BV421-A	BV510-A	BV605-A	BV711-A	FITC-A	PerCP-Cy5-5-A	PE-A	PE-CF594-A	APC-A	Alexa Fluor 700-A	APC-H7-A
BV421-A	100	14,3909	0,6668	0,0668	0,0407	0,0045	0	0	0	0	0,0047
BV510-A	8,4523	100	23,4137	3,6787	0,4586	0,0393	0,0009	0,0032	0,0247	0,0028	0,0043
BV605-A	5,4309	1,2446	100	19,7713	0,0142	1,5939	9,477	14,0861	0,3504	0,1267	0,0628
BV711-A	5,4173	1,5379	0,192	100	0,0212	11,4506	0,021	0,0002	2,4559	38,5973	15,4388
FITC-A	0	2,5416	0,2277	0,012	100	0,5655	0,0051	0,0013	0,0017	0,0028	0
PerCP-Cy5-5-A	0	0	0,0032	36,7195	0,0028	100	0,0416	0	19,4491	19,6146	9,292
PE-A	0	0,1159	3,0514	0,2855	0,9502	3,9821	100	17,8996	0,1219	0,0214	0,0058
PE-CF594-A	0	0,0165	10,296	2,6721	0,335	39,5894	27,3771	100	1,4696	0,2893	0,124
APC-A	0,0011	0	0,0032	1,0972	0	0,3107	0,0035	0,0431	100	18,6532	6,2168
Alexa Fluor 700-A	0,0242	0,064	0,0149	5,7792	0,1564	1,3752	0,0373	0,0614	2,0472	100	30,9377
APC-H7-A	0,0265	0,0125	0	0,0518	0,0308	0,0352	0,0251	0,0278	2,3777	4,7291	100

Day six compensation matrix

Show All	BV605-A :: CD4...	BV711-A :: CD8	FITC-A :: HLA-...	PerCP-Cy5-5-A...	PE-A :: CD26	APC-A :: CD25	Alexa Fluor 700-...	APC-H7-A :: Du...	Pacific Blue-A :: ...
BV605-A :: CD45RO	100	19,8578	0,0066	1,5393	10,2739	0,3194	0,0927	0,0401	5,7483
BV711-A :: CD8	0,3582	100	0,0532	10,1154	0,0267	2,2869	32,8996	13,7173	6,6925
FITC-A :: HLA-DR	0,324	0,0922	100	0,6477	0,0275	0,0304	0	0	0,0629
PerCP-Cy5-5-A :: CD4	0,0935	38,6739	0,2476	100	0	24,1171	22,9514	10,7047	0,0362
PE-A :: CD26	2,5991	0,2457	0,9131	3,5424	100	0,0951	0,0178	0,0059	0,0022
APC-A :: CD25	0,0126	1,1313	0,0001	0,3269	0,0034	100	18,098	6,2309	0,0002
Alexa Fluor 700-A :: CD3	0,3299	6,1026	0,7009	2,9485	0,5395	5,0962	100	35,0214	0,1136
APC-H7-A :: Dump channel	0,1318	0,1817	0,1173	0,285	0,1868	3,3043	5,6442	100	0,0436
Pacific Blue-A :: CellTrace	2,5656	0,1891	0,5951	0,0559	0,0316	0	0,0049	0,0137	100

Figure 4.2: The compensation matrices for day one and day six assay

Day one assay compensation matrix shows the percentage of leakage into other channels; the spectral overlap. Day six assay compensation matrix shows the corresponding spectral overlap.

The compensation matrices represent how spectral overlap manifested in this project. More staining antibodies/dyes were used in the day one assay, and higher spectral overlaps can be seen here. Spectral overlaps should not exceed 50 %, and even if there is more spectral overlap from the day one matrix, none of them exceeds 40%. The lineage markers were deliberately put on markers with a high degree of spectral overlap. Usually a distinct cell population was possible to discern for these markers. Bright fluorochromes with less overlap were preferred for the cytokine antibodies.

4.1.2 Fixation and permeabilization; comparison of Cytoperm/cytofix vs. Formaldehyde, Triton X-100 and methanol

Many different reagents can be used for fixation and permeabilization of cells. The order of the staining and the chemicals can influence the result obtained in such a method. Two different protocols with two different staining sequences were compared in order to gather information on how the cytokines would be stained, by the fixation and/or permeabilization. $CD8^+$ cells and their expression of cytokines were tested in these protocols. $CD4^+$ cells could not be gated and $CD3^+$ cells appeared different from one another (Figure 4.3) when using these

two protocols. Rearrangement of antibody clones and panel was done later on.

The first method consisted of using cytoperm/cytofix kit, while the other one was an alternative method where formaldehyde, Triton X-100 and methanol were used. All the PBMCs were stimulated for 4 hours with PMA and IC and the cytokine secretion inhibitor agent Brefeldin A was added at the same time.

The staining order was done by either staining the cells in two steps or one step. When the cells were stained in two steps, surface markers FABs was added to the cells. Next the cells were fixated and permeabilized, followed by intracellular staining. For the one-step staining order the cells were fixated and permeabilized followed by surface- and intracellular staining simultaneously. The percentages of cytokines can be seen in Table 4.3.

Table 4.3: Comparison of percentage of Lymphocytes and cytokines in $CD8^+$ cells by using cytoperm/fix kit or Formaldehyde, Triton X-100 and methanol.

	Staining order	$CD8^+$ cells					
		Of total Lymphocytes (n)	IFN-γ (%)	TNF-α (%)	IL-10 (%)	IL-17A (%)	IL-4 (%)
Cytoperm/cytofix kit	1 step	54066	2.83	1.34	0.097	0.29	0.019
	2 steps	22565	1.97	1.06	0.024	0.97	0.018
Formaldehyde, Triton X-100 and methanol	1 step	4636	0.47	8.75	0.12	0.25	0.02
	2 steps	13292	5.26	9.09	0.14	0.51	0.13

As can be seen by Table 4.3, the number of $CD8^+$ T cells differed for the two methods. This could be due to cell loss when washing and re-suspending and the different reagents used.

The percentage of IFN-γ, using cytoperm/cytofix, was increased when the staining was done in one step, while fewer cells got stained if two separate steps were used. The opposite seemed to be the case when using formaldehyde, Triton X-100 and methanol, where more IFN-γ got stained when doing the staining in two steps For this cytokine both a two step and a one step staining order could be use, depending on which reagents were used for the fixation and permeabilization.

TNF-α^+ cells found using the two different methods seemed to vary a lot. One staining step using the cytoperm/cytofix protocol gave a higher percentage of TNF-α^+ cells than

separating the staining into two steps. Using the Formaldehyde, Triton X-100 and methanol on the other hand, gave significantly higher percentages of TNF-α^+ cells and two staining steps gave most positive cells.

For IL-10, a smaller quantity of cells got stained when using the cytoperm/cytofix compared to the formaldehyde, Triton X-100 and methanol protocol. A two step staining order using this protocol gave more positive cells.

For IL-17A both of the different methods gave best results when the staining order was divided in two. The cytoperm/cytofix protocol however, gave more IL-17A$^+$ cells than the formaldehyde, Triton X-100 and methanol protocol.

The formaldehyde, Triton X-100 and methanol method resulted in more IL-4$^+$ cells than cytofix/cytoperm, and was best noticed when the staining was done in two steps.

For most of the cytokines the formaldehyde, Triton X-100 and methanol protocol done in two staining steps seemed to be better. For IL-17A on the other hand, the best protocol was using cytofix/cytoperm.

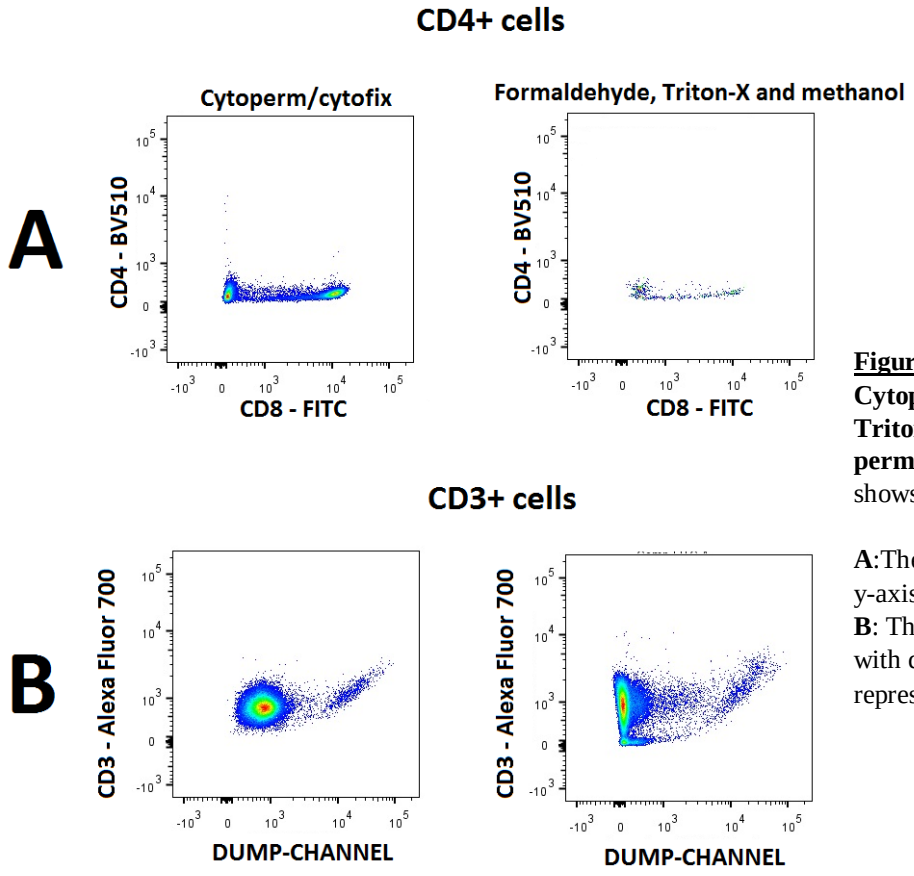


Figure 4.3: T-cell populations using Cytoperm/cytofix or Formaldehyde Triton X-100 and methanol fixation and permabilizaton protcols. This figure shows stimulated and stained PBMC.

A:The x-axis represents CD8$^+$ cells and the y-axis represents CD4$^+$ cells.
B: The x-axis represents the Dump-channel with dead and CD14$^+$ cells and the y-axis represents the CD3$^+$ cells.

Figure 4.3 represents flow cytometric plots of the CD4$^+$ vs. CD8$^+$ cells and the CD3$^+$ cells vs. the dump channel. The CD4 antibody was in this experiment coupled to the fluorochrome

Brilliant™ Violet 510 and had the clone OKT4 (Biolegend), while the final one was coupled to PerCP-Cy5.5 and expressed the clone L200 (BD Biosciences). The clone tested in this experiment was not compatible with intracellular staining, as the fixation and/or permeabilization made it impossible to separate the $CD4^+$ cells from the negative ones.

The CD3 antibody used in this experiment was coupled to the fluorochrome Alexa Fluor 700. The same antibody and fluorochrome has been used in the final panel, but the CD3 used in this experiment expressed the clone HIT3a (Bio legend), while the final one expressed UCHT1 (Bio legend). The CD3 antibody clone tested in this experiment did not show compatibility with the cytofix/cytoperm, as the $CD3^+$ cells could not be separated from $CD3^-$ cells. The CD8 used in this panel was coupled to FITC and had the clone aRPA (Bio legend).

The panel for which these two protocols were tested did have a compensation matrix where some of the spectral overlap was over 80 %. This panel was changed in order to avoid spectral overlap, but also due to the importance of having dim markers on bright and medium fluorochromes and use dim fluorochromes on markers that are highly expressed (such as surface markers). The lineage markers used in the final panel were put on fluorochromes with higher spectral overlap, and not placing fluorochromes targeting cytokines on these fluorochromes.

4.1.3 Exploring markers for CD8+ cells

To assess the importance of $CD8^+$ cells in immune responses against *Giardia*, another panel of FABs were tested. The CD8 cell toxicity was investigated by using the early T cell activation marker, CD69 on day one and perforin. On day six CD107a, a marker for degranulation in $CD8^+$ cells was investigated. The fluorochrome panels were initially developed to investigate both CD4 and CD8 responses, but could not assess effector memory $CD4^+$ T cells on day one. The FAB CD197 was included in the day six assay panel to assess its expression in activated and proliferating cells. The information about the FABs tested and the day one and day six panels are included in Appendix D.

During the method development, perforin positive cells percentages were similar for both unstimulated and stimulated cells. As a result no specific responses were found for this protein linked to cytotoxicity on $CD8^+$ cells. The staining had to be done during the last 6 hours of stimulation in the medium. Due to the above considerations, the staining with CD107a was not done. The effector memory $CD4^+$ T cells were thought to be a better approach in order to analyze specific immunity. CD69 was therefore excluded from the day one panel and replaced by CD45RA, while CD197 was removed from the day six panel and put into the day one assay

panel. In this way we could identify the memory effector $CD4^+$ T cell populations, and exclude the naïve and central memory T cell populations.

4.1.4 CellTrace failures and triumphs

4.1.4.1 Uneven labeling of CellTrace

CellTrace is useful for monitoring proliferation of cells during stimulation with antigens. When labeling with CellTrace, it is important to include a good positive control,that labeling concentration is optimal for viability of cells and for separation of generations, and that the cells are evenly labeled initially.

A problem seen when labeling cells with CellTrace, was that the unstimulated cells could appear to be proliferating, and this was not expected for a negative control (Figure 4.4). Even labeling, giving a bright peak and good separation of the positive cells from the negative cells is essential for monitoring proliferation.

A new labeling protocol was therefore of high priority to develop.

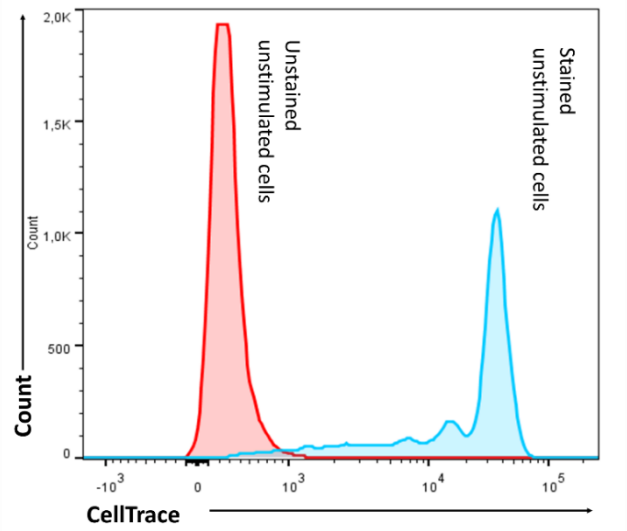


Figure 4.4 Overlayed histograms of an unstimulated unstained cell population (pink) and unstimulated CellTrace labeled cells (blue) collected after 3 days in culture.

Uneven staining of the unstimulated cells falsely showing cell division, and weakly positive cells gradually down to the level of unlabeled cells.

4.1.4.2 Labeling media for CellTrace

To fix uneven labeling, different staining solutions were tested. The two different staining solutions tested for CellTrace was PBS and Medium. Following the manufacturer's protocol, staining should only be done in protein-free media such as PBS, because CellTrace could bind to proteins in the solution instead of diffusing into the cells and label them brightly and evenly.

Labeling of the cells with CellTrace was done on day 0, when the cells were harvested. Next the cells were counted, and suspended in medium. It would seem bothersome and unfavorable to wash the cells out of the X-vivo medium, stain in PBS, wash and re-suspend in X-vivo medium again. An alternative labeling would be ideal in order to save time, and use less X-vivo medium. Figure 4.5 shows how the staining results were when labeled in medium and in PBS.

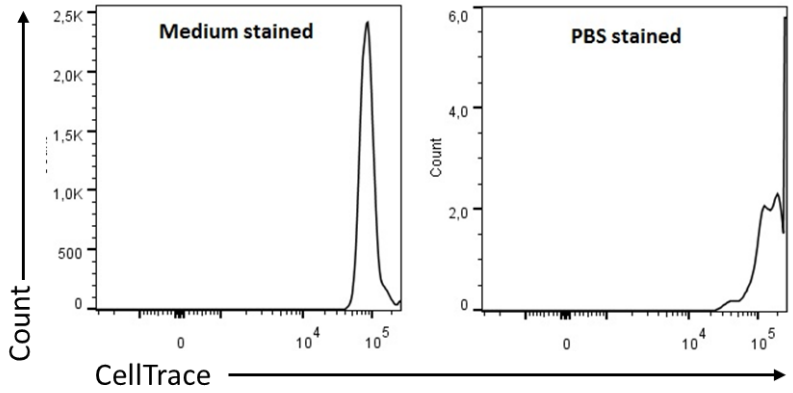


Figure 4.5: Histograms of CD3$^+$ cells stained with 20 mmol/mL CellTrace for 10 minutes in medium or in PBS.

The x-axis represents the intensities of CellTrace in the wavelength channel and the y-axis represents cell count.

This demonstrates a more even and bright labeling of cells when stained with CellTrace in medium compared to staining in PBS. Medium was chosen as the best staining media in this project, and the voltage and CellTrace labeling consentration was adjusted to a peak intensity distributed evenly around 10^5, and provided good separation of the stained cells from the negative ones. Staining in PBS gave a peak intensity so bright that it cannot be seen in the plot. The optimal voltage for the negative population (not shown in figure) was used during the testing of CellTrace. The voltage used in this experiment placed the unstained cell population evenly around zero and showed to not be compatible with CellTrace labeling in PBS. Both of the cell samples used in the test was stained with CD3 in order to test the performance of labeling specifically for T cells.

4.1.4.3 Positive proliferation controls

Different antigens were tested to show their corresponding proliferation. PPD was a good candidate as a positive protein control, because this antigen could provide a clean antigen-specific response, and study participants were all previously immunized with the BCG-vaccine against *Mycobacterium tuberculosis*. Figure 4.6 shows how some of the tested antigens proliferated when using CellTrace proliferation dye.

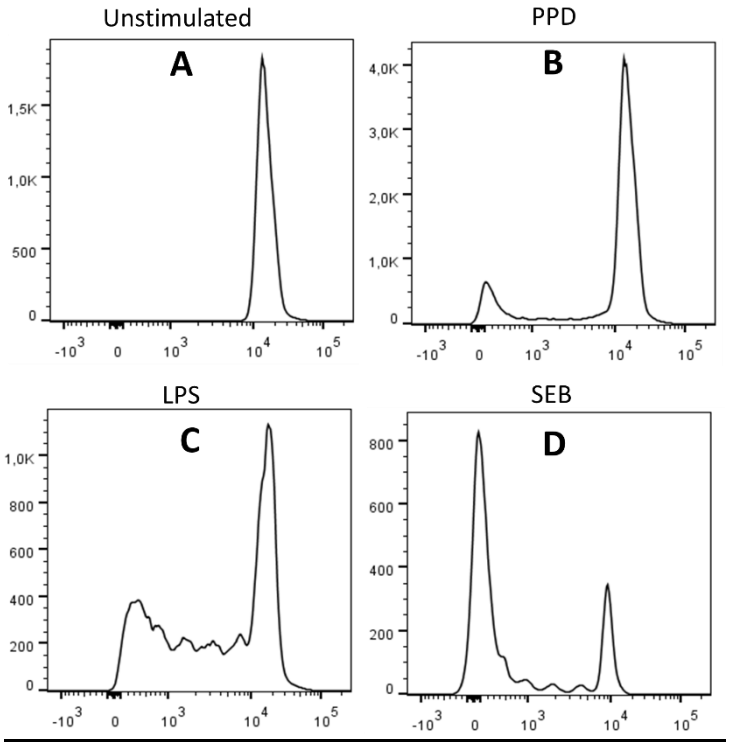


Figure 4.6: Histograms of various candidates for positive controls for CellTrace after 6 days of stimulation. The x-axis represents the intensities of CellTrace in the wavelength channel and the y-axis represents cell count. A) Unstimulated cells, B) PPD, where a small peak can be seen towards left in the plot. C) Next LPS stimulated cells are shown where 5 generations of the cells can be spotted. D) SEB stimulated cells are shown in the next plot where 5 generations of cells can be spotted.

In addition to using PPD as a positive control, SEB was also seen as a good candidate for proliferation responses. The reason for this was that SEB gave a high percentage of proliferating cells, where many cell generations could be seen (Figure 4.6). The individual responses to PPD could vary and sometimes be weak, and SEB would therefore provide information regarding cell proliferation. LPS could have been included as a positive control as well, but SEB was chosen due to stronger proliferative responses.

4.1.4.4. Concentration of CellTrace

The concentration of CellTrace is important for distinguishing between positively stained and negative cells. According to manufacturer's protocol, the concentration of CellTrace should be 5 mmol/mL in order for it to carry out its function as proliferation marker. The incubation time should in addition be 20 minutes in a CO_2 incubator followed by repeated washing steps.

In this project the staining medium was changed, and as a result of this, the concentration of CellTrace had to be increased as well. The concentration found to be optimal was 20

mmol/mL in addition to using an incubation period of 10 minutes at room temperature. This provided even labeling and gave a bright stained population, which could be separated from the negative cells at the optimal voltage.

The peak for CellTrace labeled unstimulated cells was uneven and it appeared to be proliferating (Figure 4.4), and looked like this before the optimized staining protocol done in medium was established. Figure 4.5 demonstrates even labeling when changing the staining media.

Labeling in PBS could be a contributor to the wide peak seen for the unstimulated stained cells in Figure 4.4.

4.1.5 Prevention of cell loss

A problem that was experienced during the entire method developing period, was cell loss. A good analysis of antigen-specific cytokine producing T cells cannot be done with very few cells, as these are rare events. Preparation of cells for flow cytometric analysis is a lengthy process involving many washing, staining and analysis steps where cells are lost. Optimizing of the procedures to avoid cell loss was therefore given high priority. 96 U-wells plates were at first used for the entire protocol for flow cytometric analysis. The problem with these plates was that it is easy to disturb the cell pellet when removing supernatants and re-suspending cells. Switching to using 96 V-wells plates made it easier to avoid disturbing the cell pellet. However, it rarely resulted in more than 1/10 of the starting cell population being available for analysis.

Removing supernatants and re-suspension of cells were critical steps in order to avoid cell loss. Making the pellet more firm makes it easier to discard cells when removing supernatant. The centrifuge speed used for plates was originally 400 RCF. The RCF was adjusted up to 450 for all steps, except after the fixation and permeabilization, where the RCF was increased further up to 600 RCF. These settings on the centrifuge generally yielded more cells available for analysis. The higher RCF on the centrifuge made it possible to flick the supernatants into a biohazard trash, instead of pipetting. Cells can adhere to the tips of pipettes, and some volume seemed to be left in the tips after re-suspension. By flicking the plate, more cells could be obtained for the flow cytometric analysis.

Another crucial step to make sure that the cell loss was kept to a minimum was using a plate reader instead of flow tubes on the flow cytometer. This avoided cell loss in the 96 V-wells plate and remnants in the pipettes. Using the plate reader also saved a lot of time.

The fixation and permeabilization done before intracellular staining was a contributor to cell loss. Cytofix/cytoperm gave less cell loss than the formaldehyde, Triton X-100 and

methanol method. Generally 16 % of cells were available for analysis when using cytofix-/cytoperm, and only 2 % of cells were generally available when using formaldehyde, Triton X-100 and methanol (Table 4.3 shows available cells for analysis using this protocol). Cytofix/cytoperm was therefore chosen to be used prior to intracellular staining in this project, as the number of cells for analysis was of high priority.

4.1.6 Quality control of cell gating

When analyzing data obtained by flow cytometry, populations of cells of interest were gated and investigated further. Gating of a population can be especially difficult if the two populations with negative and positive cells overlap due to continuous expression of the specific marker of interest. Cytokines can be produced in small amounts, and the gate threshold is of crucial importance, as it can heavily influence the percentage of cytokine producing cells.

Fluorescence minus one (FMOs) controls were included in the piloting of exposed and unexposed persons, and for every participant in the comparative experiment conducted. The FMOs showed how background and spectral overlap would be in a specific channel, when that FAB is missing. Figure 4.7 and Figure 4.8 shows how the FMOs looked like for the day one and day six assays. In addition to FMOs, an unstimulated stained cell sample (medium only) was always included for every participant in the two groups. These cells showed how the background production of cytokines would be, so that this background could be subtracted for each participant. It was also a helpful tool to set gating. To control for autofluorescence, an unstained sample of cells for all the antigens used was included. These cells could show how background autofluorescence signals occured, and assist gating.

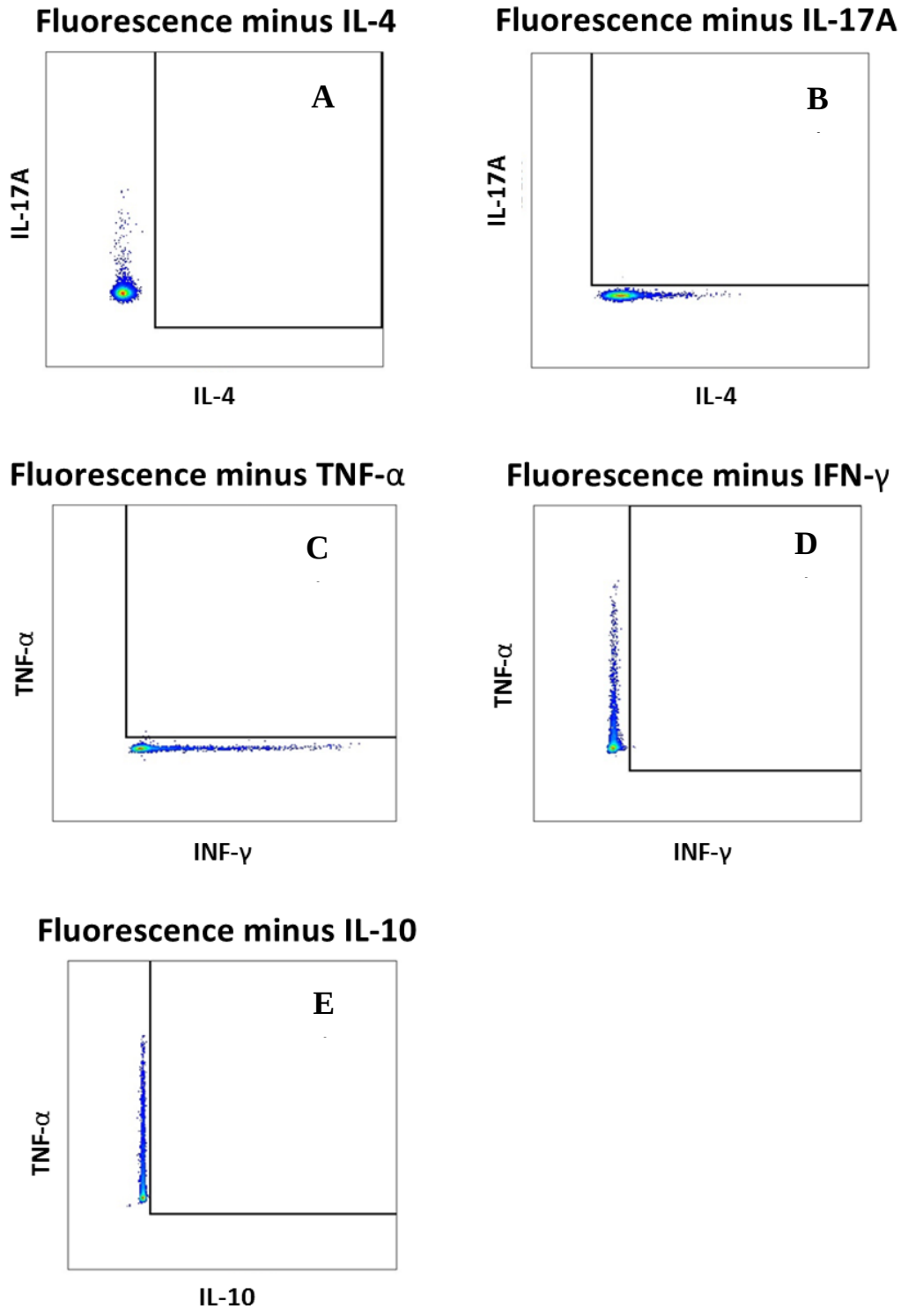


Figure 4.7: Fluorescence minus one (FMOs) controls stimulated for 24 hours with PMA/IC in day one assay. A) IL-4 FMO, B) IL-17A FMO, C) TNF-α FMO, D) IFN-γ FMO and E) IL-10 FMO. The gates set here were used as the standard gating for the cytokines in the day one assay.

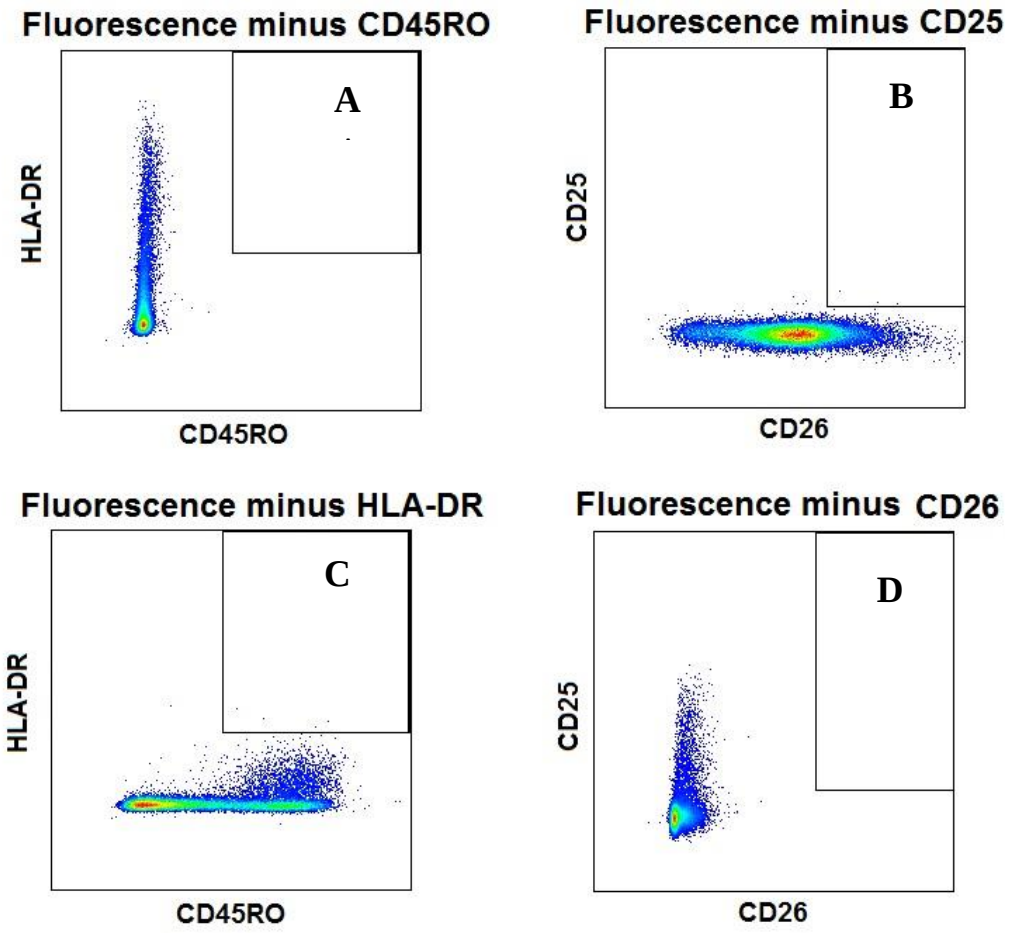


Figure 4.8: Fluorescence minus one (FMOs) controls stimulated for 6 days with SEB in day six assay. A) CD45RO FMO, B) CD25 FMO, C) HLA-DR FMO and D) CD26 FMO. The gates set here were used as the standard gating for the day six assay.

The FMOs (Figure 4.7 and 4.8) show that background staining and spectral overlap will not contribute to false positives to a certain extent.

4.2 The pilot study of Giardia-specific T cell responses

4.2.1 Participants

In total, fifteen *Giardia* exposed persons and ten low risk healthy controls were included in the study. In the *Giardia* exposed group 13.3 % (N=2) were unexpectedly still *Giardia* positive. We found generally stronger responses in these two individuals than in the rest of the *Giardia* exposed group, and therefore they were analyzed separately. The correlation of responses between SSA and SSB include all the participants from both groups. Characteristics for the three groups are displayed in Table 4.4.

P-values for all the $CD4^+$ T cell responses, when stimulated with SSA and SSB. compared between the two groups measured in SSA and SSB is attached in Appendix E.

Table 4.4: Characteristics of the study participants by group.

Characteristics	Current giardiasis	Recent giardiasis	Low risk controls	P-value
Individuals in each group (n)	2	13	10	-
Female gender, n (%)	1 (50.0)	8 (61.0)	7 (70.0)	1.0
Age (years), mean (SD)	39.5 (23.3)	42.5 (18.3)	46.8 (17.0)	0.57
Infection duration, weeks (range)	8.5 (5-12)	10.3 (1-54)	NA	0.48
Mean time since giardiasis to sampling, weeks (range)	NA	51.5 (1-112)	NA	NA

There were no significant baseline differences between the groups (Fisher's exact test for categorical, non-parametric Kruskal-Wallis test for continuous variables).

NA: not applicable.

4.4.2 Day one findings

4.4.2.1 Lymphocytes and $CD4^+$ cells

The average number of lymphocytes analyzed in this project was important. A larger number of lymphocytes analyzed, would give a more accurate estimation of rare cell events. Table 4.5 shows the average number of lymphocytes and $CD4^+$ cells found for all individuals in the *Giardia* exposed group (Ag) and the all the low risk healthy control group (LR).

Table 4.5: Average number of lymphocytes and $CD4^+$ cells acquired in the day one assay.

	Stimulation media	LR	Ag
Lymphocytes (n)	MED	736100	661902
	SSA	431736	391069
	SSB	435447	393742
	PPD	405088	354742
	LPS	392635	312894
	PMA/IC	307202	249088
Average	All	451368	393906
$CD4^+$ T cells (n)	MED	291145	281931
	SSA	179273	178380
	SSB	182278	176904

	PPD	171069	158379
	LPS	156777	132200
	PMA/IC	103134	94358
Average	All	180613	170359

4.4.2.2 Flow cytometric plot of IL-17A producing cells

The cytokine responses were compared between the study groups. We explored cytokine producing cells both in the whole $CD4^+$ T cell population and in the smaller $CD4^+CD197^-$ $CD45RA^-$ effector memory T cell population. We found antigen activated cytokine producing cells to be concentrated if the effector memory population (Figure 4.9). We concentrated the analysis of cytokine producing cells in this population, as this is likely to reflect a recall response.

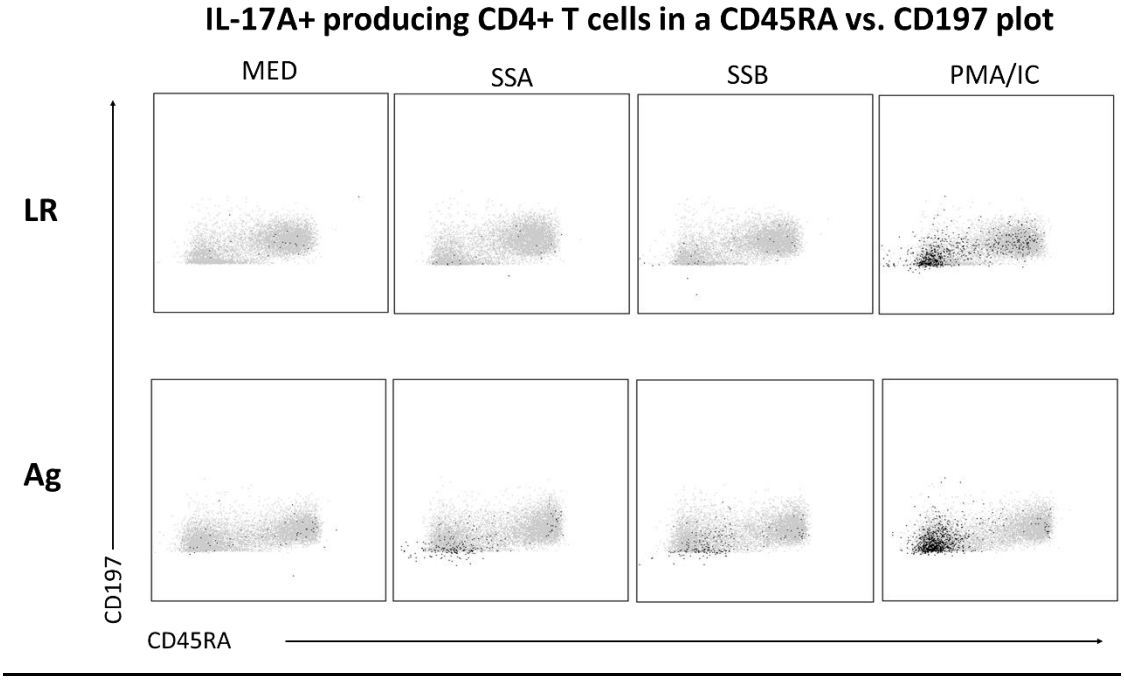


Figure 4.9: IL-17A$^+$ expressing CD4$^+$ T cells in a CD197$^-$CD45RA$^-$ plot

IL-17A$^+$ cells are represented by black dots in a gray CD4$^+$ T cell population. Cell plots represent one participant from the recent giardiasis group (Ag) and one participant from the low risk healthy control group (LR). The flow cytometric plots represent cells in medium only (MED), and cells stimulated with SSA, SSB or PMA/IC. The IL-17A producing cells are concentrated in the CD197$^-$CD4RA$^-$ effector memory cells population.

4.4.2.3 Percentages of cytokines expressed by effector memory CD4$^+$ cells

The cells were gated according to the strategy shown in Methods. Analysis of cytokines producing antigen-specific effector memory CD4$^+$CD197$^-$CD45RA$^-$ T cells, was the main focus for the day one assay. The assay allowed analysis of TNF-α, IFN-γ, IL-17A, IL-4 and IL-10 secreting cells after 24 hours antigen stimulation. We also analyzed multifunctional cells able to produce both TNF-α and IFN-γ. Data from FlowJo was used to make bar charts in SPSS of responses seen in each study group. In the recent giardiasis and low risk control group, the mean percentages of theses cytokine producing cells, with bars showing the 95 % confidence interval (CI), can be seen in Figures 4.10-4.14. Because there were only two participants in the current giardiasis group, these are represented by their measured values, rather than a mean value and 95 % CI bars. Correlation of IL-17A responses in SSA and SSB stimulated effector memory CD4$^+$ T cells is presented in Figure 4.15.

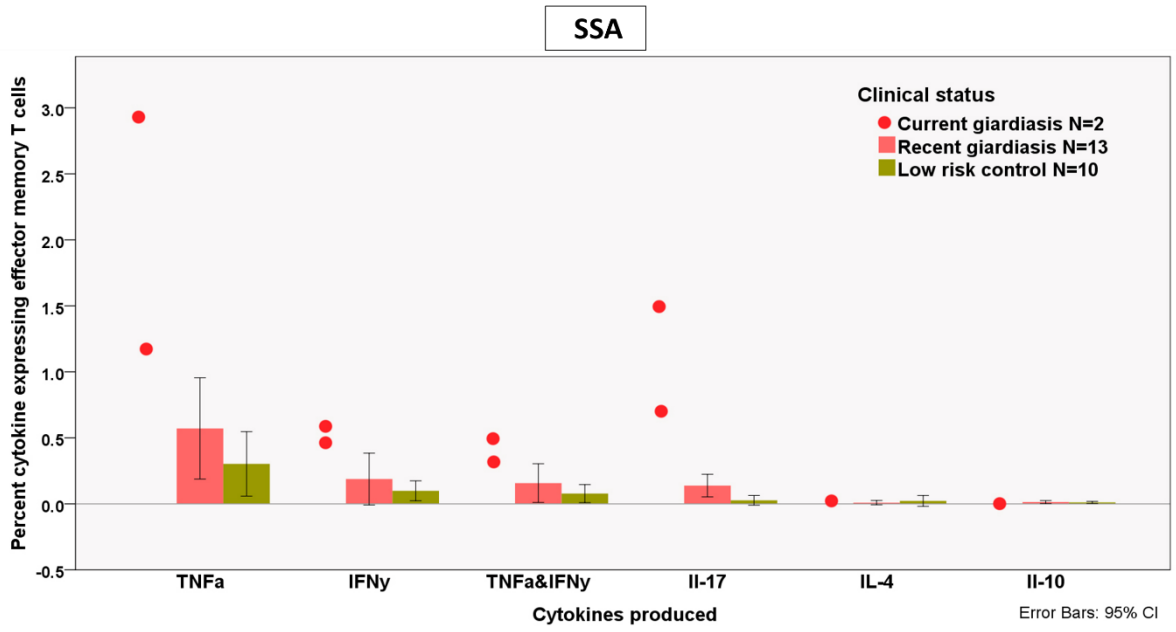


Figure 4.10: Cytokines produced in the effector memory CD4$^+$ T cell subsets when stimulated with SSA. Average percentage expression of cytokines in the effector memory CD4$^+$ T cells are represented by the y-axis and the various cytokines measures are presented on the x-axis.

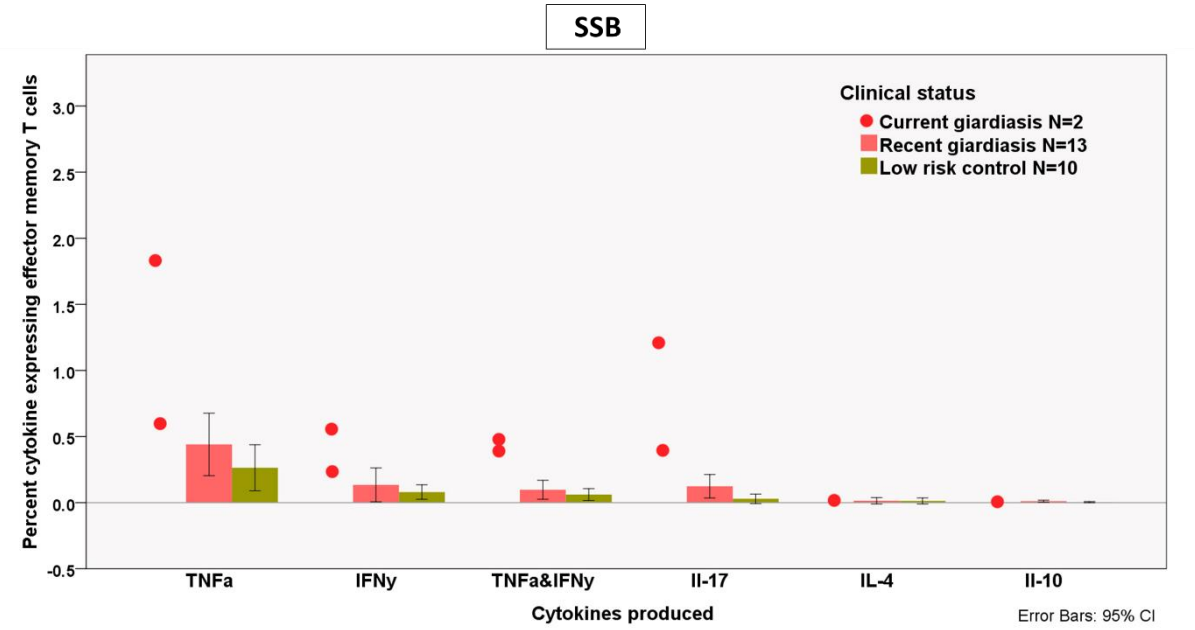


Figure 4.11: Cytokines produced in the effector memory CD4$^+$ T cell subsets when stimulated with SSB. Average percentage expression of cytokines in the effector memory CD4$^+$ T cells are represented by the y-axis and the various cytokines measures are presented on the x-axis.

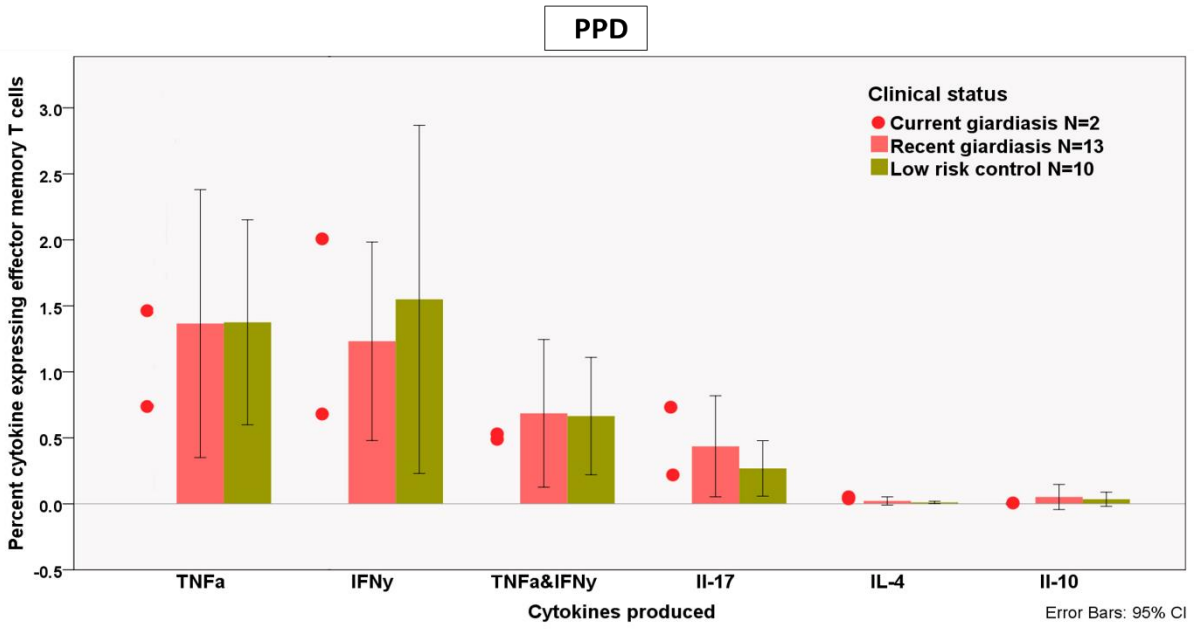


Figure 4.12: Cytokines produced in the effector memory CD4$^+$ T cell subsets when stimulated with PPD. Average percentage expression of cytokines in the effector memory CD4$^+$ T cells are represented by the y-axis and the various cytokines measures are presented on the x-axis.

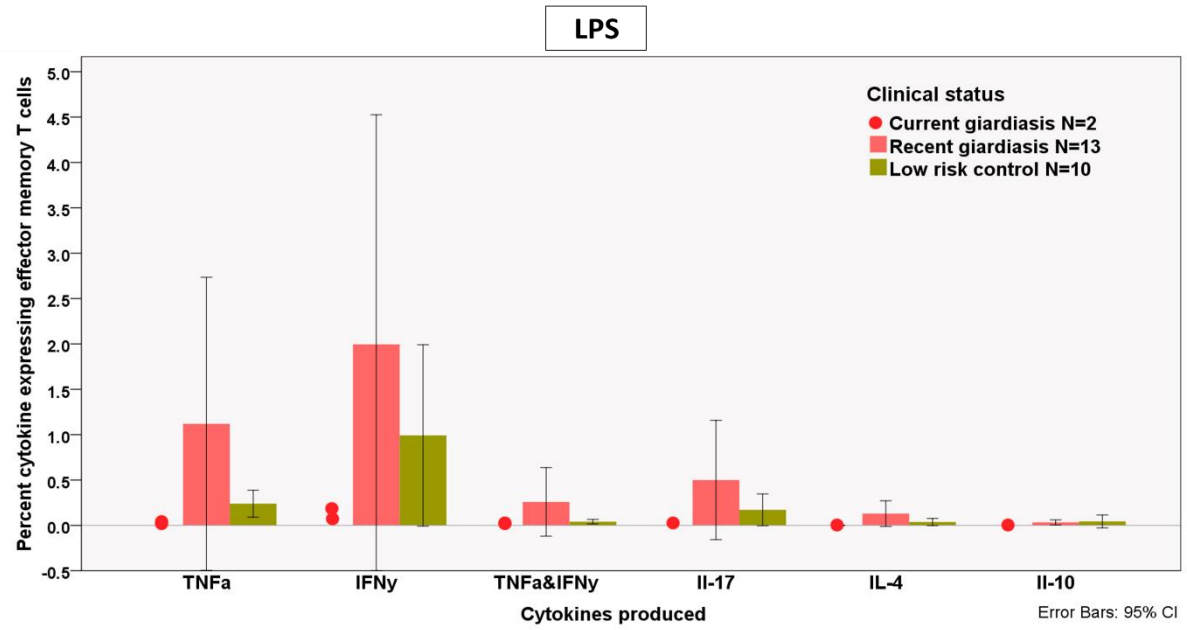


Figure 4.13: Cytokines produced in the effector memory CD4⁺ T cell subsets when stimulated with LPS.
Average percentage expression of cytokines in the effector memory CD4⁺ T cells are represented by the y-axis and the various cytokines measures are presented on the x-axis.

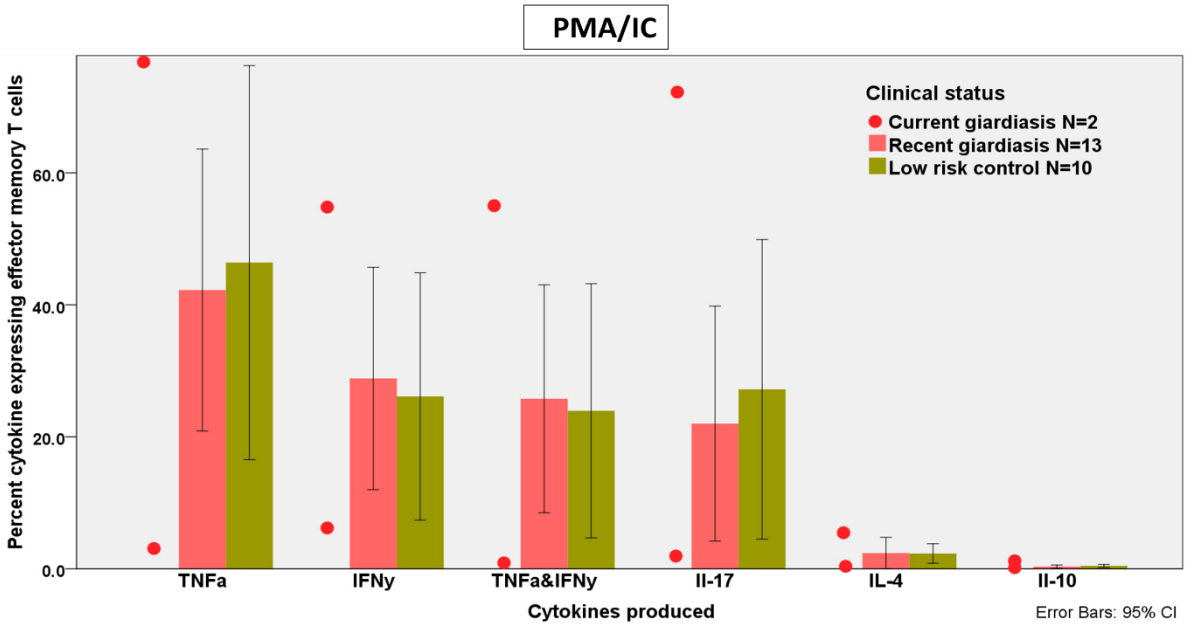


Figure 4.14: Cytokines produced in the effector memory CD4⁺ T cell subsets when stimulated with PMA/IC.
Average percentage expression of cytokines in the effector memory CD4⁺ T cells are represented by the y-axis and the various cytokines measures are presented on the x-axis.

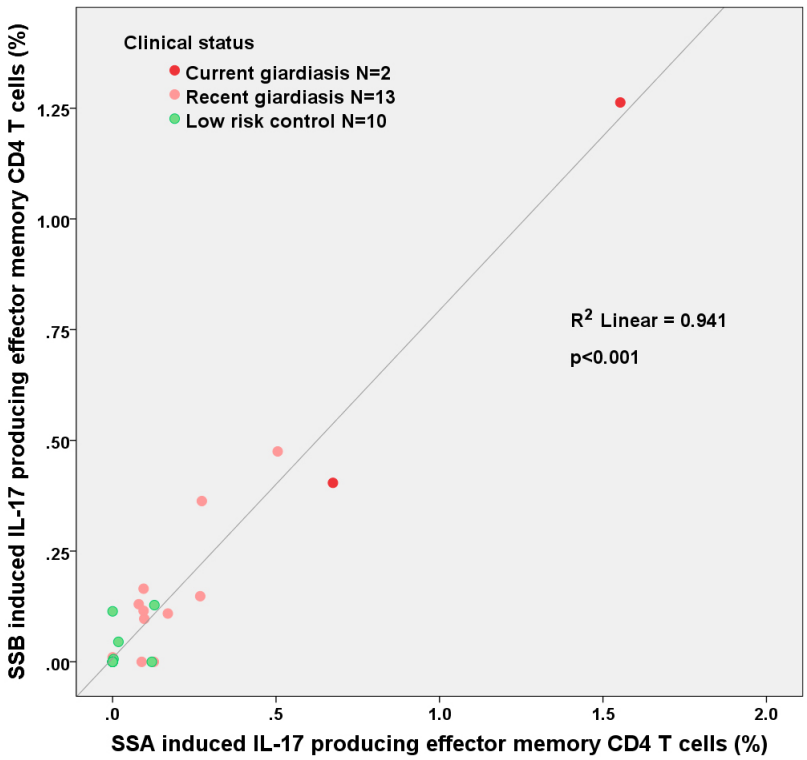


Figure 4.15: Scatter plot of the correlation of IL-17A responses in SSA and SSB stimulated effector memory CD4$^+$ T cells.

The x-axis represents SSA stimulated percentages of IL-17A$^+$ effector memory CD4$^+$ T cells and the y-axis represents SSB stimulated percentages of IL-17A$^+$ effector memory CD4$^+$ T cells.

Data for the two participants found to have current giardiasis were not statistically compared to the other two groups. However, we qualitatively evaluated their responses compared to the other groups.

The cytokine IL-17A was statistically more expressed in effector memory CD4$^+$ T cells in the recent giardiasis group, compared to low risk controls, when stimulated with SSA (P=0.035) but not with SSB (p=0.062). For comparison we also compared cytokine producing cells in the general CD4$^+$ T cell population and found P=0.043 for SSA and P=0.037 for SSB. Figure 4.10 and 4.11 also demonstrated that the two individuals with current giardiasis have higher percentages of cells producing TNF-α, IFN-γ, doubly producing TNF-α and IFN-γ, and IL-17A in response to *Giardia* soluble proteins SSA and SSB.

Figure 4.15 represents correlation between IL-17A memory effector CD4$^+$ T cell responses when the cells were stimulated with SSA and SSB. By linear regression analysis, we found a significant positive correlation between these two antigen solutions made from the two distinct isolates of *Giardia*. All the cytokines tested for correlation between SSA and SSB (TNF-α, IFN-γ, IL-17A, IL-4 and IL-10) gave P-values of 0.001 or less. The R^2 value, a measure

of goodness-of-fit, for the correlation of IL-17A responses between SSA and SSB was 0.941. The percentages of IL-4 and IL-10 positive cells were low in all of the tested stimulation media.

4.4.3 Day six findings

4.4.3.1 Lymphocytes and CD4$^+$ cells

The average number of lymphocytes found in the project was important. Surface markers analyzed for day six were not considered to be rare events such as cytokines. Fewer cells were therefore used in the day six assay. Table 4.6 shows the average number of lymphocytes and CD4$^+$ cells found for the Ag group and the LR group.

Table 4.6: Average number of lymphocytes and CD4$^+$ cells acquired in the day six assay

	Stimulation media	**LR**	**Ag**
Lymphocytes (n)	MED	60786	105385
	SSA	63370	60091
	SSB	63605	55432
	PPD	59344	63299
	SEB	86241	72434
Average	All	66669	71328
CD4$^+$ T cells (n)	MED	60786	61932
	SSA	34800	32374
	SSB	35834	29460
	PPD	27633	31053
	SEB	34091	30212
Average	All	38629	37006

4.4.3.2 Flow cytometric plots of activation markers and proliferation on day six

The cells in this assay were gated according to the strategy shown in Methods. The surface activation responses and proliferation rates were compared between the study groups. We explored up-regulation of CD25$^+$/CD26^{++}, CD45RO$^+$/HLA-DR$^+$ and combination of CD25$^+$/CD26^{++} and CD45RO$^+$/HLA-DR$^+$ CD4$^+$ T cells and proliferation of CD4$^+$ T cells. We found antigen activated CD45RO/HLA-DR (Figure 4.16) and quadruple positive cells for the surface markers (Figure 4.17) to be up-regulated in the analysis. Proliferation of CD4$^+$ T cells is displayed in Figure 4.18.

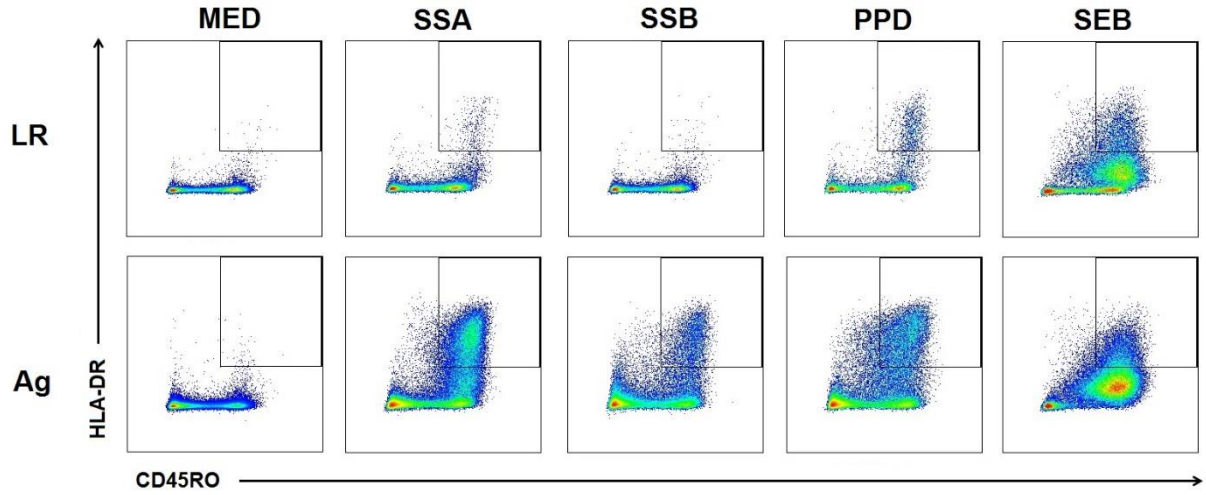


Figure 4.16: HLA-DR$^+$CD45RO$^+$ in CD3$^+$CD4$^+$ T cells.

Cell plots represent one participant from the recent giardiasis group (Ag) and one participant from the low risk healthy control group (LR). The flow cytometric plots represent cells in medium only (MED), and cells stimulated with SSA, SSB, PPD or SEB. The x-axis represents CD45RO, and the y-axis represents HLA-DR. The square in each cell plot represent the gate used to look at the double positive cells.

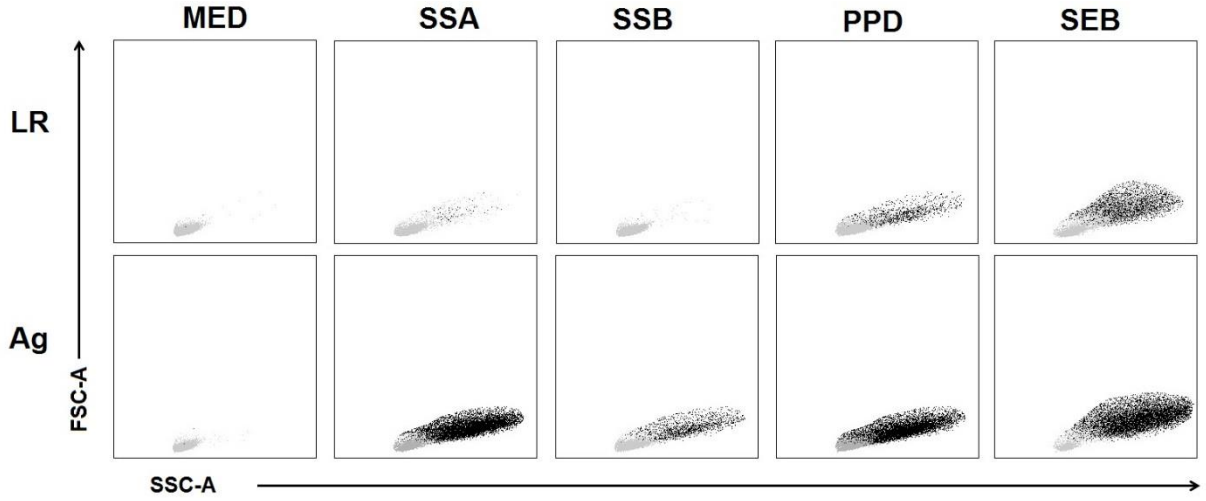


Figure 4.17: Quadruple positive cells of CD25/CD26 and HLA-DR/CD45RO

Cell plots represent one participant from the recent giardiasis group (Ag) and one participant from the low risk healthy control group (LR). A gray CD4$^+$ T cell population is represented, and the x-axis represents SSC-A and the y-axis represents FSC-A. The black dots represent quadruple positive cells of CD25/CD26 and HLA-DR/CD45RO. The flow cytometric plots represent cells in medium only (MED), and cells stimulated with SSA, SSB, PPD or SEB.

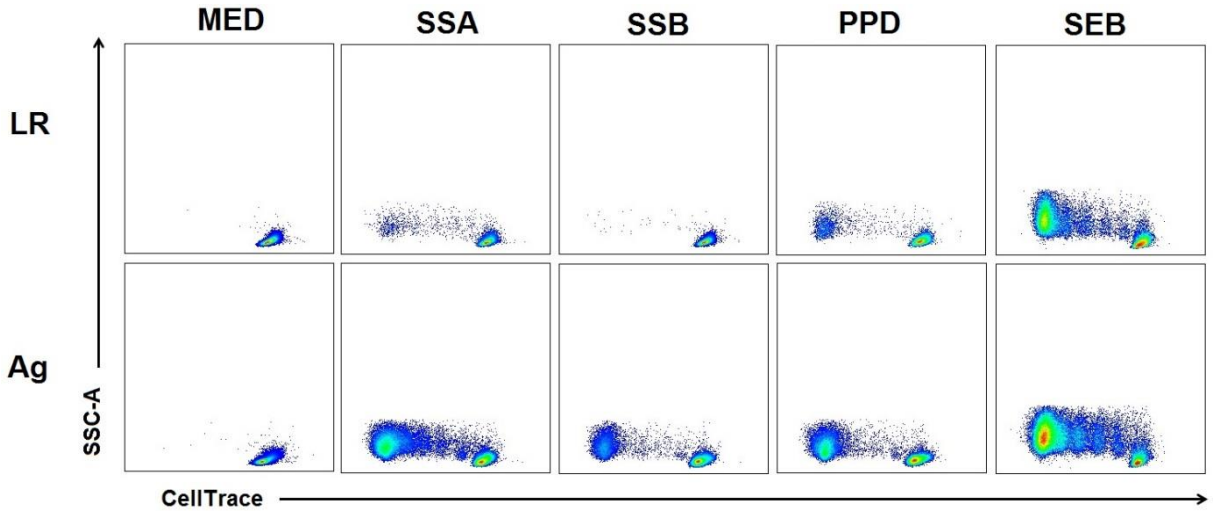


Figure 4.18: Proliferating CD4$^+$ T cells in the two groups

Cell plots represent one participant from the recent giardiasis group (Ag) and one participant from the low risk healthy control group (LR). The x-axis represents CellTrace proliferation dye and the y-axis represents SSC-A. The flow cytometric plots represent proliferating cells in medium only (MED), and cells stimulated with SSA, SSB, PPD or SEB.

Figure 4.16 represents HLA-DR and CD45RO positive cells found in the day six assay from an individual from the LR group and an individual from the Ag group. The stimulation with SSA gave significant findings for the HLA-DR/CD45RO activation markers. The negative control, MED, gave no positive responses in neither of the participants. The SSA stimulated cells gave some weak responses in the LR individual and a strong response in the Ag individual. SSB stimulation have less positive cells in the LR individual, and in the Ag individual a positive cell population can be found in the HLA-DR/CD45RO gate. The positive cells found for the SSB stimulated cells can correspond to specific responses. The PPD and SEB, positive controls, gave responses in both of the individuals, but the cells from an individual in the Ag group seem to be more activated than the individual from the LR group. No differences of statistical significance were found for PPD stimulation between the groups.

Figure 4.17 demonstrates black quadruple positive cells of the four surface activation markers (CD25, CD26, CD45RO and HLA-DR) displayed in black in a grey CD4$^+$ T cell population shown in gray.

4.4.3.3 Percentages of activated and proliferating CD4$^+$ T cells

Analysis of surface activation markers and proliferation was the main focus for the day six assay. The percentages of CD4$^+$ T cells showing activation and proliferation were compared between the Ag and the LR group. The assay allowed analysis of doubly positive CD25/CD26 and CD45RO/HLA-DR cells, quadruple positive cells CD25/CD26 and CD45RO/HLA-DR

and proliferation after six days of antigen stimulation. Data from FlowJo was used to make bar charts in SPSS of responses seen in each study group. In the recent giardiasis and low risk control group, the mean percentages of the surface activation responses and proliferation are displayed with bars showing the 95 % CI (Figures 4.19-4.22). Because there were only two participants in the current giardiasis group, these are represented by their measured values, rather than a mean value and 95 % CI bars.

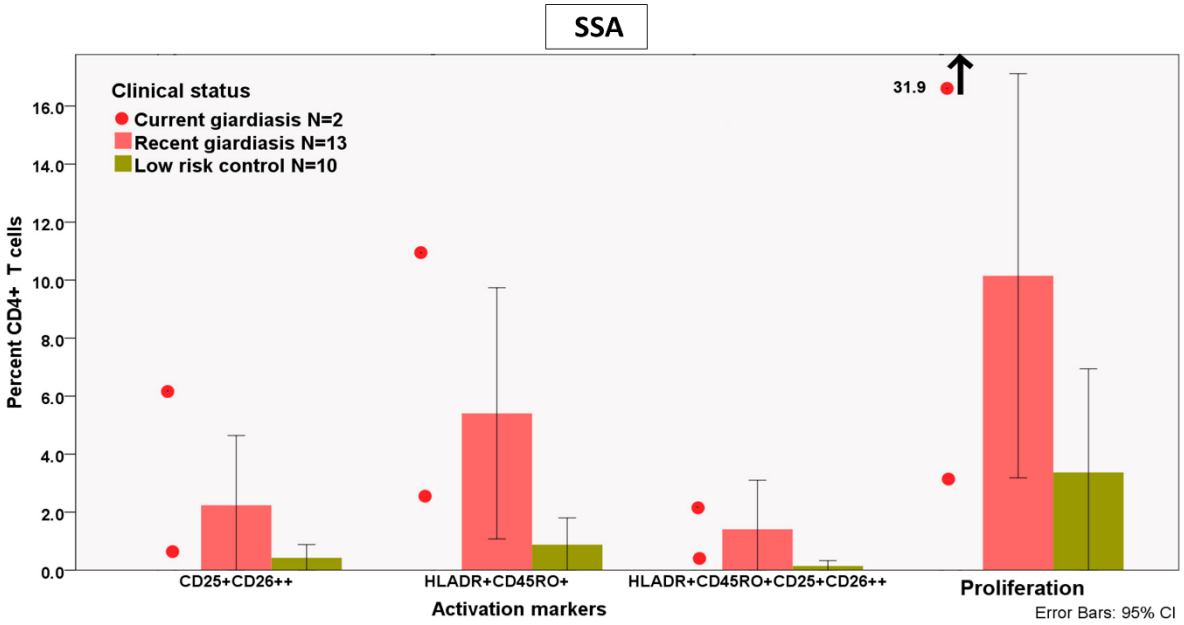


Figure 4.19: Activation markers and proliferation of $CD4^+$ T cells when stimulated with SSA.

Average percentage expression of surface activation markers and proliferation of $CD4^+$ T cells are represented by the y-axis and the various markers and proliferation measures are presented on the x-axis.

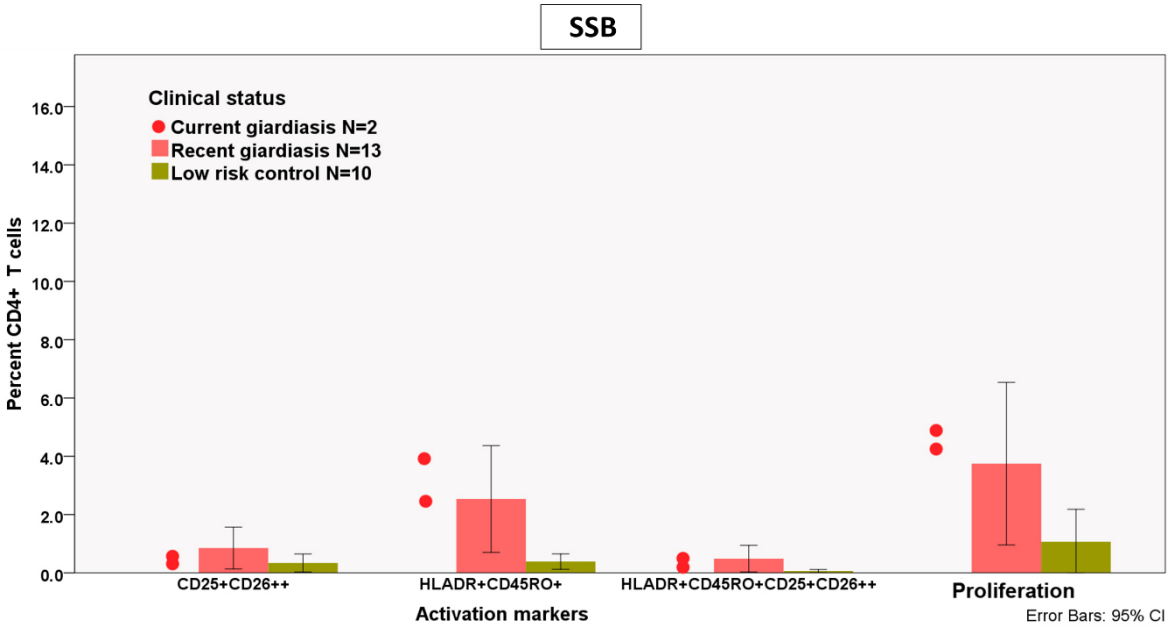


Figure 4.20: Activation markers and proliferation of CD4$^+$ T cells when stimulated with SSB.

Average percentage expression of surface activation markers and proliferation of CD4$^+$ T cells are represented by the y-axis and the various markers and proliferation measures are presented on the x-axis.

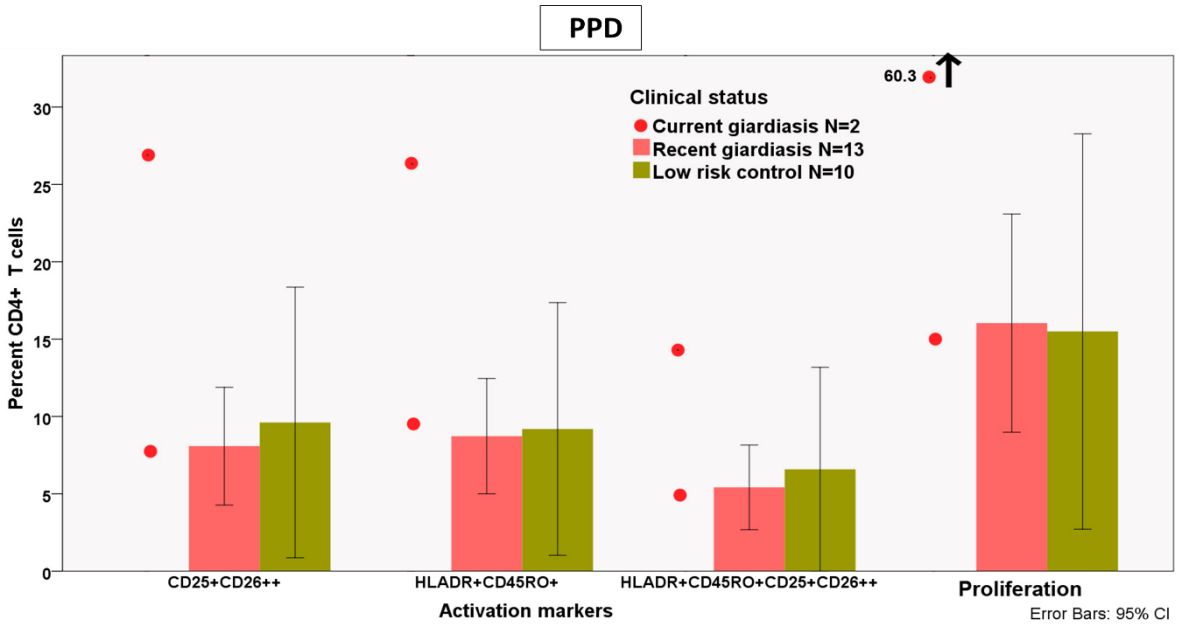


Figure 4.21: Activation markers and proliferation of CD4$^+$ T cells when stimulated with PPD.

Average percentage expression of surface activation markers and proliferation of CD4$^+$ T cells are represented by the y-axis and the various markers and proliferation measures are presented on the x-axis.

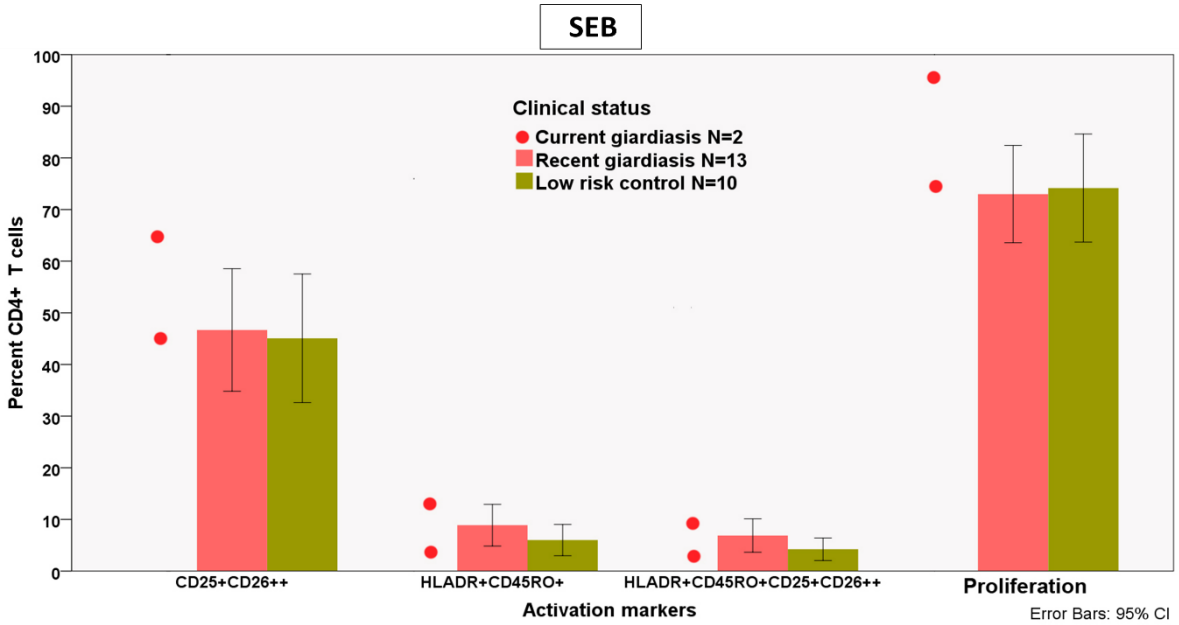


Figure 4.22: Activation markers and proliferation of CD4$^+$ T cells when stimulated with SEB.

Average percentage expression of surface activation markers and proliferation of CD4$^+$ T cells are represented by the y-axis and the various markers and proliferation measures are presented on the x-axis.

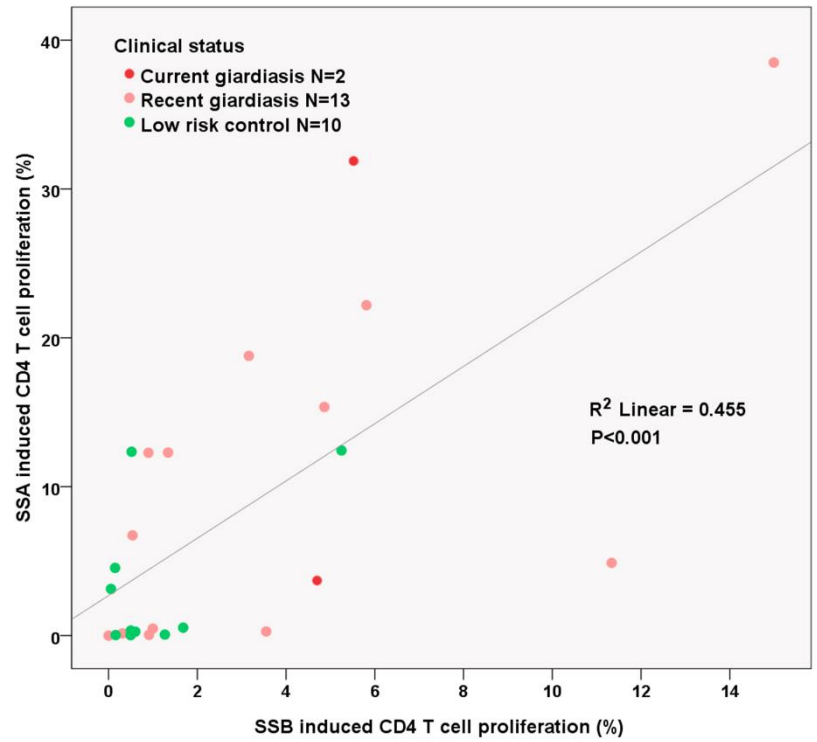


Figure 4.23: Linear regression analyses of correlation of proliferation percentages between SSA and SSB stimulated CD4$^+$ T cells.

The x-axis represents SSA stimulated percentages of proliferating CD4$^+$ T cells and the y-axis represents SSB stimulated percentages of proliferating CD4$^+$ T cells. Current giardiasis individuals are represented by red dots, recent giardiasis individuals are represented by pink dots and low risk controls are represented by green dots.

For the SSA stimulated cells, CD45RO and HLA-DR positive cells were found to be significantly up-regulated in the Ag group with a P-value of 0.036. Quadruple positive cells for CD25, CD26, CD45RO and HLA-DR did also differ between the groups giving a P-value of 0.041. The CD25/CD26 alone did not show statistical significance, P=0.088. Figure 4.18 demonstrates proliferating $CD4^+$ T from the two groups. The proliferation did not show statistical differences between the two groups P=0.208

Figure 4.23 presents the correlation between proliferation of $CD4^+$ T cells when the cells were stimulated with SSA and SSB. By linear regression analysis, we found a significant positive correlation with a P-value under 0.001, and an R^2 value of 0.455.

SSB stimulated cells did not show statistical differences between the two groups.

5.Discussion

5.1 Methodology

5.1.1 The voltages and compensation matrices

The voltages and the optimal concentration of a fluorochrome is important for setting up compensation. When the voltage set up for one fluorochrome is adjusted, the corresponding relationship between detectors will change, and the values of the compensation will change accordingly [77].

The voltages used in this study were based on repeated analysis and adjustments using single stained beads for flow cytometry, and also during titrations with live cells. It was noticed that the voltage values were important for how the spectral overlap manifested. A good compensation matrix should show as little spectral overlap as possible, but when multicolor fluorochrome panels are used, more spill over will be seen. By optimizing the voltages for the different channels, spectral overlap being 80 % and over, was adjusted to under 40 % by continued adjustment. Lineage makers being easy to separate from one another should therefore be placed on channels were spectral overlap is expected to manifest, whereas one should be careful with rare event populations such as cytokines.

Day-to-day variations in a flow cytometer can result in different results for a compensation matrix. A CST was done every day the flow cytometer was used, as a quality control for these variations. Considering day-to-day variations, compensation acquisition using single stained beads with FABs should be run prior to every flow cytometric experiment. A new compensation matrix was not made for every new experiment in the present study. However, several compensation matrices were tested and one for the day one assay and one for the day six assay showed to be reasonably stable over time. Figure 4.2, which represent the compensation matrices in this study, may have manifested differently according to the day the compensation was run.

5.1.2 The permeabilization and fixation reagents

As demonstrated earlier, two separate protocols were tested for the fixation and permeabilization of the cells. Formaldehyde, Triton X-100 and methanol did show to be a promising protocol for intracellular staining. However, more cells were lost with this method

compared to cytoperm/cytofix. The CD4 FAB clone tested in this experiment did not give good lineage separation, so $CD8^+$ T cells were used to compare cytokine expression instead. We later settled to analyze only $CD4^+$ T cells, and the cytokines produced by $CD8^+$ T cells when testing fixation and permeabilization reagents, could be misleading. The testing of these reagents was also performed at an early stage in the study, before we started using FMOs and negative controls with unstimulated stained cells and unstained cells. Some caution was therefore taken about the gating and measurements in this testing setup. Cell loss during the staining and permeabilization procedures was our major problem in the start. Cytoperm/cytofix gave a reasonable number of cells available for flow cytometric analysis, and these are the reagents most widely used in published method papers and intracellular T cell assays. In addition, the formaldehyde, Triton X-100 and methanol protocol takes 60 more minutes to conduct. The cytoperm/cytofix protocol was therefore chosen as the fixation and permeabilization reagent for further experiments.

5.1.3 Validation of CellTrace assay

Measurement of proliferation of antigen-specific cells can give a good indication of memory immune responses [65]. Cell division can be tracked using different methods, both flow cytometric and by nucleoside uptake of proliferating cells [65].

An earlier study investigating proliferation in response to SSA and SSB [10], used ^{3}H-thymidine assay to evaluate proliferation. The ^{3}H-thymidine was in this study added to the PBMCs after 5 days of stimulation with SSA and SSB. ^{3}H-thymidine incorporation in dividing cells could then be measured.

The flow cytometric assay for investigating proliferating cells is more specific than incorporation of ^{3}H-thymidine. The reasons for this is that in addition to proliferation tracking, specific cells exhibiting phenotypic or functional characteristics can be gated and investigated further when using flow cytometry [78].

Flow cytometric dye dilution with CellTrace was chosen as the proliferation tracking agent in this study and to investigate the percentages of proliferation together with the surface activation markers. Being able to analyses the proliferating of $CD4^+$ T cells to investigate *Giardia*-specific immune responses was one of the aims for this study. To avoid working with radioactive substances, and to be able to analyze the specificity of the proliferating subsets, ^{3}H-thymidine assay was not chosen in this study.

CellTrace labeling of PBMCs was done on day 0 in the study, and therefore proliferation

would be recorded during the entire culturing period. Incorporation of ^{3}H-thymidine, on the other hand, would just have investigated cells still proliferating after 5 days, since only these cells would take up ^{3}H-thymidine. The proliferation ratio seen in a ^{3}H-thymidine assay will be based on an entire population of mixed cells and proliferation of specific subsets cannot be assessed.

The proliferation of $CD4^{+}$ T cells in this study was not significantly different between *Giardia* exposed and unexposed. A larger study population could perhaps have given more information regarding proliferation as the p-value for the SSB proliferation was close to 0.05.

Measuring proliferation with CellTrace investigation has its limitations. Using too high concentration of this dye can be toxic to cells and result in reduced proliferation. Cells that are activated without starting to proliferate will be missed. Also the lack of differential proliferation responses might be due to cross-reacting immunity, or unknown previous *Giardia* infection, in the low risk controls. No significant p-values seen for proliferation measurements does not necessarily mean that *Giardia*-specific immunity does not exist for $CD4^{+}$ T cells [78].

5.1.4 The number of cells per well for the two different assays

The number of cells per well and if duplicates or triplicates were used, could be variable. For some of the individuals recruited, lower numbers of PBMCs were obtained from the four CPT tubes of blood, which was drawn from each participant. The number of PBMCs needed for the day one assay was 21 million, where two triplicates were used for the negative control, and one triplicate per antigen/positive controls. In the day six assay 2×10^{5} cells were added to all the wells. A typical set-up consisted of 3 million PBMCs in the day six assay in order to fill triplicates to the 96 V-wells plate. If fewer cells than the required 24 million cells were obtained, duplicates were set up instead, resulting in fewer cells in the final analysis. Still, the lowest number of cells obtained from SSA or SSB stimulation in the day one assay was 68104 and 30818 in the day six assay. Keeping all participants in the analysis, and using a cell concentration as close to 1×10^{6} as possible for day one, where rare events were investigated, was considered to be important The standard cell concentration per well in the day one assay was 1×10^{6} per well, and always higher than 6×10^{5}.

The differences in the cell concentrations used for the day one and day six assays were different due to the expected cell loss in the day one assay caused by the fixation and permeabilization steps and more washing steps. Additionally, cytokine producing cells are rare events, and require a larger cell population to be analyzed to achieve reliable data.

Figures 4.10-4.14 demonstrates the percentages of cytokine producing cells obtained in this study. The percentages of cells producing IL-4 and IL-10 are generally lower than the percentages of the other cytokines. Percentages of IL-4^+ effector memory $CD4^+$ T cells for the stimulation media SSA and SSB were generally between 0.00-0.02 and too few cells were collected (Table 4.5) to give a CV of 5 %. Percentages of IL-4^+ cells in the positive control PMA/IC, was however over 2 % and a CV over 5 % can be found. IL-4^+ cell percentages can thus be unreliable and only gave a CV of 5 % in the PMA/IC positive control.

Percentages of IL-10^+ cells were generally below 0.01 % for the stimulation media SSA and SSB not giving a CV of 5 %, while stimulation with PMA/IC gave 0.4 % IL-10^+ cell or more. The average number of $CD4^+$ T cells stimulated with PMA/IC were 103134 for the LR group and 94358 for the Ag group (Table 4.5). 100.000 total events should have been collected to give a CV of 5% for IL-10. The IL-10^+ result detected in this study can therefore be unreliable.

An evaluation of how many cells are needed for analyzing the cells of interest should be done before a flow cytometric experiment is done, to avoid too few cells in the final sample.

Surface markers were investigated on day six, and these are more abundant and therefore more easily stained than cytokines. Because of fewer washing steps, cell loss was less of a problem. The responses being analyzed were also expected to be amplified through the proliferation of responding cells, thus positive events are not that rare. The percentages for responses found for the day six assays were generally over 1 %, meaning that at least 40 000 cells had to be acquired to get a CV of 5 %. The average number of $CD4^+$ T cells acquired in the day six assay in this study (Table 4.6) was 38629 for the LR group and 37006 for the Ag group. The numbers obtained for $CD4^+$ T cells in this study are very close to 40 000. If a CV of 6 % was accepted for the day six assay, 27777 $CD4^+$ T cells had to be aquired.

2 x 10^5 PBMCs were added to each well in this assay, and if a higher concentration had been used in the 96-well plates, the 200 µL medium could have been spent by growing cells before the sixth day was reached. This could have led to cell death or inhibition of proliferation and activation. For these reasons, a smaller T cell population was deemed adequate for analyzing activation and proliferation responses.

5.1.5 Pitfalls in flow cytometry

Several factor can influence the quality of a flow cytometric analysis and have to be taken into consideration.

The importance of having a good compensation matrix with minimal spillover has been discussed earlier. Even if the compensation matrix is good, spectral overlap may still happen. The calculated compensations in the software program can be misleading. The fluorescence and scattered light recorded by the flow cytometer may change due to day-to-day variations in the laser [79]. The CST is therefore needed and important to do before analysis, to be aware of changes and failures in the flow cytometer.

Cell loss was seen during the development of the flow cytometric method. Having enough cells in the wells to compensate for cell loss during staining and permeabilization was important.

The acquisition of data during flow cytometry can be challenging. If an accessary plate reader for a flow cytometer is used, the wells have to be filled with a volume compatible with the flow cytometer, to avoid air in the machinery. Air can disturb the analysis, resulting in no valid data for the analysis.

Fresh cell samples were always used in this study, to avoid freezing and thawing of cells, leading to more cell death [61]. Dead cells should always be excluded from the analysis to avoid false positive non-specific binding by dead or dying cells [64].

The clones for a specific FAB is important for the affinity of a target [61] and as experienced in this study, the clones can also be incompatible with fixation and permeabilization reagents. The FABs and their antibodies should therefore be tested for such incompatibility.

Due to FABs capacity to be excited they are sensitive to light [61] and caution should be taken when staining cells, washing and analyzing to avoid degradation.

Some fluorochromes such as phycobiloproteins (APC and PE) have large molecular sizes compared to other FABs, and steric hindrance should be considered for the analysis of intracellular cytokines [61]. The IL-10 and IL-4 used in this study were coupled to, respectively, PE and APC, and low percentages of these cytokine producing cells obtained, can thus be speculated to be a result of both inadequate positive controls and steric hindrance. Still APC and PE are bright fluorochromes [61], and are often used for intracellular targets in flow cytometric analyses. Bright fluorochromes have an advantage for staining cell population of

rare events.

5.2 Quality control

When testing with flow cytometry, is essential to include positive and negative controls, as internal quality controls. Cytokine producing cells are as mentioned rare events, and in order to be able to investigate *Giardia*-specific $CD4^+$ T cells, a negative control without cytokine producing cells is needed, as well as a positive control expected to give many positive events. These two internal controls can validate the status of the cells. If responses are seen in the negative control, contamination can have happened, and the results cannot be trusted. On the other hand, no responses for a positive control may indicate that the cells are not responding well to stimulation and the results may be weak for the tested antigens as well.

Figure 4.14 shows the percentages of the cytokine producing cells when stimulated with PMA and IC in this study. Large confidence intervals for the percentages are presents for all of the cytokines, and one individual with current giardiasis had weak responses overall. The other individual with current giardiasis has high responses for all of the respective cytokines compared to the other one. The unexpectedly low reaction to PMA/IC should raise caution that perhaps the PBMC of this participant were responding weakly to all of the antigens tested. Another reason could be that the weak response was due to the dilution of PMA and IC and the storage. New solutions were not made for every experiment, and the solutions may have lost some reactivity during storage. A standard procedure should have been used in this study for the PMA/IC positive control, by either using fresh made solutions for every new experiment, or using one-week-old solutions for every experiment. However, PPD and LPS were also included as positive controls in this study, and the individuals with current giardiasis shows responses in the expected range towards both PPD and LPS, and we concluded that the weak PMA/IC response was due to variations in its reactivity. Thus, the results seen for SSA and SSB could be trusted.

All of the 96 V-wells plates with antigens and negative control were made the same day and kept at -20 °C to keep day-to-day variations to a minimum. PMA and IC were added after 18 hours of PBMC stimulation and therefore, and these reagents could not have been added to the 96 V-wells plate and kept frozen to avoid variations.

FMOs were used to set gates for the day one and day six assays. The FMOs were particularly important for the gating strategy for continuous surface markers such as CD45RO,

HLA-DR with where no definite separation of positively and negatively stained cells could be seen (Figure 4.8 and Figure 4.16).

5.3 Evaluation of the immune responses

5.3.1 Why use flow cytometry to look at immune responses?

Flow cytometry has the advantage of recording multiple cellular markers at the same time. Cellular responses can be investigated according to surface markers or other phenotypic markers expressed on cells. Flow cytometry is unique in this respect compared to i.e. ELISA, where specific cell cannot be sorted and multipotent cells cannot be distinguished from other cells [78]. More specific responses can therefore be examined using flow cytometry [61].

5.3.2 Stimulation times

The PBMCs were stimulated for a total of 24 hours in the day one assay. The duration of stimulation is essential, as different cytokines can have their highest levels of expression at different time points.

The cytokine IL-17 has showed to reach its peak level after 24 hours when stimulated with several reagents, including PMA and IC. Levels of IFN-γ and TNF-α were showed to be persistent after more than 24 hours for activated cells [80].

IL-10 producing cells have been examined in an earlier study [81], looking for the dominant cytokine responses in malaria exposed children. The PBMCs were stimulated for 24 hours, and it is known that a stimulation time of 24 hours is needed to elicit IL-10 responses [63]. TNF-α and IFN-γ were also investigated in the malaria study and supports the stimulation time we used.

Due to IL-17A, IL-10, TNF-α and IFN-γ having been found after 24 hours of stimulation before, we decided use the same stimulation time. The duration of stimulation can affect the cytokine production, and optimally different stimulation periods could have been tested in this study. Surface activation markers and proliferation in response to SSA and SSB have been investigated before, and suggested good responses after six days of culturing [10]. The same stimulation period was therefore chosen in the present study.

5.3.2 $CD4^+$ T cell responses against Giardia

An effector response mediated by T cells have earlier been seen in mice studies. This indicates that control and eradication of an infection may be facilitated through an antibody-independent pathway [58].

The general $CD4^+$ T cell production of IL-17A seemed to be a better approach to investigate *Giardia*-specific $CD4^+$ T cell responses, as statistical significant differences between the recent giardiasis group and the low risk control group were found for both SSA and SSB. IL-$17A^+$ cells were only found to be statistical significant in the $CD4^+$ effector memory T cells when stimulated with SSA.

In cattle with current, on-going *Giardia* infection, IL-17A has recently been found to be up-regulated in proliferating PBMCs when stimulated with *Giardia* trophozoites [53]. FoxP3 was also investigated in this study, which measured responses using qPCR. The IL-17A responses found in this cattle study suggest that T_h17 responses may play a role in the protection and eradication of *Giardia*. Still, a larger human study population, preferably with current infection, is needed to validate our findings.

The surface activation markers HLA-DR, CD45RO, CD25 and CD26 on $CD4^+$ T cells in addition to proliferation using a ^{3}H-thymidine assay have been studied in individuals 5 years after initial *Giardia* infection [10]. Up-regulation of the surface markers were found in this study and the proliferation was also up-regulated. The expression of HLA-DR and CD45RO correlates with our findings and might suggest that up-regulation of these surface markers are specific for cellular immunity against *Giardia*. Proliferation and CD25 CD26 did not, however, show to be statistically significant in our study. A larger study population exposed to *Giardia* should be used in order to assess proliferation and CD25 and CD26 up-regulation in

Correlation of IL-17A $CD4^+$ effector memory T cell responses when stimulated with SSA and SSB, were found in our study. Also we found a positive correlation between SSA and SSB induced proliferation of $CD4^+$ T cells. Immune responses have been shown to be assemblage independent before [10], and our findings support this.

Quadruple positive $CD4^+$ T cells for all the surface markers, CD25, CD26, CD45RO and HLA-DR were significantly increased in the recent giardiasis group compared to the low risk control group in our study, when stimulated with SSA. These findings suggest a *Giardia*-specific immunity.

5.3.2.1 Responses not seen in the project

Up-regulation of some of the cytokines, that can be connected to a Th1 response, TNF-α and IFN-γ, were not statistically significant between the two groups, recent giardiasis and low risk controls. This result can be due to a small study population and small but unspecific responses in low risk healthy controls, large individual differences, undiagnosed previous *Giardia* in the low-risk group, as well as and time since the initial infection. The current giardiasis group did

however show up-regulation of TNF-α, IFN-γ and doubly positive cytokine producing TNA-α and IFN-γ. More individuals with current giardiasis are needed to investigate, if TNF-α and IFN-γ are up-regulated during infection.

It can be difficult to assess protective immunity from individuals who have had giardiasis. Reasons to this can be: protective immunity may only be partial, asymptomatic cases of infection is relatively common, variations between *Giardia* isolates expressing different immunogenic proteins can give differences and comparisons between studies can be limited due to different methods, antibodies, reagents and equipment [26].

5.3.3 The status of the low risk healthy controls

Some of the low risk healthy controls had small but unspecific responses against the *Giardia* soluble proteins we tested. Characteristics in the individuals, such as immune system, underlying ailments, earlier infection with other gut pathogens and/or *Giardia* assemblages can be reasons for different responses between the three groups [10]. The $CD4^+$ T cell responses seen for some of the low risk healthy controls can be due to several factors

Previous *Giardia* infection in low risk healthy controls can be difficult to assess, since a *Giardia* infection can pass unnoticed. The age of the low risk control would also be relevant for exposure to *Giardia*, as an older individual will have a greater chance of being exposed. A good method to determine whether an individual previously has had *Giardia* infection, would have been a good tool in order to find true low risk healthy controls.

Serology has can be useful to separate individuals who have had or have current infection with *Giardia* [28, 57]. *Giardia* IgM has shown to be increased in response to infection and could be useful for identifying individuals with current infection. IgG could also be useful to filter out *Giardia* exposed individuals, but cannot discriminate between past or current infection [28]. Low risk healthy controls should not be positive for *Giardia*-specific antibodies in order to be classified as an unexposed control.

5.3.4 The sonicated soluble proteins from Giardia trophozoites

The sonicated supernatant proteins from *Giardia* contains a mixture of many different soluble proteins potentially acting as antigens. As seen in an earlier study [10] we also found considerable cross reactivity between assemblage A and assemblage B *Giardia* isolates. We generally found lower responses to SSB compared to SSA. One reason for this could be that *Giardia* assemblage A parasites grow faster in culture than the B assemblage parasites [10].

Initial concentration of proteins were therefore higher in the SSA solution, than in the SSB solution. Even if we adjusted for this by measuring protein concentration and diluting to the same concentrations in stock solutions, this might have led to quantitative or qualitative differences between SSA and SSB, other than just their inherent assemblage differences.

Responses that could be observed in the low risk controls and in the exposed group in addition to cross reactivity can also have been affected by contamination of stimulatory agents in the growth medium. Bacterial or viral parts cannot be guaranteed to be absent even if all experiments were done in a sterile environment.

Trophozoites from clinical samples are difficult to culture [82]. The soluble proteins used in the present study are from trophozoites growing and replicating in culture, thus under unnatural conditions, and may have lost some of their virulence. Differences between pathogenic *Giardia* strains from clinical samples and *Giardia* strains grown in culture cannot be excluded. Strain differences may result in different proteins being up-regulated in the trophozoites and variability in virulence factors being present. The participants exposed to *Giardia* might have stronger reactions towards other variants of the extracellular *Giardia*-specific proteins on the surface known as VSPs.

The lack of differential responses between the exposed and unexposed group might be due to non-specific responses towards the large number of different proteins in the SSA and SSB mixtures. Identification and use of single *Giardia*-specific proteins or *Giardia*-specific peptides is likely to improve the specificity of the assay.

Proteins inhibiting cell activation and proliferation, such as ADI [23], could be present in the SSA and or SSB soluble protein solutions. Proliferation of PBMCs has been shown to be decreased if high concentrations of SSA and SSB were used in the stimulation [10], suggesting that proteins being able to reduce responses also can be present in the *Giardia* soluble proteins used in this study.

5.4 statistics

Multiple testing was done in this study between the recent giardiasis group and the low risk control group. Bonferroni correction can be used to adjust the level of significance when multiple comparisons are performed in a large dataset at the same time [83]. We did not do such adjustments due to the explorative nature of the study.

5.5 Limitations of the study

One limitation of the study is that a small study population was recruited. Laboratory confirmed *Giardia* infection is relatively uncommon in Norway. Individuals with recent giardiasis were included with a time span ranging from current, on-going, infection to over 2 years since successful treatment. The long time span, and the age of the participants which varied from 22 – 69 years probably cause a large variability in response measurements. Two individuals with current infection were unexpectedly included, and these were not included in statistical analysis but their data were qualitatively compared to the responses in the two larger groups.

Day-to-day variations in the flow cytometer and not running compensation controls on the flow cytometer prior to every experience could have influenced the results. Day-to-day variations for FAB concentrations, temperatures and exposure to light during the staining or after the staining could also influence the results.

δγ T cells were not excluded in this study. It has been reported that 0.02-0.4% of human δγ T cells may recognize PE as a specific antigen [84]. Thus, these cells can contribute to non-specific staining of the PE-fluorochrome and lead to false positive events.

Various cell concentrations were used in the day one assay ($6 \times 10^5 – 1 \times 10^6$) and could influence the number of responding antigen-specific cells per well and lead to different cytokine responses.

The statistics were not adjusted for multiple comparisons and caution should therefore be taken when interpreting the results.

5.6 Conclusion

In this study we show that flow cytometric assays measuring immune responses during antigen stimulation with soluble proteins from *Giardia lamblia* assemblage A and B, was possible to develop. The effector memory $CD4^+$ T cell responses seen in this study did not give more information on *Giardia*-specific immunity compared to the responses seen for the general $CD4^+$ T cell population when stimulated with SSA or SSB (Appendix E).

The increased IL-17A expression after 24 hours PBMC stimulation with SSA and SSB in the exposed group, especially found in individuals with current *Giardia* infection, could mean that T_h17 responses are dominant. However, a larger study population of individuals with current giardiasis is needed to qualify the responses seen in this study.

The up-regulation of the surface markers CD45RO and HLA-DR seen in the exposed group, when the PBMCs were stimulated with SSA for a total of 6 days, suggests that $CD4^+$ T

cells recognizes soluble fractions of proteins from *Giardia*. Up-regulation of the surface markers CD45RO and HLA-DR suggests that these surface markers can be used to investigate specific immune responses.

Combination of CD25 and CD26 to investigate *Giardia*-specific immune responses did not show statistical differences between the groups, and may not be a good marker of *Giardia*-specific immune responses.

No statistically significant differences in *Giardia* specific T cell proliferation between the two groups were seen in this study. However, the small study population could be contributing factor to the results seen, and effector cells can exert their functions without specific proliferation.

In this study we can conclude that T_h17 polarization of the T cell response may be important in *Giardia* infection and the up-regulation of surface activation markers on $CD4^+$ cells suggests that cellular immunity is important in *Giardia* infection. The *Giardia*-specific immune responses could have been improved using purified recombinant *Giardia* proteins as antigens.

Cell-mediated immunity can thus be speculated to be an important factor for protection and perhaps eradication of giardiasis. However, a larger study population is needed to strengthen these findings.

5.7 Further research

Infection with *Giardia* is mostly associated with developing countries where it can give rise to dehydration due to diarrhea and contribute to malnutrition and other serious complications. More research investigating immune responses in humans against *Giardia* is important for instance to develop a successful vaccine. A vaccine has been developed for domestics pets such as cats and dogs [85] but efficacy has been questionable. A vaccine for humans would be useful to protect against infection, decrease *Giardia* resistance to antibiotics and decrease serious complications that can follow an infection.

It would have been interesting to have stimulated PBMCs with surface VSPs from *Giardia* and compare cellular responses to PBMCs stimulated with soluble intracellular *Giardia* proteins. In order to maximize chances of recognition of a VSP, either focusing on a limited number of semi conserved VSP regions or including all the over 200 VSPs could be used in the stimulation. As *Giardia* has capacity for antigenic variation [51], only using one or a few VSPs might not be sufficient to detect immune responses.

Due to the low prevalence of *Giardia* infections in Norway, investigation of IL-17A, surface activation markers and proliferation responses in endemic areas would be fruitful. Individuals living in endemic areas probably have encountered the parasite several times, and the immune responses might be different. Other factors, including humoral immunity, might be protecting individual from *Giardia* infection.

Further, it would be interesting to compare the IL-17A responses in three different groups of *Giardia* infected individuals, where one group had current giardiasis, one group had chronic infection and another one had asymptomatic infection, to assess differences in immune responses between individuals, and get a better insight into immune responses in individuals leading to different clinical manifestations.

Next, it would have been interesting to look at the local immune responses induced in the epithelial barrier of the small intestine during a *Giardia* infection. This could have been done by culturing *in vitro* enterocytes, and thereafter stimulate the cells with *Giardia* trophozoites.

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Appendix A: Case Report Form

Pasient oppfølging skjema (Case Report Form) GSI 1

Pasient navn:

Fødseldato: **Tel nr:**

Kontakt:(sett ring) Tidl giardiasis Low risk control Studie Id:.....

Yrke: **BCG vaksinert:** ja / nei

Høyde: cm **Vekt:** kg

Helsetilstand

Kjent tarmsykdom eller familiær opphopning av dette?

Irritabel tarm/ Evt urolig mage forut for giardiasis?

Allergi / atopi / astma:

Immunsvikt?

Noen gang giardiasis før aktuelle episode? Evt når

Faste medisiner:

Utenlandsopphold siste året:

Giardiasis

Symptomdebut dato: Sannsynlig smitte fra (land):

Påvist *Giardia* Første gang dato: Siste gang dato:

Anti-giardiasis behandling påbegynt dato:

Medikament:

Helsetilstand siste to uker (for alle)

Har deltager, i løpet av siste 2 uker, hatt noen av følgende symptomer?

	Nei	Ja	Hvis ja; Ofte	Noen dager	Sjelden
Løs mage/diare?	☐	☐	☐	☐	☐
Kvalme?	☐	☐	☐	☐	☐
Oppkast?	☐	☐	☐	☐	☐
Feber?	☐	☐	☐	☐	☐
Klaget over magesmerter/ubehag?	☐	☐	☐	☐	☐
Liten matlyst?	☐	☐	☐	☐	☐
Spesielt illeluktende avføring/promping?	☐	☐	☐	☐	☐
Vekttap siden symptomdebut	☐	☐	Evt antall kg:.....		

Prøvetakingsdatoer (merknader: fastende = F, ikke fastende = IF, Spist/drukket siste time = S)

Blodprøve: **Spyttprøve:** **Avføringsprøve:**

Kommentarer:

Bristol scale:

Sucrose:

IMS:

For Low Risk controller:

	Nei	Ja
Noen gang reist ut av Norge	☐	☐
Noen gang ut av Europa	☐	☐
Vokst opp på gård?	☐	☐
Exponert under utbruddet i Bergen 2004?	☐	☐
Noen slektninger som har hatt kjent giardiasis tidligere?	☐	☐

Dato for utfylling: Initialer:

Appendix B: Informed consent

Immunresponser mot Giardia – September 2013

Forespørsel om deltagelse i forskningsprosjektet

«Immunresponser mot *Giardia* hos mennesker»

Bakgrunn og hensikt
Dette er et spørsmål til deg om å delta i en forskningsstudie for å teste og karakterisere immun-responser mot tarmparasitten *Giardia lamblia* hos mennesker. Formålet med studien er å få økt kunnskap om hvordan og hvorfor mennesker kan bli syke av denne parasitten, med tanke på bedre fremtidig behandling og vaksine. Ved å se på immunresponer hos både personer uten tidligere *Giardia* infeksjon og sammenligne med personer som har, eller har hatt, *Giardia* infeksjon med eller uten symptomer, kan man vurdere forskjeller i responsene og kartlegge dette.

Hva innebærer studien?
I denne studien ber vi om en besvarelse av et spørreskjema om symptomer og kroniske tarmsykdommer, tidligere giardiasis og medikamentbruk. Der vil tas blodprøver, spyttprøver og avføringsprøver fra personer som har eller har hatt *Giardia*-infeksjon siste to år og hos kontrollpersoner som ikke har hatt infeksjonen. Deltagelse består i fremmøte til prøvetaking en gang for kontroller og personer med gjennomgått giardiasis, eller opptil 4 ganger dersom man har pågående giardiasis siden der da legges inn kontroller ved 6 uker, 6 måneder og 12 måneder etter behandling for å evaluere varighet av responsene.

Mulige fordeler og ulemper
Blodprøver, spyttprøver og avføringsprøver er ikke assosiert med noen alvorlige komplikasjoner. De som deltar i studien vil få en kompensasjon på 200kr for reise til Universitetssykehuset samt for tiden prøveinnsamlingen tar. Deltagere vil få beskjed dersom det blir påvist parasitter i prøvene eller annet som kan være av interesse for deltager. De som får påvist sykdom får tilbud om konsultasjon og råd om relevant medisinsk behandling. Genetisk kartlegging av din evne til immunforsvar mot Giardia, samt mulige sårbarhetsgener vil bli gjort.

Hva skjer med prøvene og informasjonen om deg?
Prøvene tatt av deg og informasjonen som registreres skal kun brukes slik som beskrevet i hensikten med studien. Alle opplysningene og prøvene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger og prøver gjennom en navneliste. Prøvemateriale vil bli undersøkt og oppbevart på ubestemt tid i en godkjent biobank på Haukeland Universitetssykehus. Seksjonsoverlegen ved infeksjonsseksjonen, medisinsk avdeling er ansvarshavende for forskningsbiobanken. I tillegg kan prøvemateriale bli sendt til samarbeidspartnere i EU eller USA for ytterligere analyser, men da uten personopplysninger.
Det er kun autorisert personell knyttet til prosjektet som har adgang til navnelisten og som kan finne tilbake til de som deltar. Personidentifiserbare data slettes etter 10år. Det vil ikke være mulig å identifisere deg i resultatene av studien når disse publiseres.

Frivillig deltakelse
Det er frivillig å delta i studien. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke til å delta i studien. Dette vil ikke få konsekvenser for din videre behandling. Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Om du nå sier ja til å delta, kan du senere trekke tilbake ditt samtykke uten at det påvirker din øvrige behandling. Dersom du senere ønsker å trekke deg eller har spørsmål til studien, kan du kontakte Kurt Hanevik, telefonnummer: 9385 6690 eller e-post: kurt.hanevik@med.uib.no

Immunresponser mot Giardia – September 2013

Samtykke til deltakelse i studien

Jeg har lest informasjonen om studien testing og karakterisering av immunresponser mot parasitten *Giardia lamblia* hos mennesker, og er villig til å delta. Jeg forstår at deltakelsen er frivillig og jeg kan si nei til å delta i hele eller deler av studien. Jeg vet at jeg på ethvert tidspunkt kan trekke meg ut fra studien og videre deltakelse, uten å oppgi en grunn og uten at det blir noen konsekvenser for tilbud om helsetjenester eller trygdeytelser. På grunn av mulige oppfølgingsstudier vil personidentifiserbare data oppbevares i 10 år.

Jeg samtykker til følgende (sett kryss som markerer ditt valg)

Deltakelse i prøvetaking, undersøkelser og spørreskjemaer: JA __ NEI __

Jeg samtykker i at prøvemateriale fra meg kan undersøkes i utlandet JA __ NEI __

Jeg samtykker i at innhentede data og prøvemateriale fra meg i forbindelse med denne studien kan brukes i senere studier av *Giardia* infeksjon, inkludert spesifiserte genetiske sårbarhetsfaktorer. En forutsetning vil være at slike prosjekter godkjennes av Regional komité for medisinsk og helsefagelig forskningsetikk og/eller andre lovpålagte instanser. JA __ NEI __

Signatur (pasienten) :.....

Navn (blokkbokstaver): .....

Sted/Dato.....

Jeg bekrefter herved at jeg har informert om formålet med undersøkelsen og hva deltakelsen innebærer, og at pasienten får kopi av informasjonen for pasienter.

Signatur:.....

Navn(blokkbokstaver):.....

Sted/Dato:.....

Appendix C: Flow cytometric lasers, wavelengths (BP filter) and example fluorochromes

Core Facility, BBB BD LSR Fortessa, filter configurations and fluorochromes

LASER	PMT	LP FILTER	BP FILTER	FLUOROCHROMES (examples)
488 (blue)	A	685	695/40	PerCP-Cy5.5
	B	635	670/30	PerCP
	C	505	530/30	FITC, eGFP, eYFP, DiO, CFSE, YoPro1, Alexa488
	D	-	488/10	SSC
				FSC
561 (yellowgreen)	A	750	780/60	PE-Cy7
	B	685	710/50	PE-Cy5.5,
	C	635	661/20	PE-Cy5, 7AAD
	D	600	610/20	PE-TxRed, mCherry, mStrawberry, PE-CF594, Alexa 594, PI
	E	-	582/15	PE, PI, DiI, TRITC, DsRed, SYTOXOrange
	F	open		
	G	open		
	H	open		
635 (red)	A	750	780/60	APC-Cy7, Alexa 750
	B	710	730/45	Alexa680, Alexa700
	C	-	670/14	APC, DiD, Alexa647, Alexa633, Cy5,
407 (violet)	A	685	710/40	BV 711
	B	635	670/30	Qdot 655, BV 650
	C	595	605/12	QDot 605, BV 605
	D	570	585/42	BV570, Qdot 585
	E	505	525/50	Alexa 430, Cascade Yellow, QDot 525, BV 510, Horizon 500
	F	-	450/50	Pacific Blue, Horizon450, Alexa405, DAPI, BV 421, Hoechst

Marianne Enger Side 1 12.06.2013

Figure C.1: BD LSR Fortessa, filter configurations and fluorochromes

Appendix D: Exploration of CD8 cytotoxicity

Table D.1: Fluorochrome-conjugated antibodies tested for investigated cytotoxicity of $CD8^+$ cells.

Antibody	Clone	Fluorochrome	Isotype	Concentration	Supplier	Catalog nr.
Perforin	dG9	PE	M* IgG2b, κ	100 µg/mL	BioLegend	308105
Perforin	B-D48	PE	M* IgG1, κ	12 µg/mL	BioLegend	353303
CD69	FN50	PE-CF594	M* IgG1, κ	50 µg/mL	BD Biosciences	562617
CD107a	H4A3	PE-Cy™7	M* IgG1, κ	12 µg/mL	BD biosciences	561348

* = Mouse

Table D.2: Protein Transport inhibitor for investigating cytotoxicity of $CD8^+$ cells.

Name of reagent	Supplier	Catalog nr
BD GolgiStop™ Protein Transport inhibitor (containing Monensin)	BD Biosciences	554724

Table D.3: Alternative fluorochrome panel for day one assay looking at $CD4^+$ and $CD8^+$ T cell responses.

Antigen/dye	Fluorochrome	Supplier
TNF-α	Brilliant™ Violet 421	Bio Legend
IL-17A	Brilliant™ Violet 605	Bio Legend
CD8a	Brilliant™ Violet 711	Bio Legend
IFN-γ	FITC	BD Biosciences
CD4	PerCP-Cy5.5	BD Biosciences
Perforin	PE	Bio Legend
CD69	PE-CF594	BD Biosciences
IL-4	APC	Bio Legend
CD3	Alexa Fluor 700	Bio Legend
LIVE/DEAD	APC-H7	Life Technologies
CD14	APC-H7	BD Biosciences

Table D.4: Alternative fluorochrome panel for day six for looking at $CD4^+$ and $CD8^+$ T cell responses

Antigen/dye	Fluorochrome	Supplier
CellTrace	Pacific blue	Life Technologies
CD45RO	Brilliant™ Violet 605	Bio Legend
CD8a	Brilliant™ Violet 711	Bio Legend
HLA-DR	FITC	BD Biosciences
CD4	PerCP-Cy5.5	BD Biosciences
CD26	PE	Bio Legend
CD197	PE-CF594	BD Biosciences
CD107a	PE-Cy7	Bio Legend
CD25	APC	BD Biosciences
CD3	Alexa Fluor 700	Bio Legend
LIVE/DEAD	APC-H7	Life Technologies
CD14	APC-H7	BD Biosciences

Appendix E: SSA and SSB response data

Table E.1: Responses to SSA and SSB stimulation after one day (percentage of cytokine producing effector memory $CD4^+$ T cells and $CD4^+$ T cells), and day six (percentages of $CD4^+$ T cells with surface markers and proliferating $CD4^+$ T cells).

Assay	Current giardiasis N=2	Recent giardiasis N=13	Low risk healthy controls N=10	Recent vs Low risk p-value
SSA, Day one, Effector memory CD4 T cells				
TNF-α, mean (SD)	2.01 (1.22)	0.57 (0.63)	0.30 (0.34)	0.148
IFN-γ, mean (SD)	0.50 (0.09)	0.19 (0.32)	0.10 (0.06)	0.773
TNF-α & INF-γ, mean (SD)	0.43 (0.12)	0.16 (0.25)	0.08 (0.10)	0.512
IL-17A, mean (SD)	1.11 (0.62)	0.14 (0.14)	0.03 (0.05)	0.035
IL-4, mean (SD)	0.014 (0.008)	0.009 (0.026)	0.022 (0.057)	0.210
IL-10, mean (SD)	0.07 (0.28)	0.01 (0.018)	0.01 (0.11)	0.938
SSA, Day one CD4 T cells				
TNF-α, mean (SD)	0.48 (0.18)	0.22 (0.22)	0.15 (0.19)	0.152
IFN-γ, mean (SD)	0.07 (0.01)	0.03 (0.07)	0.03 (0.03)	0.229
TNF-α & INF-γ, mean (SD)	0.057 (0.003)	0.023 (0.034)	0.016 (0.027)	0.376
IL-17A, mean (SD)	0.10 (0.01)	0.03 (0.03)	0.01 (0.02)	0.043
IL-4, mean (SD)	0.001 (0.002)	0.004 (0.011)	0.004 (0.009)	0.431
IL-10, mean (SD)	0.01 (0.000)	0.002 (0.004)	0.004 (0.009)	0.347
SSA, Day six, CD4 T cells				
CD25+CD26+, mean (SD)	3.32 (4.08)	2.24 (3.98)	0.42 (0.64)	0.088
HLADR+CD45RO+, mean (SD)	6.82 (5.96)	5.41 (7.16)	0.88 (1.29)	0.036
CD25+CD26+ and HLADR+CD45RO+, mean (SD)	1.33 (1.51)	1.41 (2.80)	0.14 (0.25)	0.042
Proliferation, mean (SD)	17.8 (19.9)	10.2 (11.5)	3.4 (5.0)	0.208
SSB, Day one, Effector memory CD4 T cells				
TNF-α, mean (SD)	1.18 (0.86)	0.44 (0.39)	0.26 (0.24)	0.284
IFN-γ, mean (SD)	0.39 (0.24)	0.97 (0.12)	0.08 (0.08)	0.914
TNF-α & INF-γ, mean (SD)	0.32 (0.21)	0.97 (0.12)	0.06 (0.06)	0.473
IL-17A, mean (SD)	0.83 (0.61)	0.12 (0.15)	0.03 (0.05)	0.062
IL-4, mean (SD)	0.007 (0.009)	0.014 (0.040)	0.013 (0.032)	0.889
IL-10, mean (SD)	0.029 (0.022)	0.011 (0.013)	0.004 (0.007)	0.082
SSB, Day one, CD4 T cells				
TNF-α, mean (SD)	0.22 (0.17)	0.15 (0.14)	0.10 (0.12)	0.291
IFN-γ, mean (SD)	0.04 (0.16)	0.28 (0.06)	0.02 (0.02)	0.328
TNF-α & INF-γ, mean (SD)	0.03 (0.02)	0.01 (0.02)	0.01 (0.02)	0.648
IL-17A, mean (SD)	0.09 (0.03)	0.03 (0.02)	0.01 (0.02)	0.037
IL-4, mean (SD)	0.002 (0.002)	0.006 (0.019)	0.001 (0.002)	0.167
IL-10, mean (SD)	0.005 (0.001)	0.003 (0.005)	0.002 (0.002)	0.463
SSB, Day six, CD4 T cells				
CD25+CD26+, mean (SD)	0.49 (0.23)	0.85 (1.19)	0.34 (0.43)	0.291
HLADR+CD45RO+, mean (SD)	3.36 (0.98)	2.53 (3.03)	0.39 (0.37)	0.057
CD25+CD26+ and HLADR+CD45RO+, mean (SD)	0.31 (0.12)	0.49 (0.76)	0.06 (0.07)	0.067
Proliferation, mean (SD)	5.11 (0.58)	3.75 (4.62)	1.07 (1.56)	0.067

SSA = Soluble proteins form *Giardia* assemblage A; SSB = Soluble proteins form *Giardia* assemblage B