Investigation of immune cells in psoriasis patients treated with infliximab

Master thesis in Pharmacy

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Summary

Psoriasis is a chronic inflammatory disease that manifests in the skin affecting both sexes equally and all ages. In Norway, the prevalence reaches 11.43% in adults. Both genetics and environment factors are important in developing the disease. Comorbidities such as cardiovascular disease, psoriatic arthritis and psychiatric disorders are associated with psoriasis. Treatment of the disease depends on comorbidities and disease severity. For moderate to severe disease, the TNF-α inhibitor infliximab is approved. Even though the discovery of biologics revolutionized the treatment of autoimmune diseases, there is still a problem with relapsing disease and non-responders to treatment. Disease specific biomarkers that could help indicate who will respond to treatment and not at earlier stages are therefore required to optimize treatment.

Psoriasis is a T cell mediated disease and T cell subsets such as the IL-17 secreting Th17 have been identified as important contributors to disease. However, other cell types and cytokines are also important in the pathophysiology, including dendritic cells and the cytokines TNF-α and IFN-γ. Other cell types are not as investigated in the disease mechanism, and the changes in immunophenotype after treatment are also not fully known. Continued research on these fields is therefore interesting.

In this master project, flow cytometry was used to investigate immune cells in 24 psoriasis patients treated with the TNF-α inhibitor infliximab. The cells were immunophenotyped and compared to 33 healthy controls, matched for age, sex and BMI. Whilst we found no changes in frequencies of T cell populations or a trend of activated or inactivated T cells, some of the surface markers were different from the control populations. This change included a decreased expression of CD107a which was also evident on all subtypes of NK cells. NK cells did also not show a trend of increased or decreased activity, but similarly showed a changed expression of some expression markers. Both CD38 and CD27 was significantly increased in patients on $CD56^{dim}$ NK cells. The observations indicated continuous activation of monocytes even after treatment with an increase of frequency of intermediate monocytes, which is implicated in the cardiovascular disease was observed. On these cells, both HLA-DR and CD38 were increased, suggesting increased activity.

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Abbreviations

PASI	Psoriasis Area and Severity Index
CVD	Cardiovascular Disease
TNF	Tumor Necrosis Factor
IFN	Interferon
PsA	Psoriasis Arthritis
pDC	Plasmacytoid Dendritic cell
cDC	Conventional Dendritic cell
IL	Interleukin
Th cell	T helper cell
NET	Neutrophil Extracellular Trap
APC	Antigen Presenting Cell
PAMP	Pathogen Associated Molecular Pattern
DAMP	Damage Associated Molecular Pattern
NK cell	Natural Killer cell
PBMC	Peripheral blood mononuclear cell
CTL	Cytotoxic T Lymphocyte
MHC	Major Histocompatibility Complex
FCS	Forward Scatter
SSC	Side Scatter
BMI	Body Mass Index
PBS	Phosphate Buffered Solution
DMSO	Dimethyl Sulfoxide
PO	Pacific Orange
FMO	Fluorescence Mimus One
HC	Healthy Control
PP	Psoriasis Patient

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1 Introduction

1.1 Psoriasis

Historically, psoriasis was known as a skin disease. In recent years it has been accepted as a systemic disease, which means multiple organ systems of the body are affected (1). It can manifest in different clinical patterns and might be associated with different genetic predisposition (2, 3). The most common type is psoriasis vulgaris. Other subtypes include inverse psoriasis, guttate psoriasis, pustular psoriasis and erythrodermic psoriasis (2).

1.1.1 Epidemiology

Psoriasis affects people of all ages with men and women equally affected (4). There are two peaks of initial disease onset identified at 15-20 years and at 55-60 years, although the disease can affect all ages (4, 5). A review study revealed that the disease is more frequent in countries more distant from equator, and the prevalence is also affected by latitude (4, 6). Another review study found the prevalence worldwide ranges from 0,09% in Tanzania (1994) to 5,1% in the USA (2009) (7). In Scandinavian countries, studies restricted to adults showed even higher prevalence, one of these in Norway showed a prevalence as high as 11,43% (2008) (4, 7).

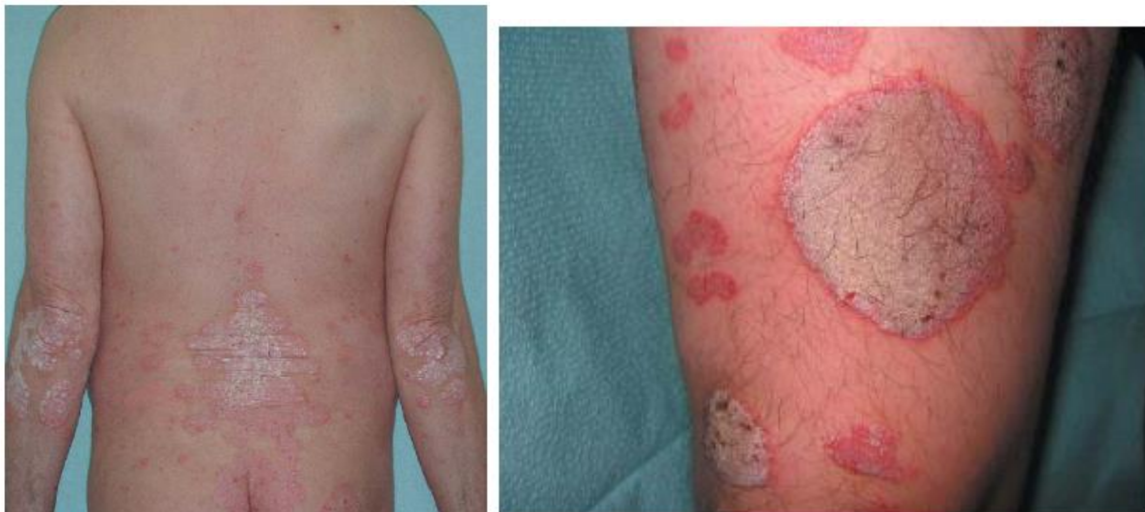


Figure 1: Clinical manifestation of psoriasis vulgaris. The picture to the left show symmetrical distribution of plaques on elbows and back. The picture to the right shows a circular lesion of psoriasis with a well-defined border. Reprinted with permission from Annals of the Rheumatic diseases (5).

1.1.2 Psoriasis vulgaris

Psoriasis vulgaris is also called plaque psoriasis. It accounts for around 85% of psoriasis cases (8). The disease manifests as lesions that are red with silvery white scales (2). They have a defined border between affected- and healthy skin as can be seen in figure 1. The lesions are typically distributed symmetrically on the scalp, elbows, knees and back (5).

1.1.3 Disease severity and quality of life

The disease may vary in severity and is often categorized as mild, moderate, and severe (9). There are a few different systems that are made to help classify the severity. One of them is Psoriasis Area and Severity Index (PASI). PASI is often used to assess treatments and the goal of successful treatment is a 75% reduction in PASI during treatment (5). It is a scoring system based on redness, thickness and scaling as well as it considers the extent of the skin lesions (10). A PASI score ≤10, is defined as mild disease, and PASI scores ≥10, is defined as moderate to severe psoriasis (11). A complementary method to assess quality of life is using the Dermatology Life Quality Index. This is a questionnaire that patients themselves answer, based on their quality of life the last week (12).

1.1.4 Risk factors

Smoking and obesity increase the risk of psoriasis. Skin trauma can trigger the disease and streptococcal throat infection can trigger or worsen the condition (13). One subtype of psoriasis called guttate psoriasis is often triggered by group-A beta-haemolytic streptococcal infections of tonsils in childhood or adolescents. Around one third of those with guttate psoriasis will later develop chronic psoriasis (5). Some medications may also increase the risk of developing psoriasis including lithium, antimalarials and beta blockers as a group of medications for blood pressure and the heart (14).

There is also a genetic factor involved which is supported by findings in studies involving twins. The concordance rate of monozygotic twins is approximately 70% and for dizygotic twins is about 20% (8, 15). Alleles related to susceptibility to psoriasis have also been found, including HLA-C*06:02, which has been found to relate to more severe disease (16).

1.1.5 Comorbidities

Psoriasis is a lifelong disease, but the disease itself does not affect the patient's life expectancy. Nevertheless, comorbid conditions like cardiovascular disease (CVD) and stroke are associated with severe psoriasis and increase mortality in these patients (1, 8, 17). The cause of this however is not widely agreed upon. Even though the disease is mainly visible in the skin, it is acknowledged to be a systemic disease as inflammatory markers like tumor necrosis factor (TNF)-α is reported to be elevated systemic in psoriasis patients (18). Enhanced levels of TNF-α and interferon (IFN)-γ links psoriasis and the development of plaques in the arteries (19). However, the risks of developing CVD and psoriasis overlap as well. It is therefore discussed whether it is psoriasis increasing the risk of cardiovascular disease, or not (1).

Around one third of patients with chronic disease go on to develop psoriatic arthritis (PsA) (8). PsA may also precede skin lesions in some cases (20). PsA is characterized by oedema, pain and stiffness of the joints, ligaments and tendons (20). A key feature of PsA is synovial membrane inflammation, which leads to joint deformity and loss of function (21).

The disease also negatively affects the patients because of stigma. The disease is associated with low self-esteem and anxiety (20). Because of this, depression, and even suicidal tendency is seen in more than 5% of patients (5). Other comorbid conditions include metabolic syndromes, chronic kidney disease, gastrointestinal disease, mood disorders and malignancy. These comorbidities can be grouped as classic (psoriatic arthritis, inflammatory bowel disease, psychological and psychiatric disorders), emerging (metabolic syndrome, cardiovascular diseases, atherosclerosis), related to lifestyle (smoking habit, alcoholism, anxiety), or related to disease treatments (hypertension, hepatoxicity, skin cancer) (20).

1.1.6 Pathophysiology

A key element of psoriatic skin is keratinocyte hyperproliferation which leads to epidermal hyperplasia. In normal skin, basal keratinocytes turn into skin cells without a nucleus in 40-56 days, but in psoriatic skin, it only takes 6-8 days, and the keratinocytes may retain their nucleus (22-24). Neovascularization, which is forming of new capillaries is also prominent in the skin. Normally, inflammation is triggered by extracellular bacteria and fungi, which triggers an immune response where neutrophils and monocytes are recruited, and the complement system is activated. This is initiated by mast cells and macrophages releasing histamine, TNF-α and prostaglandins, which leads to increased local blood flow and leakage of plasma proteins (25).

TNF-α may be secreted by most immune cells, and also works by activating epithelial cells, which then secrets adhesion molecules and chemokines which attracts more immune cells (26).

The exact mechanism of the pathogenesis is multifaceted and not fully understood. The disease is a result of an overreaction to molecules that exist naturally in the body. These molecules are called autoantigens and trigger an immune response. When keratinocytes are damaged, they secrete antimicrobial peptides that act as self-antigens. Several have been identified, like LL37 and ADAMTSL5 (27). Recognition to self-antigens like these leads to a cascade of reactions that enhance keratinocyte hyperproliferation. This cascade can be initiated by trauma or infection and is then sustained by dendritic cells (figure 2) (27).

When plasmacytoid dendritic cells (pDCs) are activated, they secrete inflammatory cytokines. pDCs are a subset of dendritic cells known to secrete high levels of type 1 IFNs (28). pDCs activate conventional dendritic cells (cDCs) by secreting IFN-α. cDCs migrate to draining lymph nodes and secrete TNF-α, interleukin (IL)-23, and IL-12. This stimulates differentiation of T helper (Th)-17 and Th1 cell subsets. Other innate immune cells including monocytes in the skin are also responsible for secreting high amounts of inflammatory cytokines. The activated Th17 and Th1 cells secrete cytokines such as IL17, IL-21 and IL-22 which activate keratinocyte proliferation in the epidermis as well as stimulation from TNF-α and IFN-γ (27).

In plaque psoriasis, the IL-23/Th17 axis is central. IL-17 increases the expression of TNF-α from macrophages and is for instance responsible for inducing cell proliferation, abnormal cell differentiation, and production of cytokines amongst others (27, 29) (26). There are several subtypes of IL-17, including IL-17A and IL-17F. They bind to the same receptor but have different potencies. Both TNF-α and IL-17 are involved in pro-angiogenetic activity as well (26). IL-23 secreted by dermal dendritic cells drives the production of IL-17 from several cell types, including Th17 (8).

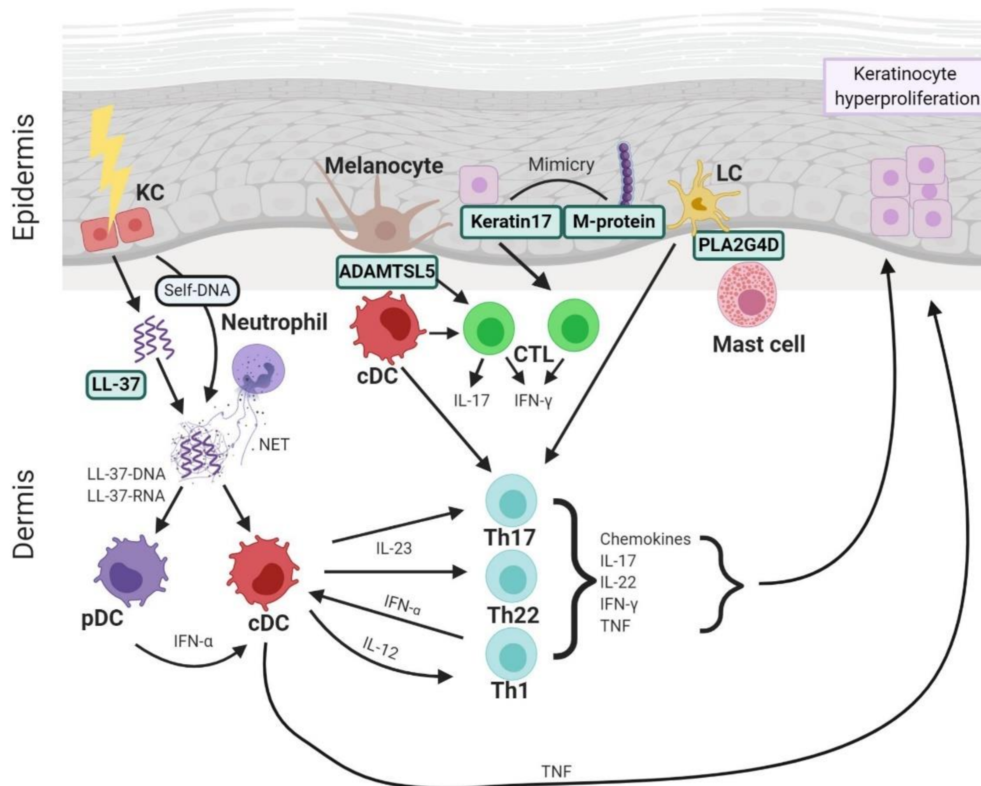


Figure 2: Overview of the disease mechanisms in psoriasis. Keratinocytes make LL-37 in response to trauma to the skin. Neutrophils release neutrophil extracellular traps (NET) that aggregate with LL-37. pDCs secrete IFN-α in response to this, which activates cDCs. cDCs in turn secrete several other inflammatory cytokines including IL-23 which drives the differentiation of a subset of T cells, Th17 cells. This starts a chain of reactions from different cells. The other autoantigens start this chain of reaction from different angles. All results in chemokines and cytokines like IL-17 and IFN-γ being secreted from different subsets of T cells, which stimulates keratinocyte hyperproliferation. Reprinted with permission from John Wiley and sons (16).

1.1.7 Current treatment

Psoriasis treatment is based on disease severity and present comorbidities. It usually requires long term treatment as it cannot be cured. For mild disease, topical treatment might be sufficient. This includes retinoids, calcineurin inhibitors, corticosteroids, and D3 analogues. For moderate to severe psoriasis, systemic treatment is usually necessary. This includes cyclosporine, methotrexate, and biologics. These are anti-inflammatory and immune modulating (30).

Biologics are proteins that target specific elements of the immune system. They cannot be taken orally as they would be degraded in the gastrointestinal tract and are therefore given as injections or infusions (31).

TNF-α is a proinflammatory cytokine secreted by several cells, including T cells, dendritic cells, macrophages, and keratinocytes (8) IFN-α and TNF-α are secreted by pDCs and upregulates secretion of IL-23 by cDCs, which leads to upregulation of Th17 (32). TNF antagonists that are approved as psoriasis treatments exist under the names etanercept, infliximab, adalimumab and certolizumab. Their effect on psoriasis is thought to be because of an indirect effect on IL-17 signalling by regulation of IL-23 (32). Of the approved TNF antagonists, infliximab is the most effective and may also be used when the patient suffers from psoriasis arthritis (30, 33). Though it is effective against psoriasis, it is also associated with a higher risk of serious infections and weight gain (30).

Ustekinumab is an IL-12/23 agonist which inhibits the subunit p40. The subunit p40 is shared by IL-12 and IL-23 and consequently blocks both cytokines (30, 34).

The IL-23 inhibitor risankizumab inhibits the p19 subunit of this cytokine. It is a humanized IgG1 monoclonal antibody (30, 34). IL-23 is one of the cytokines responsible for the upregulation of Th17. Other IL-23 inhibitors are tildrakizumab and guselkumab (8).

Three IL-17 antagonists have been approved for the treatment of psoriasis: secukinumab, ixekizumab, brodalumab. The first two both target IL-17A, while the latter one targets the receptor subunit IL-17RA (31).

When a new biologic drug is released, it has a patent for several years. When this period is over, other companies can make a similar one, called a biosimilar. To be considered as such, the amino acid sequence is expected to be the same. However biologics are usually large and complex molecules, therefore small differences in inactive components are allowed (35). These drugs should be used to treat the same disease at the same dose and interval as the original drug. The making of biosimilar allows for lower prices of otherwise expensive drugs (36).

1.2 Immunology and autoimmune diseases

A healthy immune system should not overreact to self-antigens. T-cells are dependent on antigen presentation from antigen-presenting cells (APC), such as dendritic cells, macrophages, and B cells. Cells from the innate immune system recognize molecules called pathogen-

associated molecular patterns (PAMPs) from microbes and damage-associated molecular patterns (DAMPs). PAMPs and DAMPs are general structures and innate immune receptors cannot differentiate from self-antigens and antigens from microbes. These APCs consequently present all antigens to the T cells, whether it is foreign or self-antigens. As well as tolerating self-antigens, our immune cells must also be able to co-exist with non-pathogenic microbes (25). When the immune cells recognize self-antigens for instance and do not respond by initiating an immune response, it is called *immune tolerance.*

Immune tolerance is preserved by several mechanisms. T and B lymphocyte development is subject to central mechanisms of tolerance in the thymus and bone marrow, respectively. Here they are presented with self-antigens. Cells expressing receptors that have high affinity to these undergo apoptosis. If some self-reacting cells escape this mechanism, there is extra safety in the dependence of co-stimulation. A single self-reacting cell may not react to a self-antigen without costimulatory molecules (37). In addition, regulatory T cells may inhibit self-reactive lymphocytes (25).

When this balance of reaction and tolerance is interrupted, there are different ways the immune system can overreact and cause allergies and autoimmune diseases. These reactions may be organ specific or systemic. Psoriasis is a T cell mediated autoimmune disease. The mechanisms that T cell mediate damage to host cells are the same mechanisms used to eliminate microbes. Inflammation is often caused by cytokine secretion from $CD4^+$ T helper (Th) cells. Tissue injury is predominantly caused by the macrophages and neutrophils that are recruited by cytokines from Th1 and Th17 (25).

1.3 Immune cells analysed in this study

Peripheral blood mononuclear cells (PBMC) have a round nucleus and include several immune cells, including lymphocytes like T cells, B cells and natural killer (NK) cells and monocytes. They can easily be acquired from whole blood by gradient centrifugation and are extensively used in research (38).

T cells are immune cells that can be divided into two main groups: $CD8^+$ cytotoxic T lymphocytes (CTL) and $CD4^+$ Th lymphocytes. T cell receptors recognize antigens in the form of peptides presented to them by other cells on a major histocompatibility complex (MHC) molecule. All nucleated cells express MHC class I and if infected by a virus, they present the virus antigen to a CTL which then kills them. APCs present antigens from microbes they have

phagocytosed on their MHC class II molecule. $CD4^+$ Th cells interact with this complex and may be activated. T cell receptors are specific for some proteins and will not react to all antigens they are presented to. As well as antigen presentation, T cell reactions are also dependent on costimulation. CD4 and CD8 are examples of costimulators, as well as CD3. Cytokines are also an important factor in the effector function of T cells (25).

When T cells are activated, they develop into effector or memory cells. T cells play a major role in psoriasis. Lesional psoriatic skin contains high levels of $CD8^+$ and $CD4^+$ T cells and especially Th17 cells driven by IL-23 (8). IFN-γ producing T cells (Th1) are also elevated in lesions (15) What type of T cell is developed depends on the cytokine signals they receive (25).

B cells are also part of the adaptive immune system and can develop into antibody secreting plasma cells. The first antibody secreting cells are called plasma blasts. They are short lived and are part of the early response (25, 39). The B cell receptors are membrane bound antibodies. When they recognize an antigen, they process it and present it to T lymphocytes. To be activated the B cell need stimulation from the T cell. An activated B cell can fight infections by different mechanisms; Neutralize microbes, opsonize for phagocytes, complement activation leading to lysis of microbes and antibody-dependent cellular toxicity (25). Transitional B cells are immature B cells and will develop into mature B cells. They can secrete cytokines and upregulate $CD4^+$ T cell proliferation (40).

NK cells are a part of the innate immune system, which means they can react to an infection immediately rather than relying on priming or memory like the adaptive immune cells. As the name implies, the NK cells kill other host cells that are infected or cancerous. This is done by releasing cytotoxic granules into the intracellular space that will kill the target cell. They also secrete inflammatory signal molecules like IFN-γ and in that way contribute to inflammation. Two important receptors NK cells have, are NKG2D and CD16. NKG2D can recognize molecules that resemble MHC class 1, but do not function as peptide presentation. Another NK cell receptor is CD26, which is a Fc-gamma receptor and regognition in these lead to killing of the antibody-bound cell. These two molecules are stress induced and cancer specific ligands (25).

The role of NK cells in psoriasis is not well known. What we do know is that they, as well as NKT cells, can produce inflammatory cytokines like IFN-γ, TNF-α, IL-17 and IL-22, and may contribute to inflammation (29). NKT cells are also part of the innate immune system and express both the NK cell receptor and the T cell receptor (29).

Monocytes are phagocytes and part of the innate immune system (25). Activated monocytes are involved in producing TNF-α, IL-12 and IL-23 (27, 41). Monocytes can be divided into three subtypes: classical, intermediate and non-classical monocytes. Classical monocytes are phagocytes with the ability to develop into monocyte-derived macrophages and dendritic cells and secrete more inflammatory cytokines. Intermediate monocytes express other chemokine receptors than the other types and secrete cytokines and regulate apoptosis and express more MHC II (42, 43). Non-classical monocytes are thought to be anti-inflammatory and have a protective effect in chronic disease and are positively associated with disease burden (44).

1.4 Aims of this study

The role of T cells in active psoriasis is well studied, however not necessarily after treatment with infliximab. Little is also known about other immune cells, such as monocytes and NK cells in psoriasis. In this study, flow cytometry was used to phenotype immune cells in psoriasis patients treated with infliximab or biosimilar to investigate systemic inflammation after treatment and with a focus on inflammatory markers of activation.

2 Materials and methods

2.1 Flow cytometry

Flow cytometry is a method for measuring cells so that different cell subsets can be identified. A single cell suspension flows through the cytometer and the size and granularity of the cells are measured. This is done by detecting the scattering of light. The flow cytometer consists of one or more lasers, a flow chamber where the fluid runs through, the optical system where the lights go through different filters and lenses, detectors, and a computer where the signals are transformed into information we can read (45, 46). Light scattering based on size and granularity can be done without any staining. Forward scatter (FSC) determines the cell size, as the light passes around the cell and into the detectors. Side scatter (SSC) determines granularity, as the light is detected at around 90° to the laser beam. Further analysis of populations can be done by staining with fluorochrome-conjugated antibodies targeting extracellular or intracellular molecules (46). When the laser hits the fluorochrome, the energy excites the fluorochrome and the detectors measure the fluorescence emitted. The emission from the fluorochrome on the antibody shows the level of expression on the cells (46).

2.1.1 Compensation

As seen in figure 3 below, the emission spectrum of the different fluorochromes overlaps, which means that the light emitted by one fluorochrome may pass through the filter of another. This can be compensated by subtracting a percentage of the signal detected. This is found by analysing one fluorochrome at a time so that the amount detected in another channel can be determined (46).

Compensation beads capture specific antibodies conjugated to fluorochromes and are often used for compensation instead of cells as they stain brightly, are practical in the case if limited cell samples are available and are easy to prepare (47, 48).

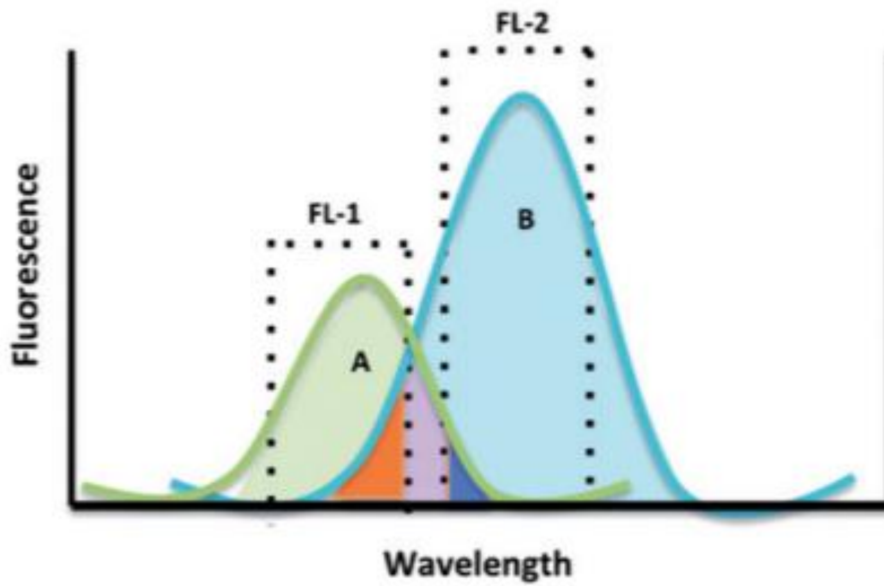


Figure 3: Overlapping of two imaginary fluorochromes, A and B. They are excited by the same laser and are measured in two different channels, FL-1 and FL-2. Reprinted with permission from Taylor & Francis (46).

2.1.2 Cell-surface markers used in this study

Table 1: Immune cell subset definitions by cell surface markers:

Population name	CD molecules
B cells	$CD3^{-}CD19^{+}$
Memory B cells	$CD27^{+}CD38^{+/-}$
Transitional B cells	$CD27^{-}CD38^{high}$
Plasma blast	$CD27^{+}CD38^{high}$
T cells	$CD14^{-}CD56^{-}CD3^{+}$
CD4+	$CD4^{+}$
Naïve	$CD4^{+}CD45RO^{-}$
Memory	$CD4^{+}CD45RO^{+}$
CD8+	$CD8^{+}$
Naïve	$CD8^{+}CD45RO^{-}$
Memory	$CD8^{+}CD45RO^{+}$
NK cells	$CD14^{-}CD3^{-}CD56^{+}$
Classical	$CD56^{dim}CD16^{+}$
Intermediate	$CD56^{+}CD16^{+}$
Bright	$CD56^{bright}CD16^{-}$
NKT-like cells	$CD14^{-}CD3^{+}CD56^{+}$
Monocytes	$CD56^{-}CD3^{-}CD19^{-}CD14^{+/-}$
Classical monocytes	$CD16^{-}CD14^{+}$
Intermediate monocytes	$CD16^{+}CD14^{+}$
Non-classical monocytes	$CD16^{+}CD14^{-}$

CD16 (Fc-gamma receptor III) and CD56 are molecules used to identify NK subclasses. NK cells are defined as $CD3^{-}$ and $CD56^{+}$ (49) (table 1). CD56 is also known as neural cell adhesion molecule (50). NK cells can be divided into different subtypes, depending on their expression of CD16 and CD56. These populations are $CD56^{bright}CD16^{-/dim}$ and $CD56^{dim}/CD16^{+}$. Those two types of NK cells have different functions. The $CD56^{bright}$ cells are significant producers of cytokines but are not as cytotoxic as the $CD56^{dim}$ NK cells (51). CD16 on $NK56^{dim}$ cells recognize antibody coated cells and signals to the NK cell to kill the targeted cell (52). $CD56^{dim}$

NK cells account for around 90% of all circulating NK cells and are called classical NK cells. $CD56^{bright}$ on the other hand, are mainly present in peripheral tissues, like the skin (29).

CD16 is also used to identify subtypes of monocytes together with CD14 which is a co-receptor for the PAMP receptor used by the innate immune system, toll-like receptor 4. Classical monocytes are defined as $CD14^{+}CD16^{-}$, non-classical are $CD14^{-}CD16^{+}$ and intermediate are $CD14^{+}CD16^{+}$. As mentioned previously, these play different roles in the immune system (42, 53).

T cells are divided into two subtypes, depending on the properties of the cell. $CD4^{+}$ T cells are defined as T helper (Th) cells and $CD8^{+}$ T cells are defined as cytotoxic T cells (CTL). These two markers are thus used to identify these two subsets. CD4 and CD8 are coreceptors and recognizes MHC class II and I, respectively (25). CD3 is also a T cell marker and part of the T cell receptor but can also be used to identify other subgroups like NK cells, by excluding $CD3^{+}$ cells and NKT cells which are positive for both CD56 and CD3 (54, 55). It is a surface molecule on T cells and is part of the T cell receptor complex, together with the T cell receptor and ζ chain. The activation of a T cell also requires stimulation of the coreceptor CD4/CD8 (25).

CD107a is a marker for both $CD8^{+}$ T cell and NK cell activation. Its level on the surface of NK cells also correlates with cytokine secretion and NK killing of cells (56). CD107a is a lysosome-associated protein. NK cells contain cytotoxic granules in lysosomes in their cytoplasm. When the cell is activated, these granules are transported to the surface and fused with the membrane to secrete the content. During this granulation, the lysosome-associated protein is transported to the cell membrane (57). A study from 2015 indicated a difference in levels of degranulation of NK cells in psoriasis patients from healthy controls. This experiment was done by measuring levels of CD107a by flow cytometry (58)

CD69 is a lectin receptor and is expressed shortly after activation on NK cells and T cells. On T cells, it is a coreceptor for proliferation and secretion of cytokines. On NK cells, CD69 triggers cytotoxic function (59, 60).

HLA-DR is expressed by antigen presenting cells like dendritic cells and B cells and monocytes. It is responsible for presentation of antigens to $CD4^{+}$ T cells (25). This molecule is upregulated in activated cells (25). It can also be expressed by activated T lymphocytes and less mature NK cells (61, 62).

CD45 exists in different forms, including CD45RA and CD45RO. Naïve T cells express CD45RA, while CD45RO is expressed on memory T cells (63). Similarly, it has been shown that NK cells expressing the memory associated molecule reacts more strongly than those that do not (64). However, unlike on T cells, $CD45RO^+$ cells are uncommon in NK cells in healthy individuals (65).

CD19 is a transmembrane protein on B cells (25). It is involved in both B cell receptor dependent and independent signalling (66). It is therefore used to identify B cells. To differentiate between naïve and memory B cells, CD27 and CD38 are being used. CD27, a member of the TNF receptor family, is a key marker for memory B cells and is a costimulatory receptor on T cells, B cells and NK cells. T cells express this marker as naïve cells, but B cells do not. $CD27^+$ B cells are therefore classified as memory cells/plasma blasts (67, 68).

CD38 is a cyclic ADP ribose hydrolase and is expressed on many immune cells. It has traditionally been used as a marker for activity in B and T cells but is also expressed on monocytes (69-72). B cells that are identified as $CD38^{high}$ are called transitional B cells (40). CD38 is also highly expressed on plasma blasts (73).

2.2 Cell counting and cell viability

Cell counting and analysis of cell viability can be used to determine cell loss after the thawing process and to measure cell death. This can for instance be practical when concentration per million cells is used. Several devices can be used to count cells, such as the flow cytometer LSR Fortessa, CASY cell counter (Schärfe System) and MUSE (Luminex). CASY cell counter is practical as it does not require dyes to analyse the cells. The instrument consists of a measuring pore with a set size, a measuring capillary, electrodes, and a lift tube. The cells flow through the measuring pore and due to their polarized membrane, create resistance. This is measured and information like cell number, cell viability and cell size distribution are generated (74). An advantage of MUSE cell analyser is that it is simple and efficient. It works similarly to the flow cytometer, by laser excitation. It provides information like cell viability and cell count (75).

2.3 Patients

In this study, 24 patients and 33 healthy control subjects were investigated (Table 2). The patients had previously been diagnosed with moderate to severe psoriasis and were stable on infliximab when the samples were collected. The healthy controls were matched for age, sex and body mass index (BMI). The study was approved by the regional ethical committee (2014/1489 and 2014/1373). All participants provided written informed consent.

Table 2: Overview of psoriasis patients and healthy donors.

	Patients (n=24)		Controls (n=33)	
	Male	Female	Male	Female
Sex	20	4	27	6
Age, years range (mean)	29-80 (52.4)	43-70 (51.5)	24-70 (48.3)	33-64 (50.2)
Height, cm range (mean)	170,5-192 (180,9)	162,176 (170.5)	170-190 (180.5)	160-178 (169.7)
Weight, kg range (mean)	65-146,8 (94.7)	64-85.6 (73.45)	67-122 (88.4)	51-90 (73.5)
BMI range (mean)	21.1-40.2 (28.9)	22.9-27.6 (25.2)	20.1-37.7 (27.1)	19.9-30.1 (25.4)
Age of onset, range (mean)	3-71 (23.6)	21-40 (28)		
PsA+	6	2		
Psa-	14	2		

PsA: psoriatic arthritis

2.4 Methods

2.4.1 Isolation of PBMCs

The patient and control samples had been acquired for å previous project and all participants provided written informed consent (76). Blood samples used in antibody titrations were taken

from healthy volunteers. Peripheral blood samples were collected in BD Vacutainer Heparin Tubes. The PBMCs were isolated immediately by density gradient using Lymphoprep (Axis-Shield) as per manufacturer instructions. Lymphoprep were added to a falcon-tube and whole blood were added gently on top of it. After centrifugation (800g, 20 minutes, 23°C), the PBMC layer was transferred by Pasteur pipette to a new tube. The PBMCs were then washed three times with sterile Phosphate Buffered Solution (PBS, Lonza #BE17516F) (centrifugation at 470g, 1x7 minutes, 2x5 minutes). The cells were counted by CASY counter and 5 million cells were prepared in freezing medium (x-vivo (Lonza #04-448Q) and 10% DMSO) and put in a CoolCell freezing container at -70°C before being moved to a rack in a -150°C freezer for long-term storage. The patient and control samples were stored in liquid nitrogen.

2.4.2 Testing thawing protocols

When working with PBMCs it must be decided whether to work with fresh cells or cryopreserved cells. Cryopreserved cells are widely used in research, as it is practical for batch testing, avoid inter-assay variability and allow for further testing if a problem appears (77). The osmotic and temperature change while freezing cells could affect the viability of the PBMCs. To avoid forming crystals and fracturing the cell membranes, the cells need to be gradually chilled (78). Dimethyl sulfoxide (DMSO) is widely used in freezing medium, however, it is toxic to lymphocytes. The thawing procedure therefore usually consist of a washing step. One disadvantage of working with cryopreserved cells is that cells are lost and viability decreases (79).

Different thawing protocols are being used in the host lab and other protocols are known as well. It is therefore interesting to test different protocols and analyse the cells, to find the one that gives the best cellular viability. Analysis was performed using the CASY counter, MUSE cell analyser and flow cytometry to measure live/dead cells using Pacific Orange (PO, 1:1000, stock concentration 0.5 mg/ml, (Thermofisher)) as stain, as well as counting with CASY counter and MUSE.

A general protocol for thawing was made. In this protocol, the vials were put into a 37°C water bath for ca 2 minutes, until there was barely any ice visible. The cells were then transferred instantly to a 50 ml falcon tube containing warm x-vivo (37°C) and nuclease (Pierce) (1:10000). The cells were then washed by centrifuging at 453 g/5 minutes at 23°C, aspirating and resuspending in x-vivo, before a new wash at the same settings.

In this experiment, the vial stayed in the 37°C water bath for 2 minutes, when there was still some ice visible. RPMI (Thermo Fisher) with 10% FCS was used to wash the cells instead of x-vivo, which was used in titrations and for patient samples. The centrifuge was set to 453 g/ 5 minutes and 23°C. Additionally, the cells were cultured (37°, 5% CO_2) for 60-90 minutes after one wash with RPMI. Cells were taken out for counting with CASY and MUSE while cultivating. One million cells were taken out for each test, as well as cells to unstained and dead control, a vial that was shaken, flicked and vortexed, to stress and kill the cells. The cells were then centrifuged and stained in PO (1:1000, stock concentration 0.5 mg/ml) and incubated for 30 minutes in the fridge.

This base protocol was then modified based on other protocols. Four experiments were done (Table 3). The parameters that were compared were: instant versus dropwise adding of warm media to the cells, 2 minutes versus 12 minutes in water bath after thawing (2 min and 12 min in total) and centrifugation speed, 150 g vs 453 g. A test where 8 vials were thawed at the same time was also performed, to see if there were a trend of declining viability within the samples over time.

Table 3: Information about the different parameters tested in this project.

Experiment number	Time in water bath (mins)	Addition of warm media (37°C)	Centrifugation speed (g)	Number of vials thawed together
1a	2	Dropwise over 2 minutes	453 g	1
1b	2	Instant	453 g	1
2a	2	Instant	453 g	1
2b	12	Instant	453 g	1
3a	2	Instant	150 g	1
3b	2	Instant	453 g	1
4	2	Instant	453 g	8

Buffy coat was ordered from the blood bank at Haukeland University Hospital and was picked up the day after. PBMCs were then isolated by Lymphoprep as previously described. 25 vials were frozen at 10 million cells per vial, first in a CoolCell freezing container at -70°C before being moved to a rack in a -150°C freezer for long-term storage.

A biological control is analysing different biological samples, while technical control is analysing the same biological sample several times. The biological control will account for the biological variation in individuals while the technical control will account for the variation in technique or equipment (80). In the thawing project, three technical controls for each parameter were included.

2.4.3 Antibody titration:

Antibody titration is necessary to optimize staining in flow cytometry. At ideal concentrations, the antibodies bind with high specificity to the targets on the cell. At higher concentrations, unspecific binding to low affinity targets on the cell will increase. At lower concentrations, all high affinity targets will not be able to bind and separation between the populations will be vague (figure 4) (81).

Antibody titrations were done by staining samples with one antibody at a time. The titration row was prepared before thawing cells by adding PBS in one row of a v-bottom 96 well plate. An antibody was added to the first well and then transferred down the row. The concentration was made using a twofold dilution. From least to most diluted: 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280. For each antibody, a vial was thawed and added slowly to warm x-vivo with nuclease (1:10000). It was then washed twice and counted by CASY. 1 million cells were taken out for unstained and the rest for stained. FACS buffer (PBS+5%BSA) and FcR block (Miltenyi Biotec, Gladbach, Germany) were added at a concentration of 2 µl/10^6 cells before staining. Incubation time was 30 minutes in the refrigerator, before washing and preparing the cells for analysis on the flow cytometer LSR Fortessa.

When titrating CD69, CD38, CD28 and CD107a cells were stimulated before titrating. This was done because these cell surface markers are lowly expressed in unstimulated cells. The cells were thawed as before and resuspended in warm x-vivo. IL-2 was added to give a final concentration of 50 units/ml. The cells were then transferred to a T25 flask and put in the incubator (37°, 5% CO2) for 16 hours before further washing and staining by the same protocol as before.

The appropriate concentration was chosen based on the separation between the positive and negative population and the level of unspecific binding. A complete staining panel using all antibodies was tested to see how antibodies affected each other, and final concentrations were chosen.

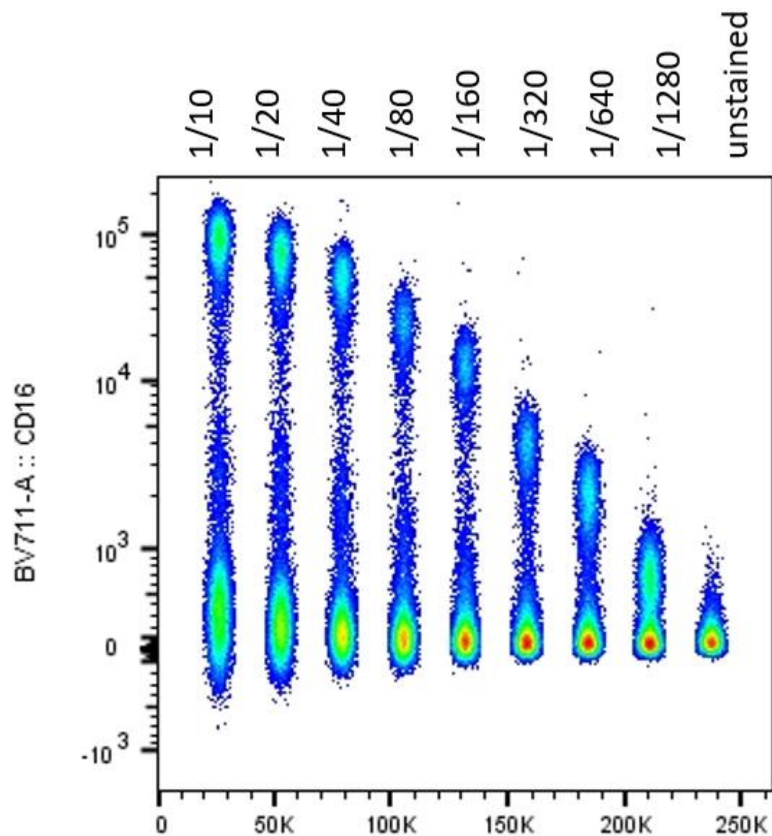


Figure 4: Concatenation of all concentrations in the titration of CD16. Concentrations are 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280 and unstained from left to right. Separation improves with higher concentrations, but unspecific binding also increases at the highest concentrations. In this case, 1:320 was chosen as the final concentration as it gave sufficient separation between positive and negative population.

Table 4: List of lasers, filters, clone, supplier, and dilution of each fluorochrome conjugated antibody.

Laser	Filter	Fluorochrome	Antibody	Clone	Supplier	Dilution
488	530/30	AF488	CD19	HIB19	Biolegend 302219	1/400
488	695/40	PerCP-Cy5.5	CD8	RPA-T8	Biolegend 301032	1/400
561	582/15	PE	CD38	HIT2	Biolegend 303506	1/80
561	780/60	PE-Cy-7	CD69	FN50	Biolegend 310912	1/80
561	610/20	PE-TR/ECD	HLA-DR	G46-6	BD Biosciences 562304	1/640
640	780/60	APC-Cy7	CD4	RPA-T4	Biolegend 300518	1/400
640	730/45	AF700	CD3	UCHT1	Biolegend 300424	1/40
640	670/14	AF647	CD56	HCD56	Biolegend 318314	1/80
407	780/60	BV785	CD45RO	UCHL1	Biolegend 304234	1/80
407	610/20	BV605	CD14	M5E2	Biolegend 301834	1/80
407	670/30	BV650	CD27	O323	Biolegend 302828	1/40
407	450/50	BV421	CD107a	H4A3	Biolegend 328626	1/80
407	710/40	BV711	CD16	3G8	Biolegend 302044	1/320

2.4.5 Compensation beads

For compensation, One-Comp Compensation beads BioScience were used. They were prepared by adding beads to 14 wells of a V-bottom plate. One antibody was added to each well, except for one unstained. The beads were then incubated for 10 minutes at room temperature in dark and washed with FACS buffer.

2.4.6 Full panel test:

The cells were thawed as before, washed, and then a 0,5 mg/ml stock of PO was added at a concentration of 1:1000 in PBS and incubated for 30 minutes in the fridge. During this incubation, the antibody master mix, consisting of all the antibodies and FACS buffer, was prepared. The cells were then washed in FACS (PBS+0,5% BSA) buffer and FcR block was added (2µL/10^6 cells) and incubated for 5 minutes. Antibodies were then added and incubated for 30 minutes in the fridge. The cells were then washed and analysed on the LSR Fortessa within one hour.

When analysing the full panel test, some of the antibodies turned out to be challenging to gate, especially the activation markers. Therefore, a new full panel test was performed on stimulated cells to see if this made it easier to differentiate between positive and negative populations. The stimulation was performed by the same protocol as used in the titration.

2.4.7 Fixing cells

Before starting with the patient samples, it was decided to do one last full panel where the cells would be fixed. Fixed cells are dead cells, which means you can no longer measure dynamic biological processes. The most commonly used fixative is formaldehyde (82). This step would provide better time to analyze after preparing the cells.

For fixation, 1,6 % PFA (Thermofischer, 16% stock glass vial format) was used by diluting stock 1:10 with FACS buffer. This was added to the cells and left for 12 minutes and then washed.

2.4.8 Fluorescence minus one (FMO) control

FMO is a sample where the cells are stained with all antibodies except one. This method is used to separate the negative population from the positive and to account for fluorescence from other fluorochrome-antibodies in the panel (83, 84). In this case, FMO controls were performed on CD27, CD38, CD45RO, CD69, CD107a and HLA-DR to confirm the gating. This was done according to the same protocol used for the full panel test with some steps changed in the staining procedure. The master mix contained all antibodies that did not require FMO. The rest of the antibodies were added individually, leaving one out for each sample.

2.5 Patient samples

Altogether, there were 57 samples to be analysed. This was done over four consecutive days. For each staining batch, one internal control and both patients and controls were included to identify batch effects making it 61 samples altogether. Internal controls are a form of biological control, to ensure that there is no inter-assay variability. The vials were taken out of liquid nitrogen and moved to a -150°C freezer prior to the experiments. The samples were prepared by the same protocol as used for full panel staining, and the samples were then directly analysed on the LSR Fortessa.

2.6 Gating strategy:

All lymphocytes were selected by size and granularity from a SSC-A FSC-A plot. Then the cells were analysed in a time gate, to check if the collecting of the data went smoothly; no clogs, or bubbles/air. Next, single cells were selected in two different plots. The first one in a FSC-H, FSC-A plot, then in a SSC-H, SSC-A plot. Pacific orange was used to stain dead cells and negative cells from this plot were selected further (Figure 5).

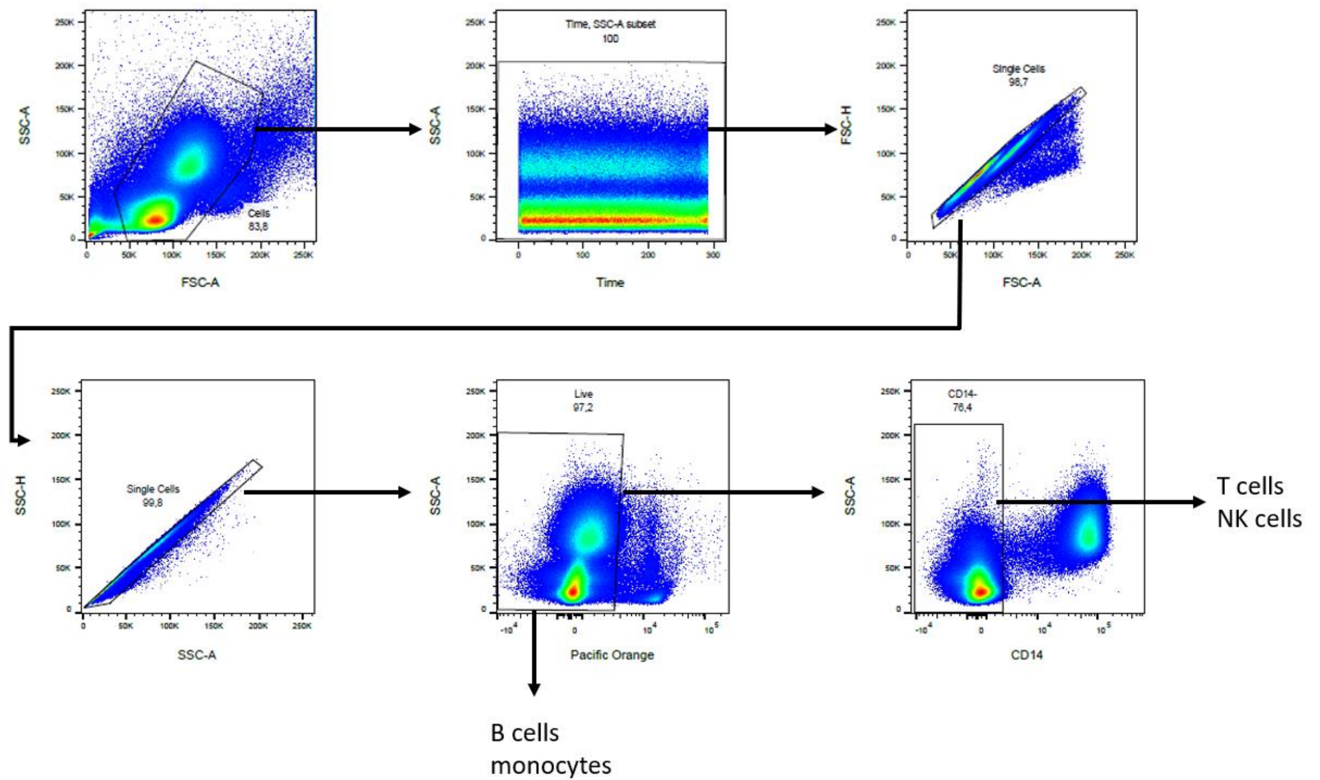


Figure 5: Representative gating strategy. From the live single cell gate, B cells and monocytes were gated, while the CD14$^-$ was used for both T cells and NK cells.

For analysis of B cells, live single cells were gated on CD19$^+$/CD3$^-$ cells (table 1). To identify memory B cells, transitional B cells and plasma blasts, CD38 and CD27 were used (figure 6).

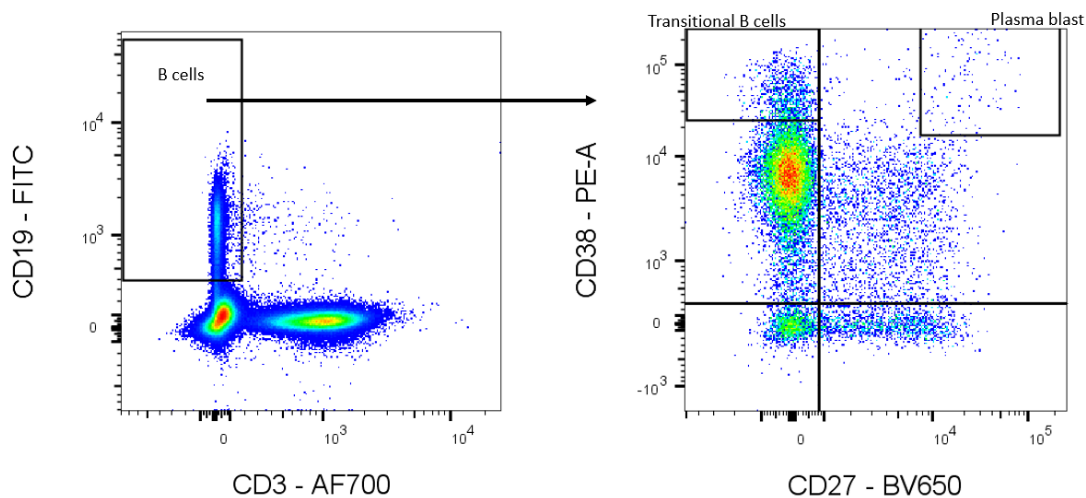


Figure 6: Gating strategy for B cells. Starting with PO^- cells shown in Figure 5, $CD19^+/CD3^-$ cells were gated. CD27 and CD38 were then used to identify memory B cells ($CD27^+CD38^{+/-}$), transitional B cells ($CD27^-CD38^{high}$) and plasma blasts ($CD27^+CD38^{high}$).

Both for T cells and NK cells, $CD14^+$ cells were excluded (figure 5). T cells and NK cells were then separated by gating on CD56 vs CD3. $CD3^+$ T cells were divided into two groups: $CD8^+$ T cells and $CD4^+$ T cells. Each of these populations was then analysed for CD45RO, to determine whether they are memory or naïve cells (figure 7). In each of these populations, expression of activation markers CD69, CD27, CD38, CD107 and HLA-DR was analysed.

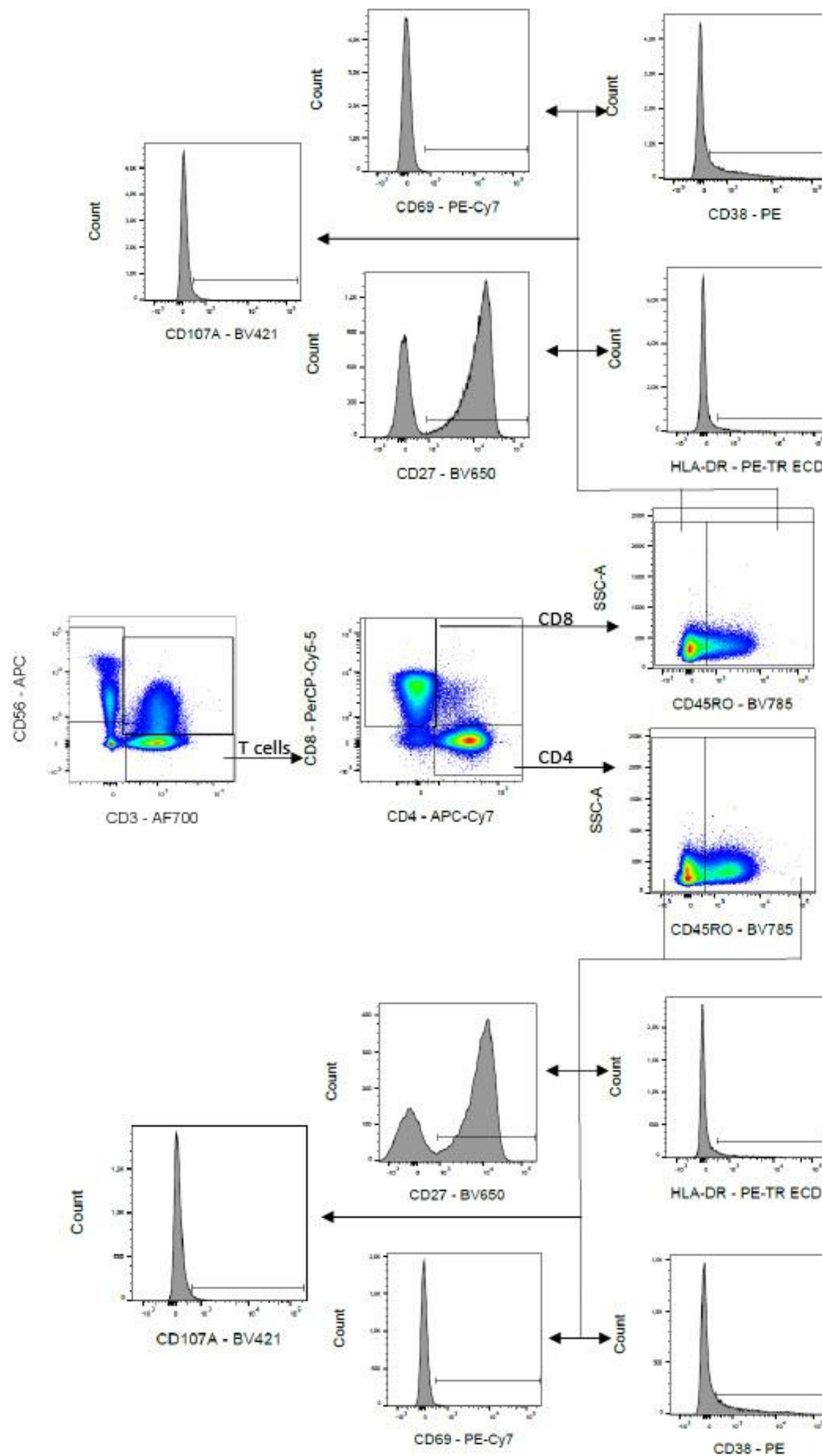


Figure 7: Representative gating strategy for T cells. Starting with $CD14^-$ cells shown in Figure 5, $CD3^+$ cells were selected. The T cells were then divided into $CD8^+$ and $CD4^+$ and within these groups, $CD45RO^{+/-}$ cells were defined and expression of CD69, CD27, CD107a, HLA-DR and CD38 were analysed.

NK cells, defined as CD56^{+} cells, were further divided into three subpopulations based on their expression of CD56 and CD16: NK CD56^{bright} (CD56^{bright}CD16^{-}), intermediate NK cells (CD56^{+}CD16^{+}) and classical NK cells (CD56^{dim}CD16^{+}) (figure 8). In each of these populations, expression of activation markers CD69, CD27, CD38, CD107a and HLA-DR were analysed.

As for T cells and NK cells, NKT-like cells are also found by gating on CD56 and CD3 within the live CD14^{-} cells. Here the double positive cells were selected. To investigate NKT-like cell activity, CD45RO, as well as activation markers CD38, HLA-DR, CD27, CD107a and CD69, were looked upon (figure 8).

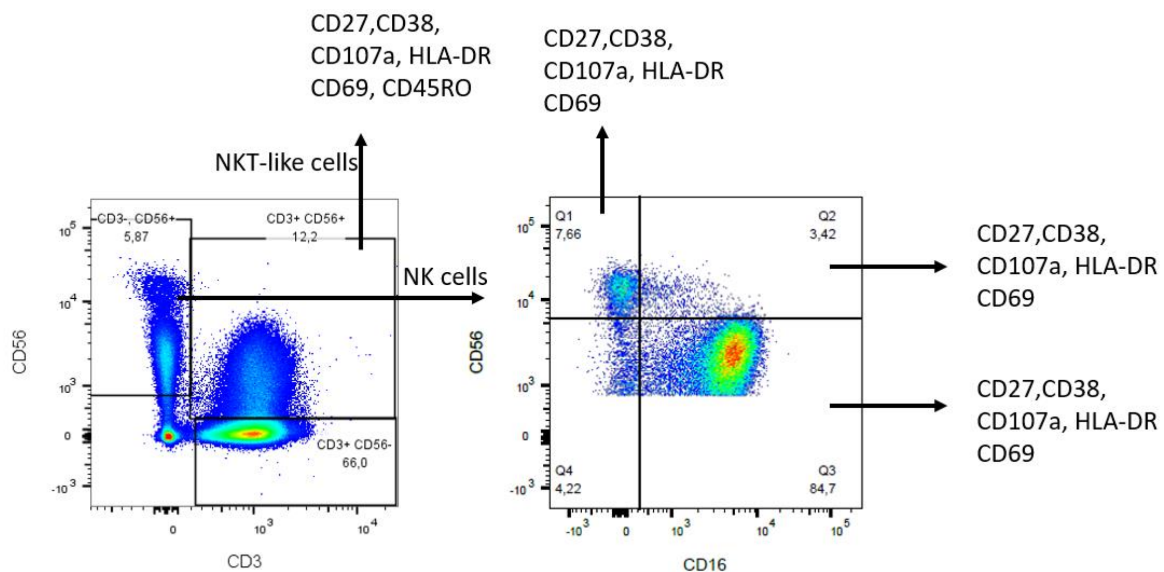


Figure 8: Representative gating strategy for NK and NKT cells. Starting with CD14^{-} cells shown in Figure 5. The first gate includes CD56^{+} cells and excludes CD3^{+} (T cells). NKT cells were then gated on activation markers CD27, CD38, CD107, HLA-DR, CD69 and CD45RO. Next, the NK cells were divided into different populations: NK CD56^{bright}, intermediate NK cells and classical NK cells (Q1, Q2, and Q3 respectively). In each of the populations, the activation markers CD69, CD38, CD27, CD107a were analysed.

To identify monocytes, live single cells were displayed as CD56 vs CD3 (Figure 9). Excluding both cell surface markers as well as CD19, cells were then displayed as CD16 vs CD14 to identify the three monocyte subtypes. Non-classical monocytes were defined as CD16^{+}CD14^{-}, intermediate monocytes as CD16^{+}CD14^{+} and classical monocytes as CD16^{-}CD14^{+}. Each of the

three subtypes was then further analysed regarding expression of HLA-DR, CD45RO and CD38.

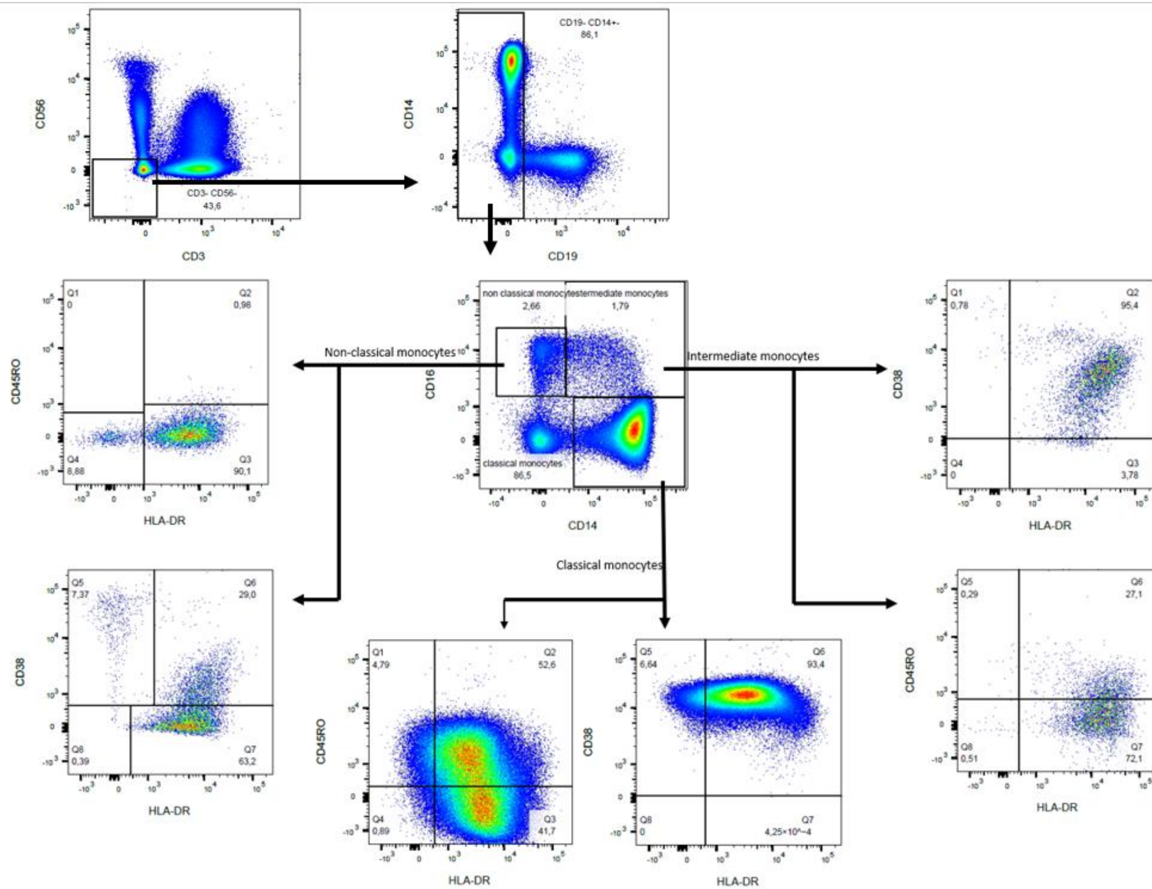


Figure 9: Representative gating strategy for monocytes. Starting with PO$^-$ cells shown in Figure 5, CD3$^+$ and CD56$^+$ cells were excluded. Then CD19$^+$ cells were excluded, and the cells were then divided into three monocyte subclasses based on their expression of CD14 and CD16. Each of the subpopulations; classical monocytes (CD14$^+$CD16$^-$), intermediate monocytes (CD14$^+$CD16$^+$), and non-classical monocytes (CD14$^-$CD16$^+$) were then looked further onto with regards to the activation markers HLA-DR, CD45RO and CD38.

2.7 Statistical analysis

For analysis of the flow cytometry data, FlowJo (TreeStar) was used. GraphPad Prism (version 9) was used for statistical analysis. The data were checked for normal distribution, and unpaired t-tests were used when normal distribution was found, otherwise, Mann-Whitney tests were used. As there were only three parallels for each experiment in the thawing project, the results were assumed to be non-parametric.

3 Results

3.1 Thawing

The patient samples to be used in this study had been biobanked over a three-year period with many having only a single vial of cells available for analysis. I therefore sought to determine the thawing protocol that provided the most viable cells whilst establishing how many patients could be batched together in each experiment. I evaluated different conditions that could affect thawing. The variables were: 1) dropwise addition of warm media to the cells compared with instant addition, 2) 12 minutes versus 2 minutes in the 37°C water bath after thawing, 3) changing the speed of acceleration of centrifugation to pellet the cells (453 g versus 150 g) and 4) 8 vials were thawed consecutively to monitor the effects of batch thawing cells (table 3).

Three methods were used to measure the percent viability of the cells, MUSE cell analyzer, CASY cell counter and flow cytometry. The cells were analysed in regard to cell viability. By flow cytometry, PO was used to identify live cells by first selecting lymphocytes in a FSC-A and SSC-A gate and then a PO gate. None of these changes in the protocol seemed to impact the viability of the lymphocytes (figure 10). A downward trend in viability was also not observed with an increasing number of vials (Figure 11).

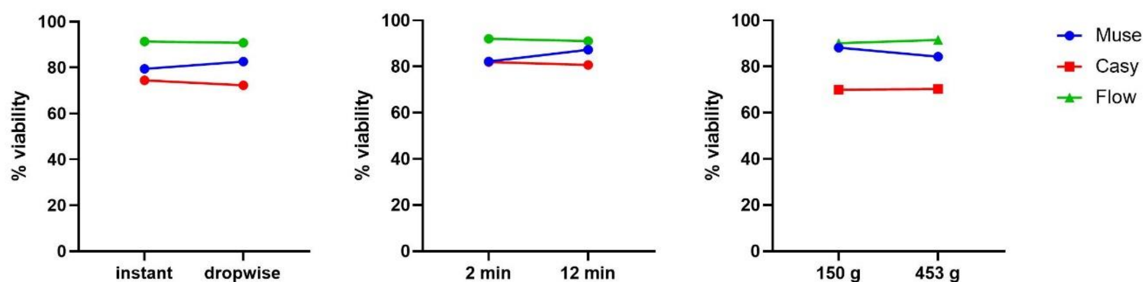


Figure 10: Percent viability of lymphocytes when the cells were thawed by instant versus dropwise adding of warm media and 2 minutes versus 12 minutes in the water bath, 150 g versus 453 g on centrifugation, respectively.

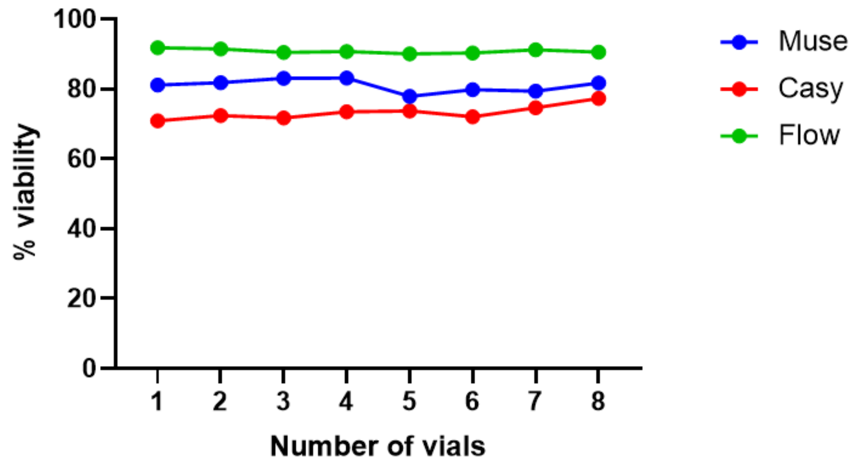


Figure 11: Percentage viability with increasing number of vials. Vial nr 1 was the first to be prepared and vial 8 the last vial to be prepared.

3.2 Immunophenotyping

3.2.1 B cells

The immunophenotyping panel consisted of subset specific markers to distinguish B cells, T cells, NK cells and monocytes together with functional markers to monitor potential activation status or maturation of cells. B cells were gated based on a live gate followed by $CD19^{+}/CD3^{-}$ gates and the percentage of B cells ($CD19^{+}$) were calculated. No difference in frequencies was observed between healthy donors and psoriasis patients (figure 13). CD27 and CD38 were then used to identify memory B cells ($CD27^{+}CD38^{+/-}$), transitional B cells ($CD27^{-}CD38^{high)}$ and plasma blasts ($CD27^{+}CD38^{high}$). Healthy controls displayed a higher portion of transitional B cells than psoriasis patients did (median healthy controls (HC): 3.6, median psoriasis patients (PP): 2.7, p=0.0116), but no difference was seen in the frequency of memory cells or plasma blasts (Figure 12).

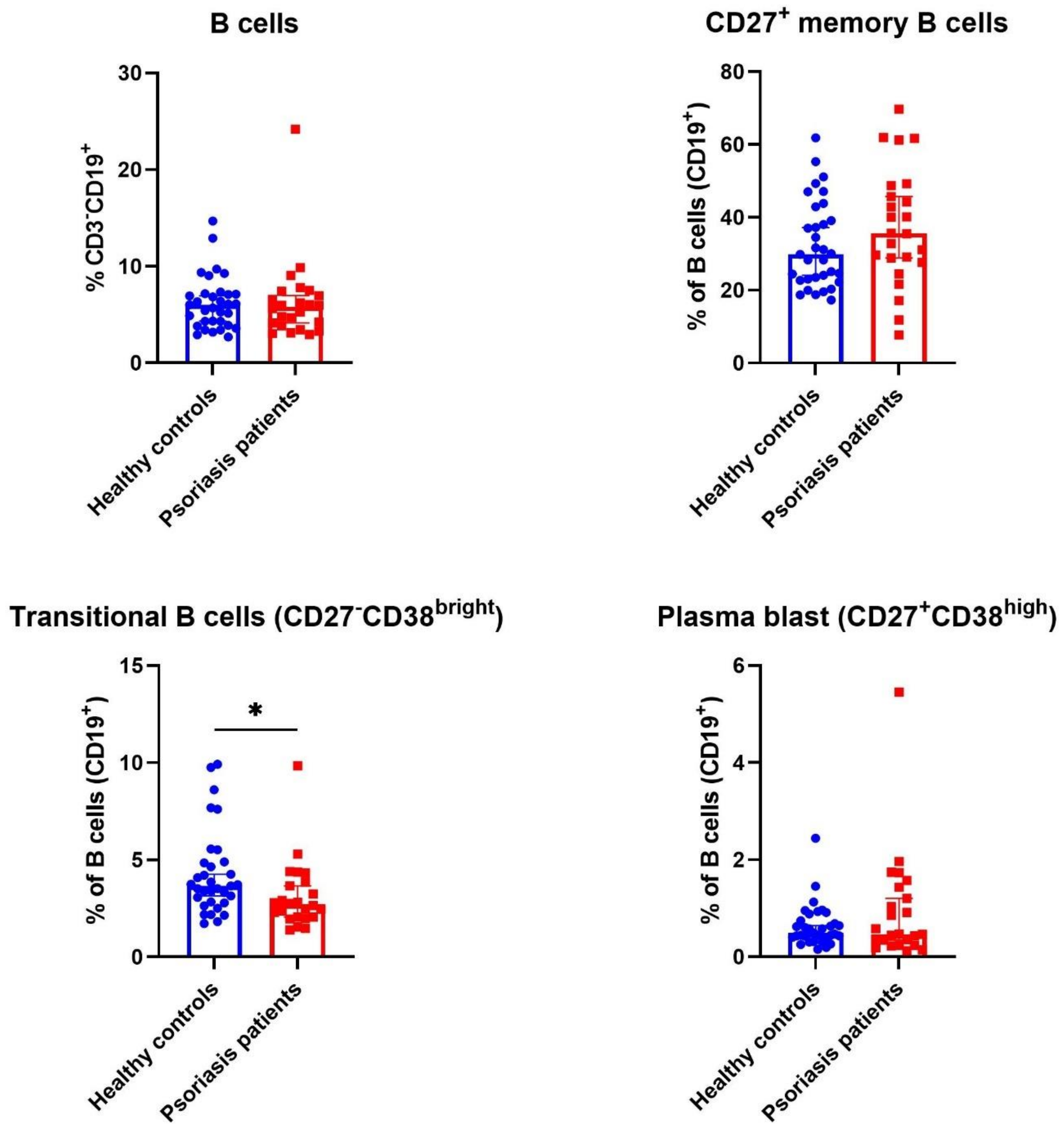


Figure 12: B cell subsets in psoriasis treated patients compared to controls. Transitional, memory cells and plasma blasts were determined as overall percentage of $CD19^{+}CD3^{-}$ B cell compartment. Healthy control (n=33): blue circles; psoriasis patient (n=24): red squares, *:$p<0.05$. Shown with median and 95% confidence interval.

3.2.2 T cells

Psoriasis disease is an autoimmune disease driven by activated T cells (8). Here, different subsets of T cells were investigated for signs of activation or differentiation. Frequencies of T cells were analysed by gating on $CD3^{+}CD56^{-}$ cells. No differences were found in total numbers of T cells between psoriasis patients and healthy control subjects. T cells were then divided into two subpopulations, T helper $CD4^{+}$ and cytotoxic $CD8^{+}$, no differences were found in frequencies of these (figure 13).

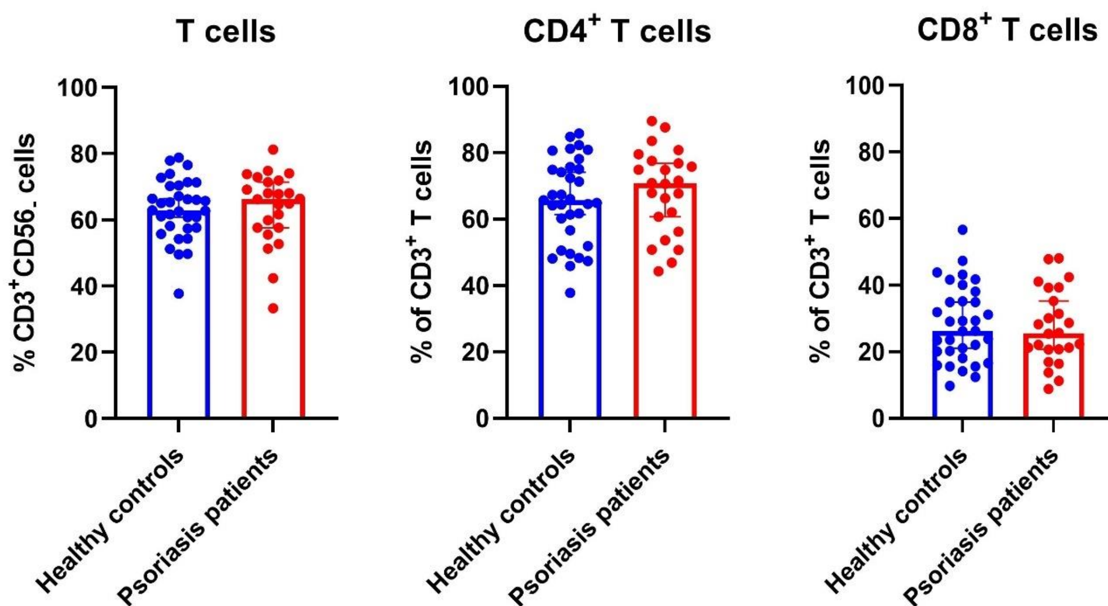


Figure 13: Frequencies of T cells were found by gating and $CD3^{+}CD56^{-}$ and were further divided into two subpopulations: T helper $CD4^{+}$ and cytotoxic $CD8^{+}$ cells. Frequencies of these populations were compared in psoriasis treated patients and control subjects. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval.

Frequencies of memory- and naïve T cells were broadly separated by looking at the expression of CD45RO. $CD45RO^{+}$ T cells were defined loosely as a memory T cell compartment, whilst $CD45RO^{-}$ cells were defined as naïve cells. Similar levels of memory and naïve T cells were found in psoriasis patients and healthy controls in both CD8 and CD4 T cell populations (figure 14).

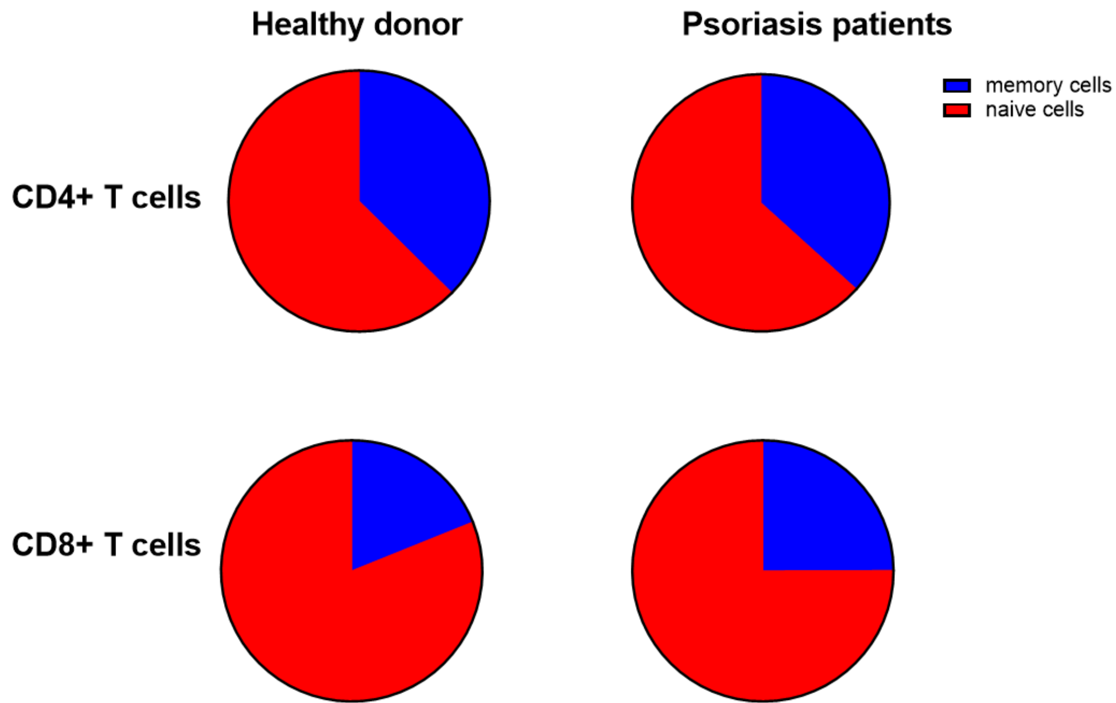
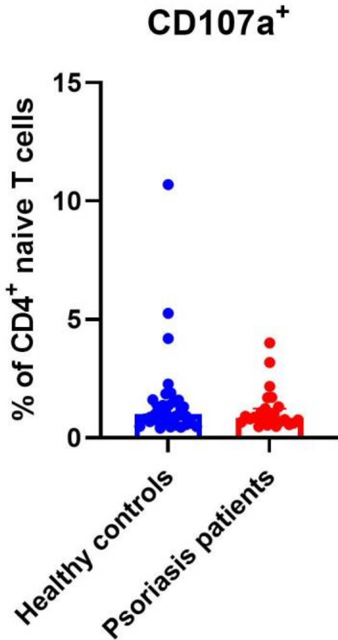
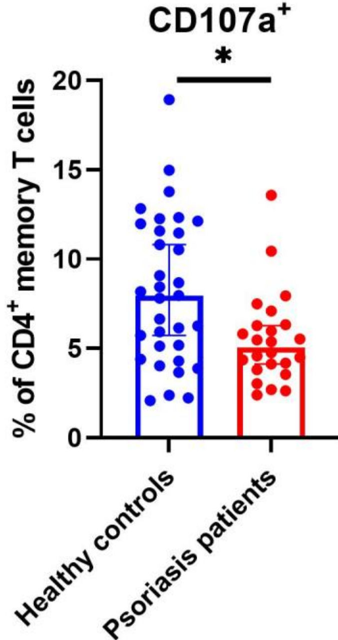


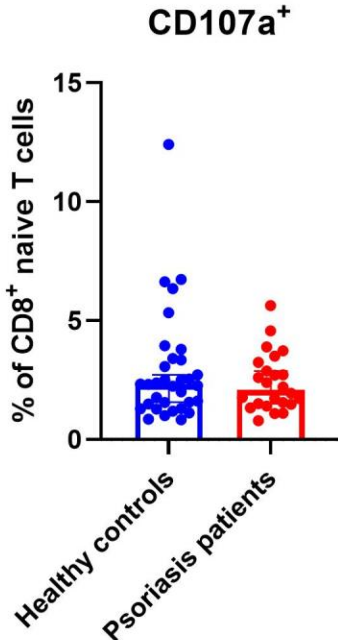
Figure 14: Memory and naïve T cells compartment in psoriasis patients and controls were compared. T helper CD4 and cytotoxic CD8 T cells were broadly separated into memory and naïve cells by their expression of CD45RO. $CD45RO^+$ were loosely defined as memory T cells and $CD45RO^-$ as naïve cells. Healthy controls: n=33, left side, psoriasis patients: n=24, right side. Memory cells in blue and naïve cells in red. Median $CD4^+CD45RO^+$ memory T cells: PP/HC: 36.7%/37,70%, $CD4^+CD45RO^-$ naïve T cells: PP/HC: 63.30%/62.60%, $CD8^+CD45RO^+$ memory T cells: PP/HC: 24.95%/18.90%, $CD8^+CD45RO^-$ naïve T cells: PP/HC: 75.05%/81.10%.

In all populations of T cells, the differentiation markers CD38, CD27, CD69, CD107a and HLA-DR were analysed. Both $CD4^+$ and $CD8^+$ memory ($CD45RO^+$) T cells showed higher expression of CD107a on healthy controls compared to psoriasis patients ($CD4^+$: median HC: 8.0, median PP: 5.1, p=0,0112, $CD8^+$: median HC: 7.7, median PP: 5.8, p=0,0147). CD107a is a lysosomal membrane protein typically associated with lymphocytes with the capacity to release perforin containing granules. However, it is also expressed by many other cell types potentially acting in cell-cell adhesion where it serves as a ligand for selectin (57, 85). No difference was seen in these activation markers on naïve T cells (Figure 15). Surprisingly, there did not seem to be a difference in expression of this marker for cytotoxicity between $CD8^+$ and

$CD4^+$ memory T cells, however, it appeared that naïve CD8 T cells express more of this marker compared to naïve $CD4^+$ T cells (median HC CD8: 2.320, median CD4: 0,990).







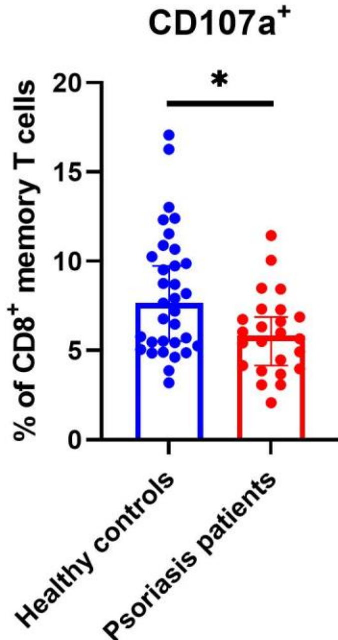


Figure 15: The expression of CD107a on T cells in psoriasis treated patients were compared to controls. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval, *: p<0.05.

Regarding the other activation markers on T cells, CD38 was expressed in high frequencies on most of the naïve T cells ($CD4^+$ T cells: median HC: 94.39%, median PP: 93.35%, $CD8^+$ T cells: median HC: 68.35%, median PP: 54%). On memory T cells, median of frequencies was 52.85% and 51.90% for $CD4^+$ T cells and 41.79% and 41.57% on $CD8^+$ T cells for healthy controls and patients, respectively. Similar frequencies of CD38 were seen on both psoriasis patients and healthy controls. However, higher MFI of CD38 was observed on healthy controls compared to psoriasis patients on $CD4^+$ naïve T cells (Figure 16) (median HC: 4882, median PP: 3984, p=0.0331).

CD27 was highly expressed on most cells for both memory and naïve CD4 and CD8 T cells (median >90% for CD4+ T cells, >80% for CD8+ T cells). MFI of CD27 was higher on psoriasis patients $CD4^+$ memory T cells compared to healthy subjects, indicating higher expression of this (median HC: 13989, median PP: 16214, p=0.0021) (Figure 17). CD69 was expressed only in low frequencies on all T cells. Naïve T cells expressed HLA-DR in low frequencies (median <5% for both subtypes), and memory cells expressed this slightly more (median <10% for both CD8 and CD4 T cells).

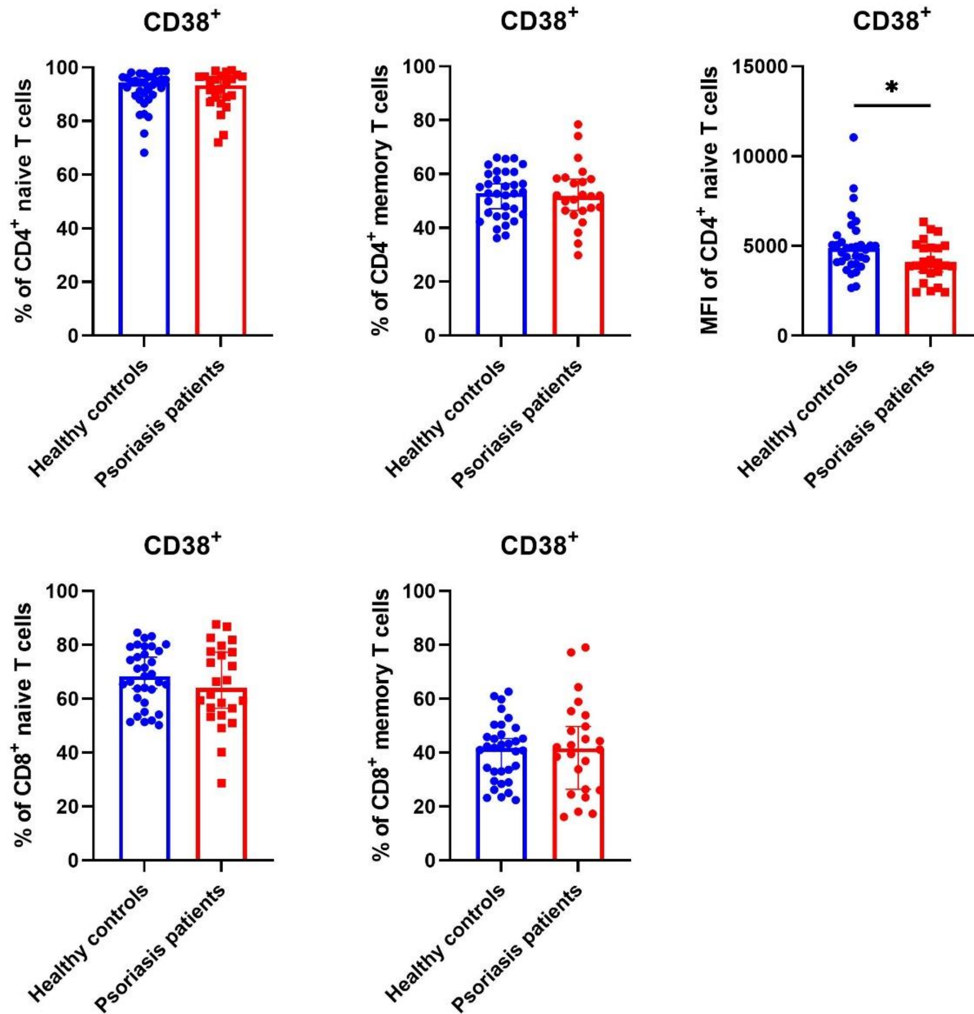


Figure 16: Expression of CD38 on T cells in psoriasis patients were compared to controls, both by comparing frequencies and MFI. Healthy control (n=33): blue circle; psoriasis patient (n=24): red square. Shown with median and 95% confidence interval, *: $p<0.05$.

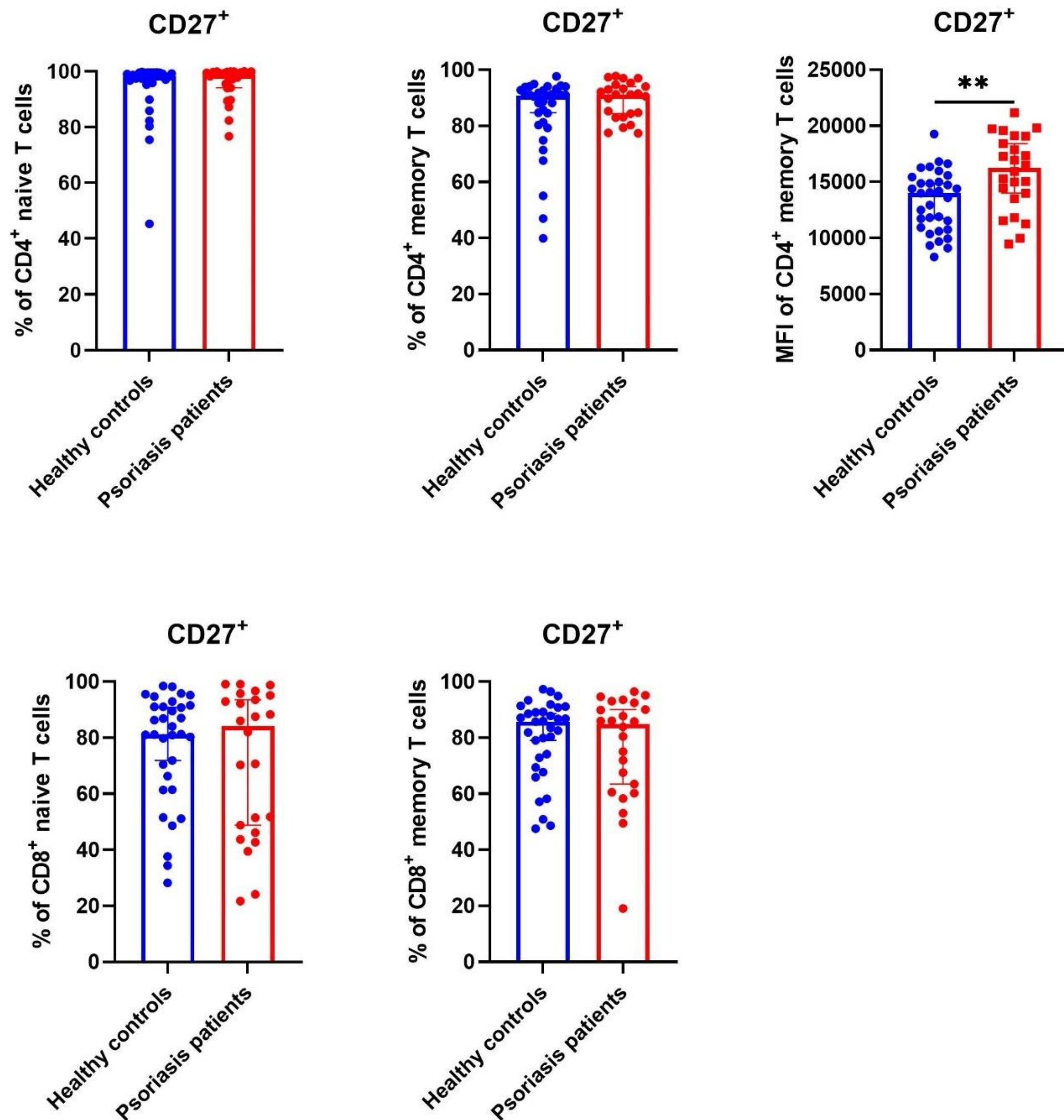


Figure 17: Expression and MFI of CD27 was analysed on all subsets of T cells and compared on psoriasis patients and controls. Healthy control (n=33): blue circle; psoriasis patient (n=24): red square. Shown with median and 95% confidence interval, **: $p<0.01$.

3.2.3 NK cells

Frequencies of NK cells and NK cell subpopulations were analysed by gating first on $CD56^{+}CD3^{-}$ cells, then dividing the NK cells further by their expression of CD56 and CD16. No difference between psoriasis patients and healthy controls were observed in NK cells ($CD56^{+}CD3^{-}$) frequencies (Figure 18). Healthy control subjects had more NK $CD56^{dim}CD16^{+}$

cells (median: HC: 84.7, median PP: 82.2, p=0.0331), which have greater cytotoxic capacity than CD56^{bright} NK cells.

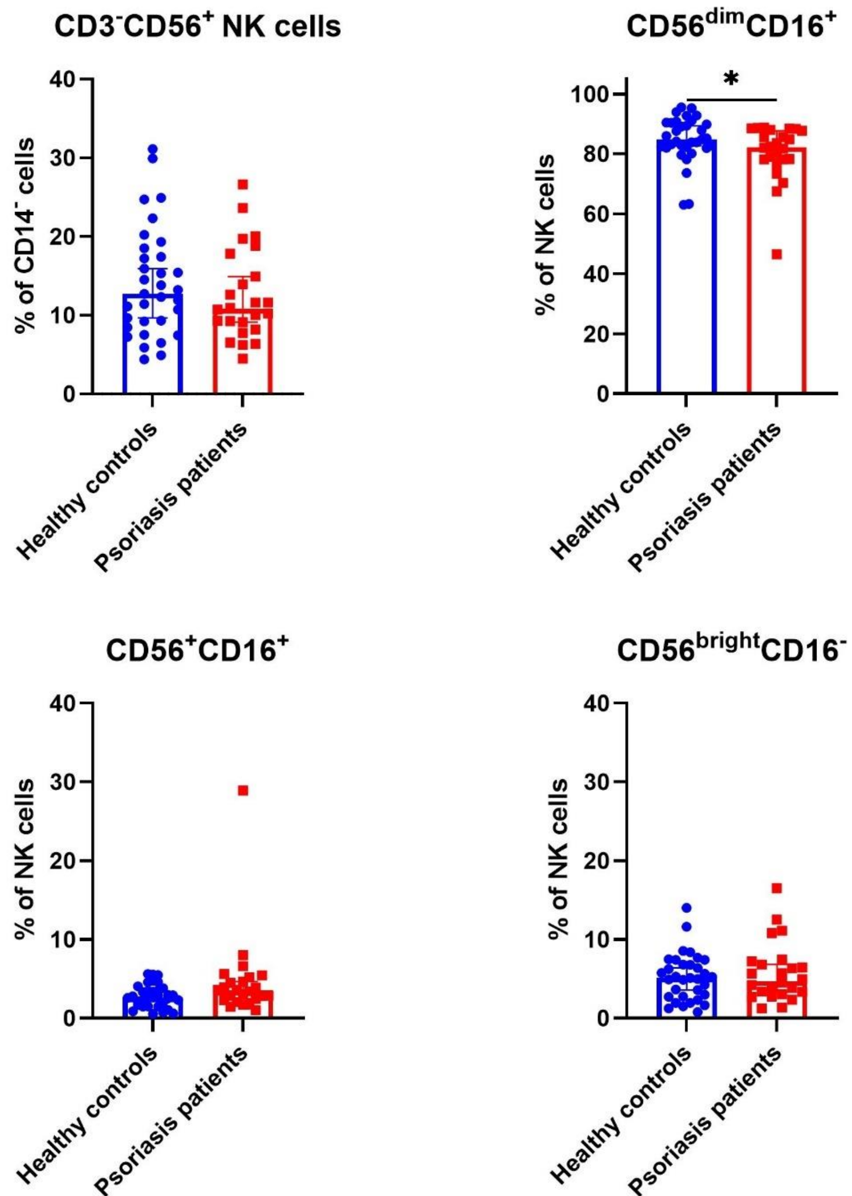


Figure 18: Frequencies of NK cells and distribution of NK subpopulations in healthy control subjects and psoriasis patients were compared. Each symbol represents one individual. Healthy control (n=33): blue circles; psoriasis patient (n=24): red squares. Shown with median and 95% confidence interval, *: $p<0.05$.

To further analyse the activation status of the different NK cell populations CD38, CD27, CD107a, HLA-DR and CD69 expression levels were analysed. CD38 was highly expressed on all NK cell populations (median >95 for all NK subtypes) in both patients and controls, however, the MFI were significantly higher on patients' cells (median: 22173) than on healthy subjects (median: 19748) on $CD56^{dim}CD16^{+}$ NK cells (p=0.0480) (figure 19).

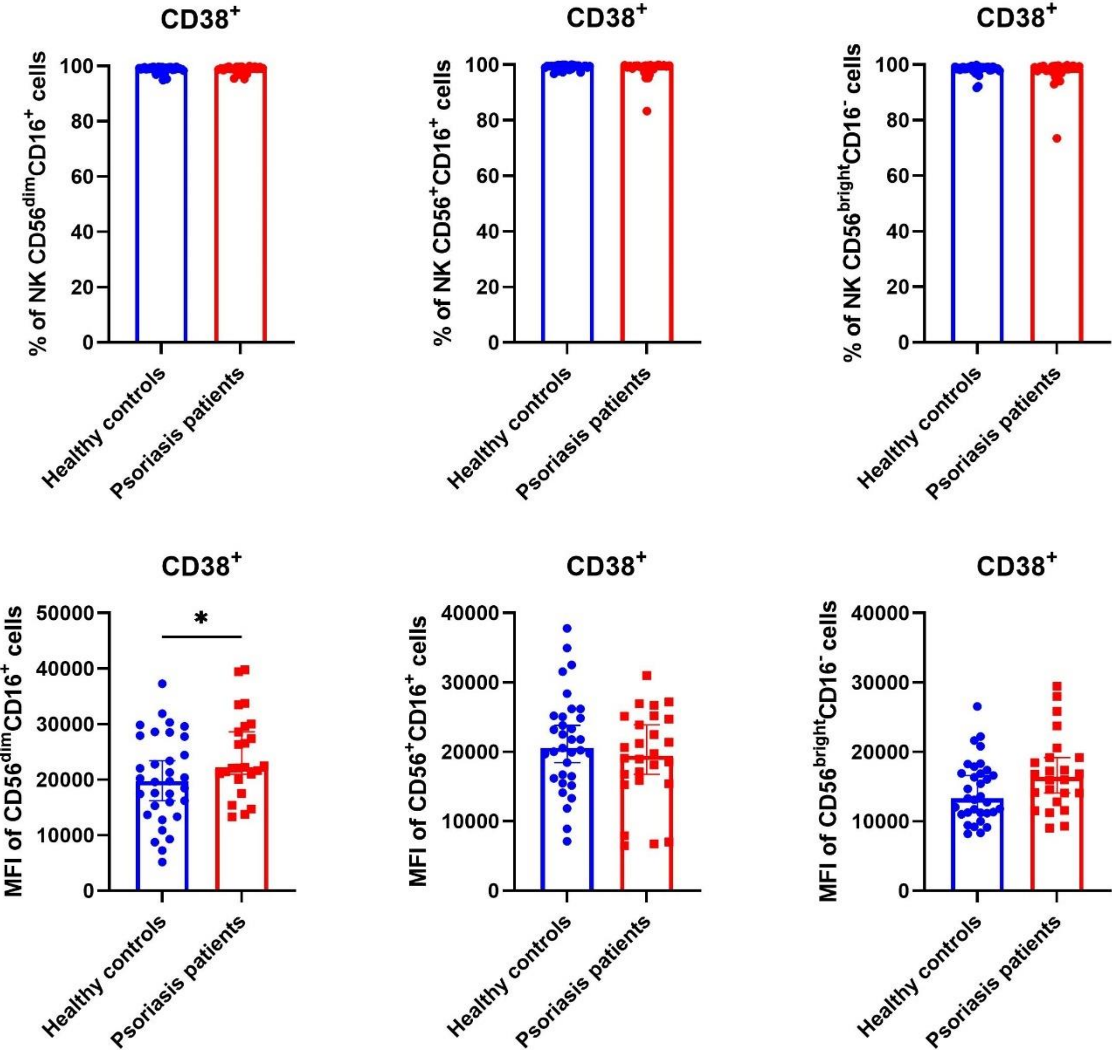


Figure 19: Expression of CD38 on subsets of NK cells in psoriasis patients and controls were compared by frequencies and MFI. Each symbol represents one individual. Healthy control (n=33): blue circle; psoriasis patient (n=24): red square. Shown with median and 95% confidence interval, *: $p<0.05$.

CD27 was higher expressed on NK CD56^{bright}CD16^{-} cells and NK CD56^{+}CD16^{+} cells compared to NK CD56^{dim}CD16^{+} cells. Psoriasis patients expressed CD27 in higher levels on their NK CD56^{dim}CD16^{+} cells compared to healthy subjects (median HC: 1.820, PP: 3.725, $p<0.0001$), though in low levels (Figure 20).

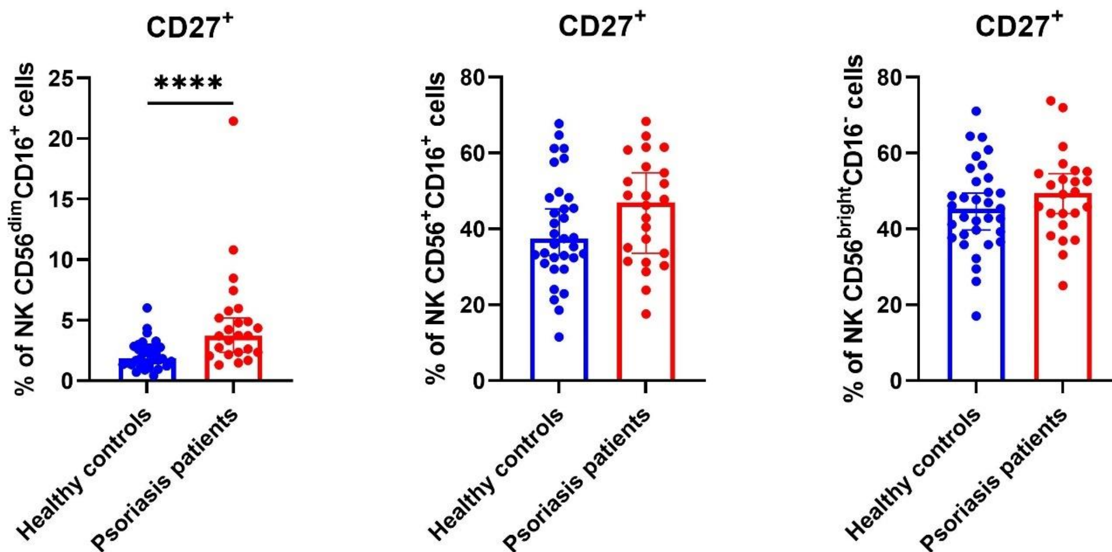


Figure 20: Expression of CD27 on NK cell subsets in psoriasis patients and controls were compared. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval, ****: $p<0.0001$.

Interestingly, aged matched control subjects expressed significantly more CD107a on all of the NK cell subtypes analysed (figure 21). The percentage of cells expressing CD107a was relatively low which was expected in the absence of target cells such as during a viral infection, therefore CD107a expression here could also possibly reflect cell-cell interactions supported by CD107a binding to selectins or else spontaneous uptake of lysosomes by the plasma membrane (85) (NK CD56^{dim}CD16^{+} cells: median HC: 6.550, median PP: 3.345, $p<0.0001$, NK CD56^{+}CD16^{+} cells: median HC: 1.820, median PP: 0.8600, $p<0.0001$, NK CD56^{bright}CD16^{-} cells: median HC: 1.350, median PP: 0.9450, $p=0.0050$). Supporting this, activation markers HLA-DR and CD69, which often strongly co-ordinate with inflammatory cytokine receptor or surface recognition receptor engagement, showed similar levels in psoriasis patients and healthy controls (figure 22).

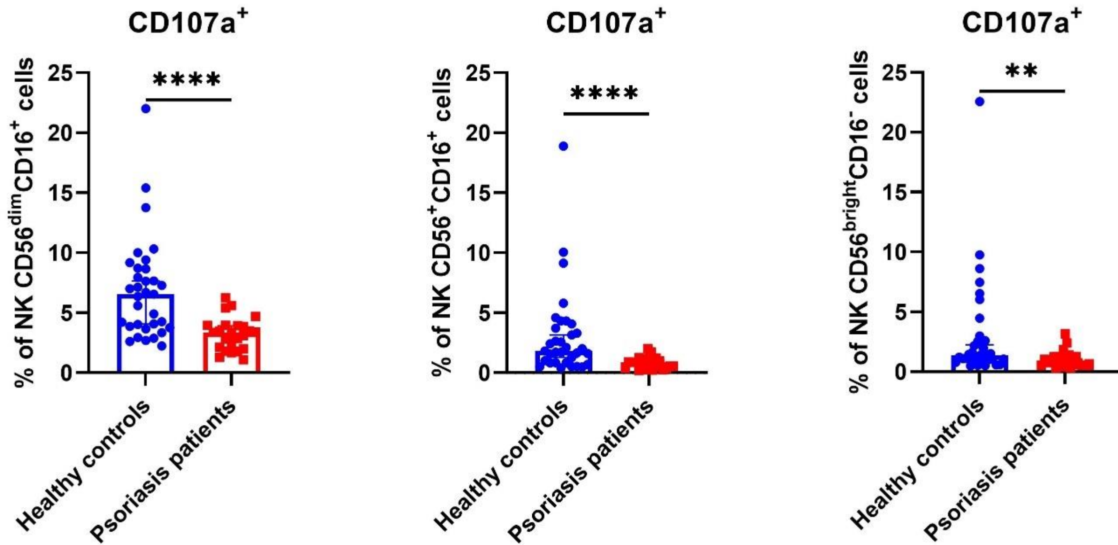


Figure 21: Expression of CD107a was analysed on all subsets of NK cell and compared on patients and controls. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval, ****: $p<0.0001$, **: $p<0.01$.

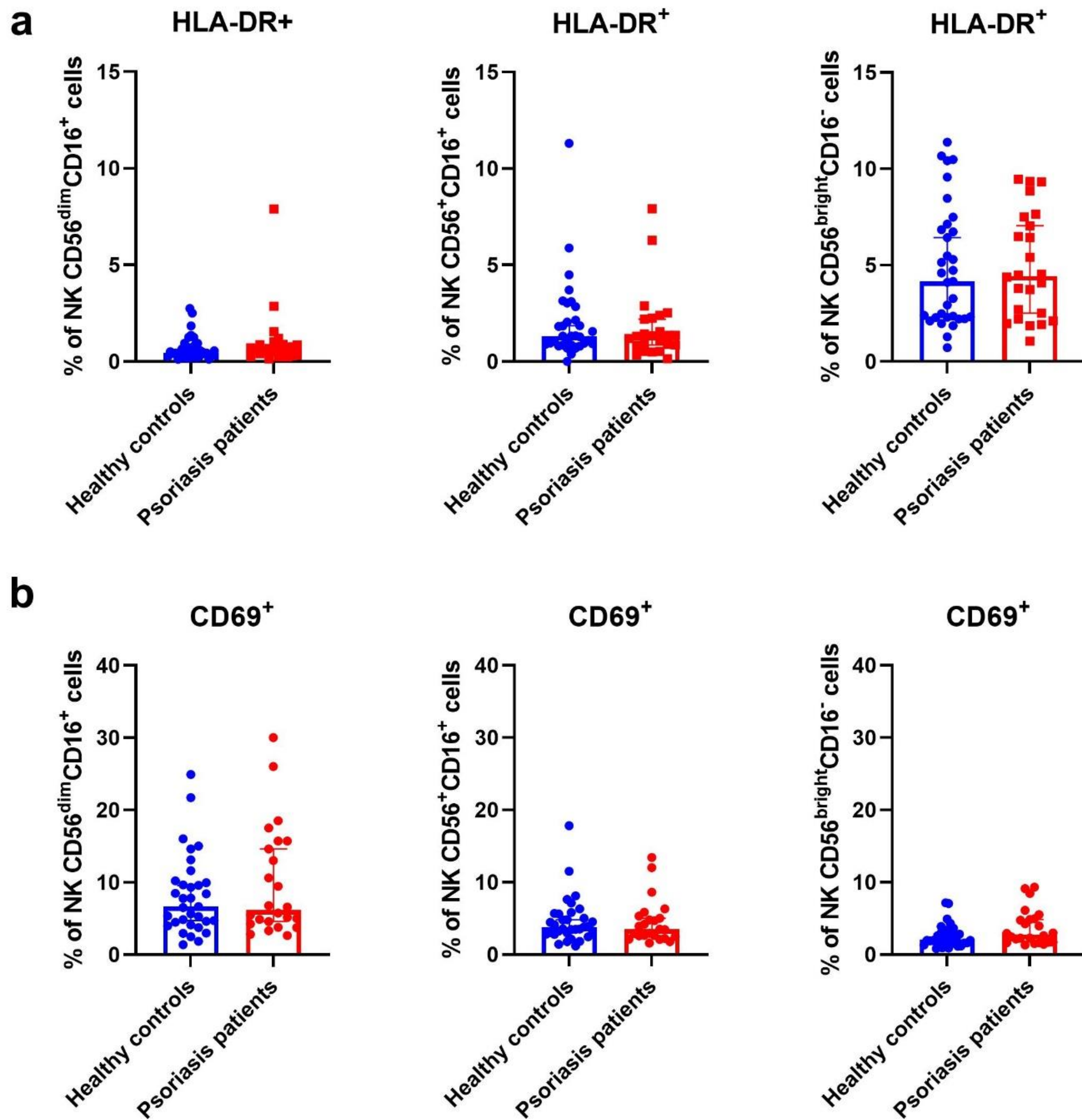


Figure 22: HLA-DR and CD69 showed similar levels in psoriasis patients and healthy controls. A) expression of HLA-DR was analysed on all subsets of NK cells and compared in patients and controls. B) Expression of CD69 in all subsets of NK cells were analysed in patients and compared to controls. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Frequencies of CD69 shown with median and 95% confidence interval.

3.2.4 NKT-like Cells

The frequency of cells gated on $CD3^+CD56^+$ comprises of NKT cells, as well as cytotoxic γδ T cells and less often αβ T cells (86). Here, these compartments were analysed as a whole and similar levels of these cell types in psoriasis patients and healthy controls were found (figure 23).

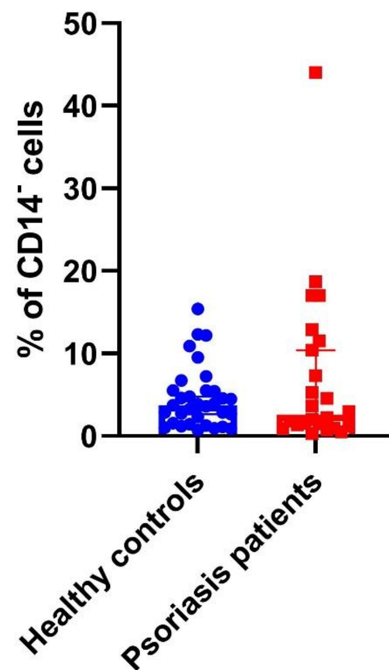


Figure 23: The frequency of NKT-like cells gated on $CD3^+CD56^+$ were compared in patients and controls. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval.

To evaluate the activation state of the NKT-like cells, surface markers HLA-DR, CD38, CD27, CD107a and CD45RO expression levels were analysed. Whilst no differences were observed with the other functional markers, HLA-DR displayed a higher expression in psoriasis patients than on healthy controls (median HC: 4.980, median PP: 6.300, $p=0.0386$) (figure 24).

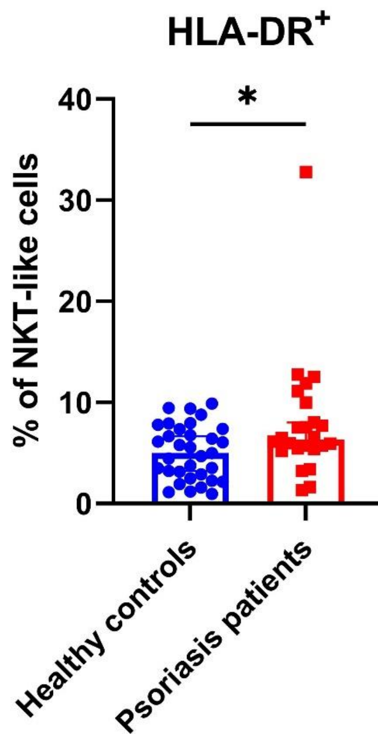


Figure 24: Expression of HLA-DR on NKT-like cells on psoriasis patients were analysed and compared to controls. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval, *: $p<0.05$.

Neither of the activation markers CD38, CD69, CD27 or CD45RO, showed any difference in frequencies between patients and controls (figure 25-26). CD107a was expressed in low levels and were also similar in psoriasis patients and healthy donors (data not shown).

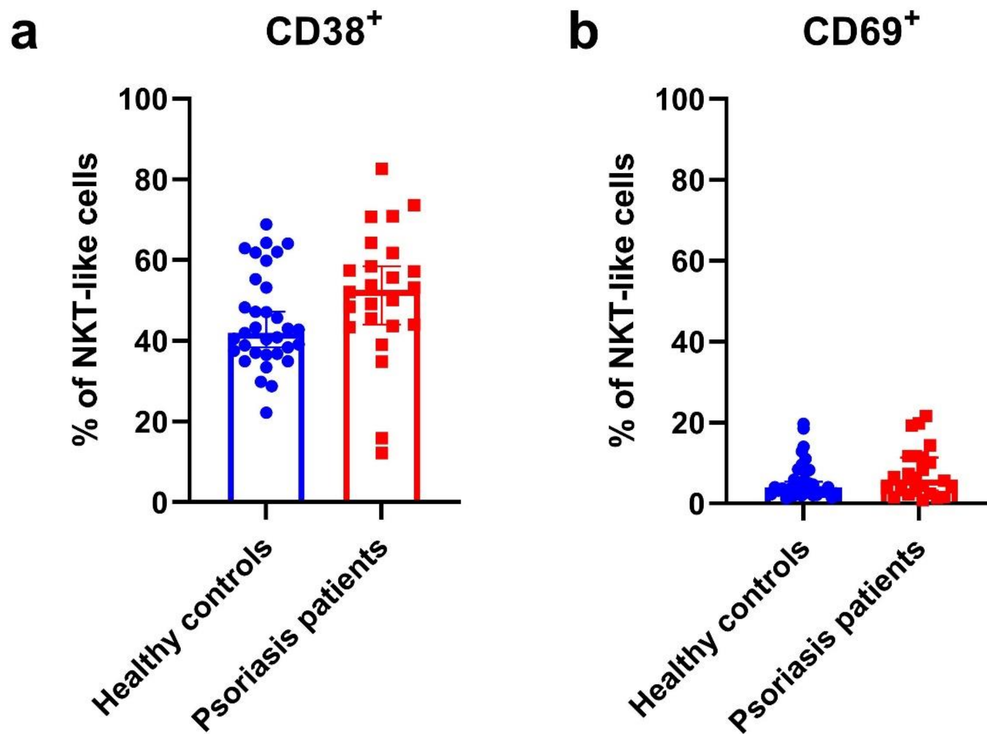


Figure 25: A) Expression of CD38 on NKT-like cells was analysed in psoriasis patients and compared to controls. B) Expression of CD69 on NKT-like cells was analysed in psoriasis patients and compared to controls. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval.

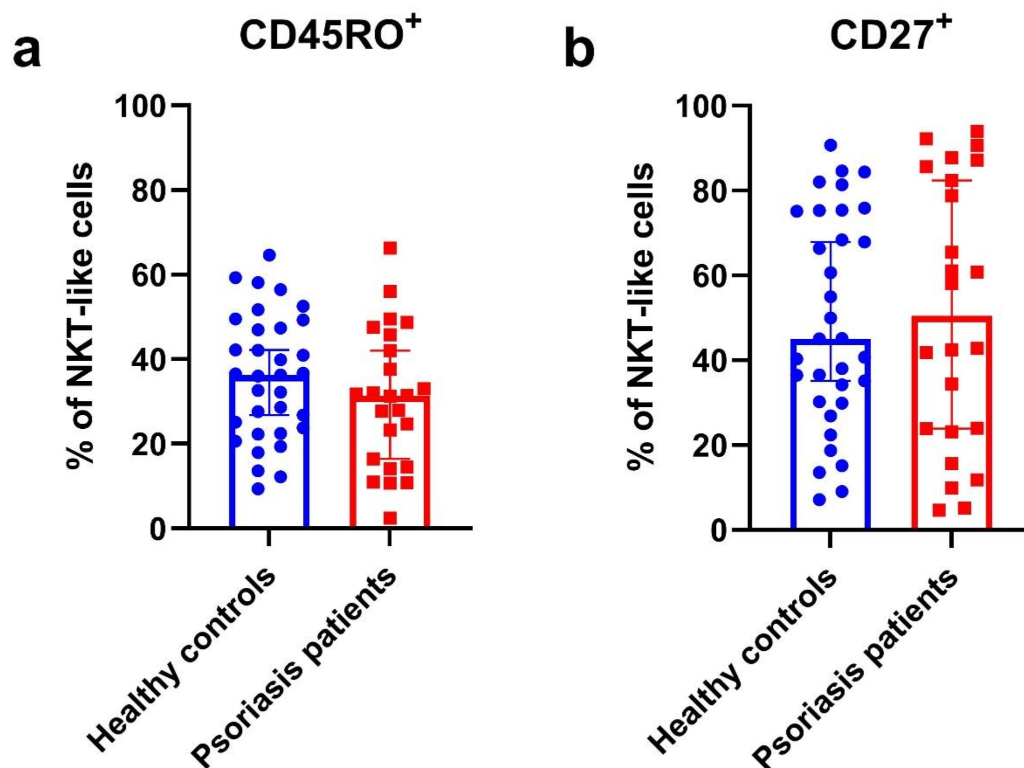


Figure 26: Similar frequencies of CD45RO and CD27 were found in NKT cells in psoriasis patients and healthy controls. A) Expression of CD45RO on NKT-like cells was analysed in psoriasis patients and compared to controls. B) Expression of CD27 on NKT-like cells was analysed in psoriasis patients and compared to controls. Healthy control (n=33): blue circle; psoriasis patient (n=24): red square. Shown with median and 95% confidence interval.

3.2.5 Monocytes

Frequencies of monocyte subpopulations were determined based on their expression of surface markers CD14 and CD16. Classical monocytes are $CD14^{+}CD16^{-}$, whilst intermediate monocytes are $CD14^{+}CD16^{+}$ and here non-classical monocytes are classified as $CD14^{-}CD16^{+}$. Psoriasis patients and healthy control subjects had similar levels of classical and non-classical monocytes, but psoriasis patients had a significantly higher portion of the intermediate monocyte subtype that is often associated with angiogenesis and have higher MHC-II compared to classical monocytes (87) (median HC: 4.240, median PP: 5.665, p=0.0053) (figure 27).

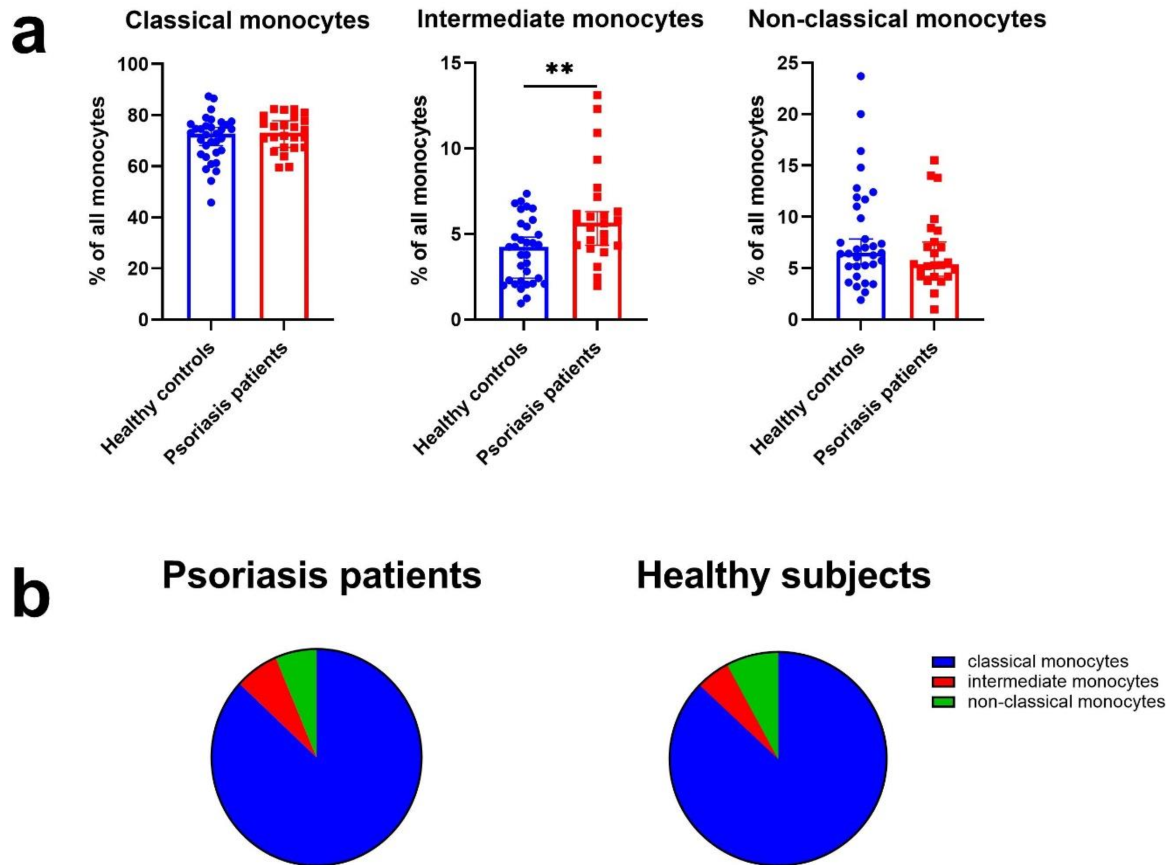


Figure 27: Monocyte subpopulation distribution in patients were compared to controls. Classical monocytes were defined as $CD14^{+}CD16^{-}$, intermediate monocytes as $CD14^{+}CD16^{+}$ and non-classical monocytes classified as $CD14^{-}CD16^{+}$. A) Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval, **: p<0.01. B) Distribution of the monocyte subpopulations in psoriasis patients and healthy subjects, respectively presented as a pie chart. Classical monocytes in blue, intermediate monocytes in red and non-classical monocytes in green.

To evaluate activation of monocytes, activation markers CD38, HLA-DR and CD45RO were analysed on these cells. The activation marker CD38 was highly expressed on classical and intermediate monocytes (>96%, median for intermediate monocytes: HC: 99.80%, PP: 99,98%) and in mediocre levels on non-classical monocytes (median HC: 48.90%, median PP: 61.03%). Psoriasis patients expressed higher levels of this surface marker than healthy donors on intermediate (p=0.0004) and non-classical monocytes (p=0.0008) (figure 28), but similar levels on classical monocytes. MFI of CD38 on intermediate cells were also higher on patients' cells (p=0.0311)

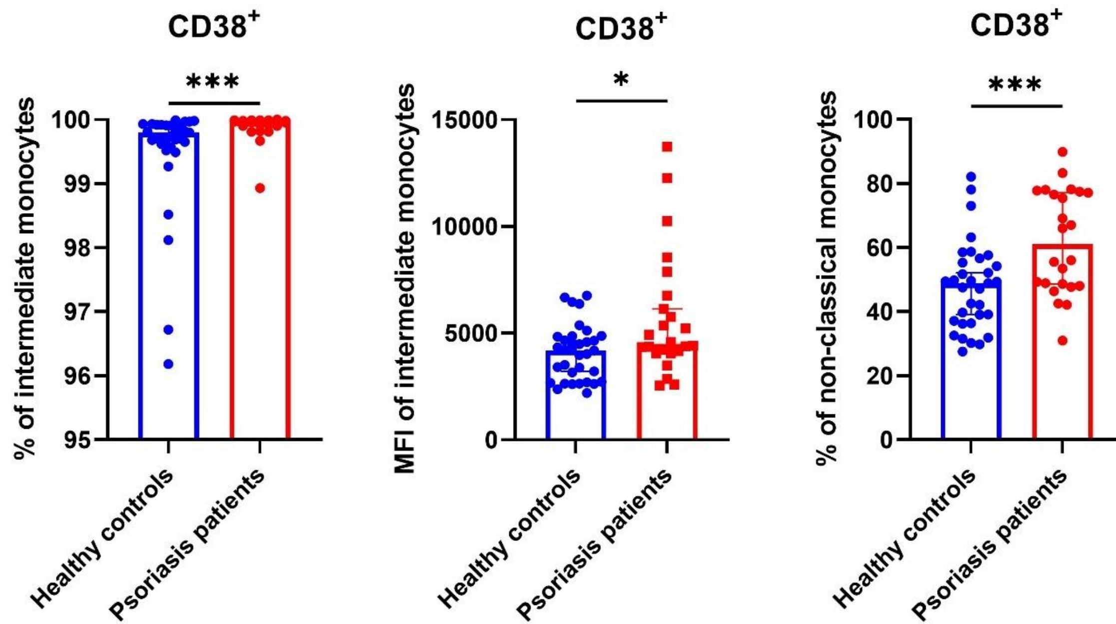


Figure 28: Expression of CD38 on monocyte subpopulations in psoriasis patients were compared to controls. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval, *: p<0.05. ***: p<0.001.

HLA-DR was highly expressed on intermediate monocytes (>90%, median HC: 98.10, median PP: 98.80). Both patients and controls also expressed high levels of this on their classical and non-classical monocytes but in a bigger range (classical monocytes, range (median): HC: 62.90-98.90% (91.10%), PP: 78.10-99.50% (90.60%,), non-classical monocytes: HC: 30.30-95.60% (80.30), PP: 18.30-98.20% (85.80%)). Psoriasis patients had higher expression of this surface marker than healthy donors on intermediate (p=0.0235) and non-classical monocytes (p=0.0067) (Figure 29).

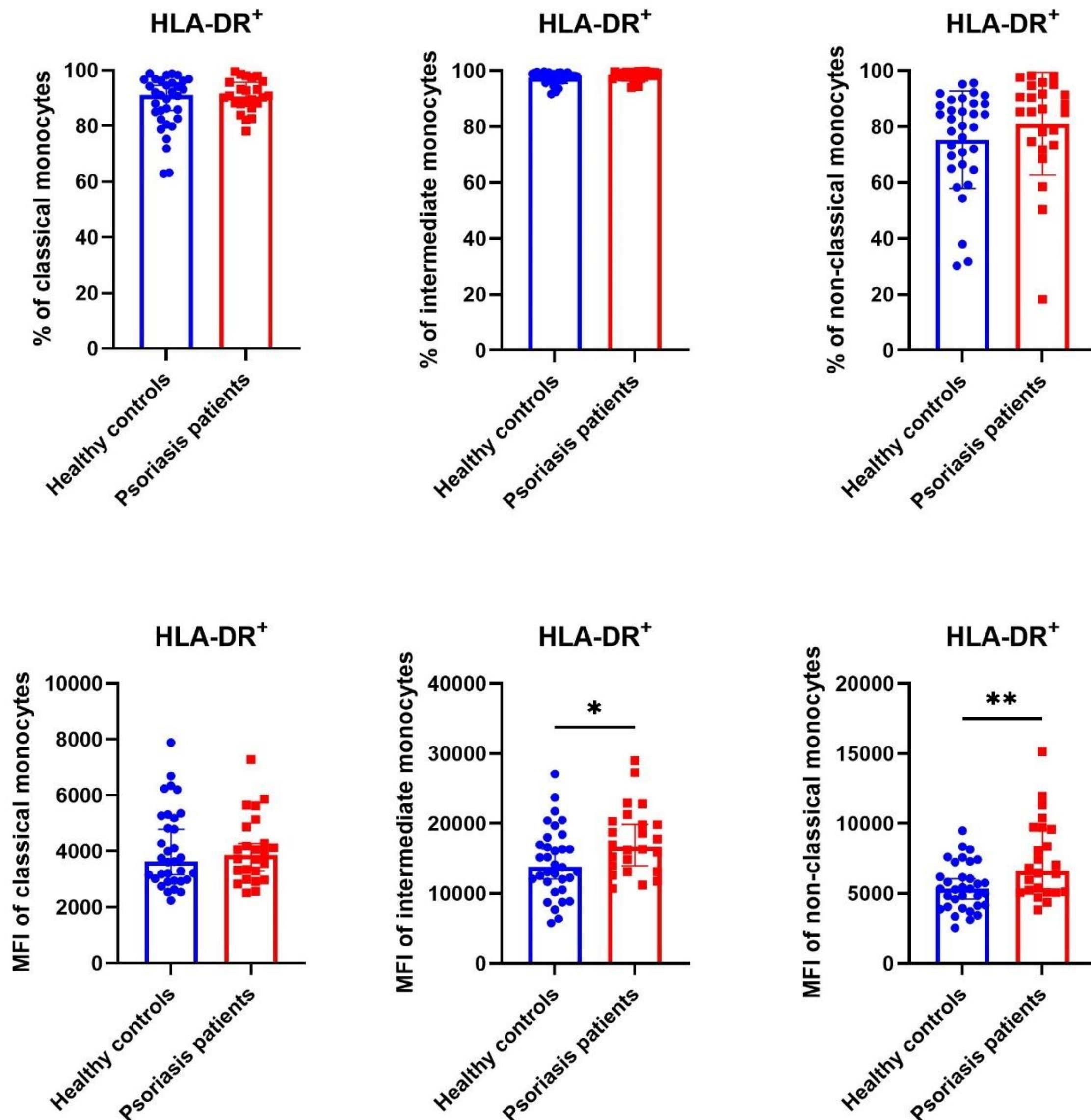


Figure 29: Expression of HLA-DR on all subsets of monocytes were analysed by frequencies and MFI on psoriasis patients and compared to controls. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval, *: $p<0.05$, **: $p<0.01$.

CD45RO was also analysed on all monocyte subpopulations (figure 30). Only on non-classical monocytes did psoriasis patients express a higher frequency than healthy donors of this marker (p=0.0180), but both groups expressed this in very low levels on these cells, while they expressed this marker in higher frequencies on the two other monocyte subpopulations.

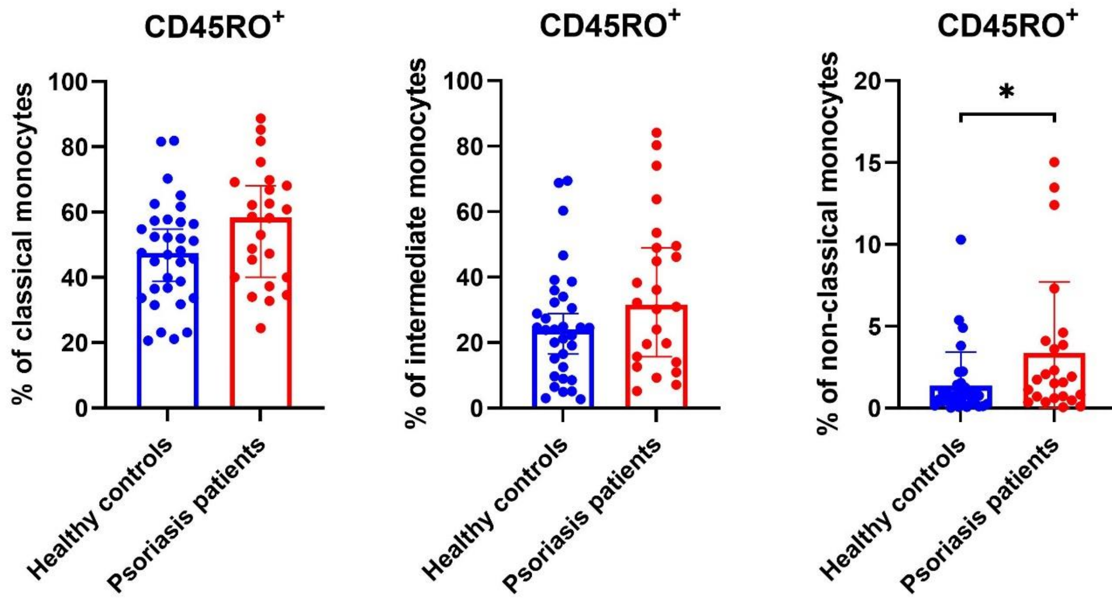


Figure 30: Frequencies of CD45RO was analysed on all subtypes of monocytes in psoriasis patients and compared to controls. Each symbol represents one individual. Healthy control (n=33): blue; psoriasis patient (n=24): red. Shown with median and 95% confidence interval, *: $p<0.05$.

4 Discussion

4.1 Immunophenotyping in psoriasis patients

This study was inspired by a pilot investigation using mass cytometry at the host lab. Aarebrot et al were investigating the effect of switching from infliximab to biosimilar and aimed to find disease specific immune profiles and biomarkers for psoriasis (76). Whilst there were no differences between infliximab and biosimilar, they noted a trend in the expression of CD38 in NK cells and T effector memory cells. This was a preliminary study to test the feasibility of using mass cytometry to monitor patient phenotypes and was done on a very small sample set (76). The present study aimed to further investigate cell surface markers, using flow cytometry. This is a good method to analyse a large sample number and smaller cell samples as it is capable of high-speed measuring multiple parameters at the same time in individual cells. It is advantageous as it does not lose as many cells in acquisition, which is important for small samples (88).

The use of biologics has revolutionised the treatment of moderate to severe psoriasis (31). Infliximab is a TNF-α inhibitor and is the most efficacious treatment in its class. At week 12, 76% reach a PASI75 reduction (30). However, up to 30% of patients do not respond to treatment, furthermore, about 50% lose response over time (89). This indicates there is a need for biomarkers to monitor treatment efficacy and to identify patients who do not respond at the earliest time possible.

Psoriasis is considered a T cell mediated disease and it might be expected to find more activated T cells in psoriasis patients compared to healthy subjects with different T cell subset distribution (90). However, the patients included in this study were all stable on infliximab with a relatively low PASI score. As such, no difference was observed in T cell frequency or memory/naïve T cell percentages. Furthermore, contradictory observations were found regarding activation of the T lymphocytes, with higher MFI of CD38 on healthy subjects as well as increased frequencies of CD107a in healthy controls. CD38 has several roles as a surface molecule on lymphocytes. It is being used as an activation marker, adhesion molecule, to regulate cytokine release, migration, and ecto-enzymatic activity. A recent review study pointed to CD38 being involved in several autoimmune diseases including rheumatoid arthritis, inflammatory bowel disease, systemic lupus, and multiple sclerosis. Increased levels of CD38 on B cells, T cells and monocytes have been observed in these diseases. The effect of this has however not been identified, and it is not clear whether it has a pathogenic or regulatory effect, and the mechanism

is not known (91, 92). Together, the decreased expression of CD107a and CD38 in patients could suggest that infliximab mediated inhibition of TNF signalling may suppress inflammatory or tonic signals below typical levels observed in healthy controls.

T cell expression of CD27 was elevated in psoriasis patients. One of the main functions of CD27 is in B cell activation and antibody production. It is a TNF receptor superfamily member, and its ligand is CD70. CD27 is also expressed in other cells like T cells and NK cells. In T cells, it is important in proliferation and cytokine production (93, 94). It works as a costimulatory receptor expressed on most resting T cells in both CD4 and CD8 compartments (95). It has also been used to analyse effector memory cells (96).

In this study, MFI of CD27 was higher on $CD4^+$ memory T cells from psoriasis patients compared to healthy controls. However, the frequency of this marker was similar in both groups, indicating that those cells expressing CD27 had a higher density of this on the cell. MFI of CD38 on CD4+ naïve cells were on the other hand higher in healthy controls, which is also contradictory to previous findings in other studies including CD38 in T cells in autoimmune disease (92). As CD69 was not changed in any of the groups, the change in CD38 and CD27 may reflect other things than activation.

CD107a is a marker for degranulation after activation of cytotoxic $CD8^+$ T cells and NK cells. Upon interaction with target cells such as a virus-infected cell, cytotoxic lymphocytes release perforin-containing granules to destroy the target cell. Degranulation of these cytotoxic granules requires merger with the plasma membrane whereby CD107a can be detected (57). CD107a was more frequent on memory T cells from healthy controls, however, unexpectedly there seemed to be similar levels of this marker on both $CD4^+$ and CD8 T cells. A subset of cytotoxic $CD4^+$ T cells does exist (97). However, in the likely absence of target cells, there were low levels of CD107a on T cells on both cell subsets and the cells are in resting state. This suggests that expression of CD107a could be from small amounts of spontaneous lysosomal uptake by the plasma membrane. Indeed, CD107a has been noted on several cell types and has been suggested to aid cell-cell adhesion via binding to E-selectin (85).

We also observed increased CD107a on all NK cells in healthy controls. Here, CD107a is likely a marker of cytotoxicity. NK $CD56^{dim}$ cells, the more cytotoxic NK subset displayed increased CD107a expression in healthy control subjects compared to patients. Earlier observations have also indicated an increased NK cell cytotoxicity in healthy subjects compared to psoriasis patients. Dunphy et al found a similar level of increased activity in both healthy donors and

psoriasis patients after IL-15 stimulation, however, they found lower degranulation in psoriasis patients in a CD107a degranulation assay. This study was done on patients with active disease (58). Our study indicates that NK cells continue to express CD107a in lower percentages of the NK cells, even after treatment and in resting state. As CD107a was increased in healthy controls on both CD8+ CD4+ memory T cells and all subpopulations of NK cells, it is possible this could be used as a biomarker. However, very few cells expressed this marker overall and more research is needed to confirm this.

In NK cells CD38 has multiple functions including it being used as an activation marker, adhesion molecule and possessing ecto-enzymatic activity. Previous work in the host lab indicated that CD38 was possibly higher in NK and $CD4^{+}$ memory T cells, however, this study included few patients (76). This present study included more patients and observed the same trend in NK cells. MFI of CD38 on $CD56^{dim}$ NK cells did imply a higher expression in patients. MFI of CD38 was however higher in $CD4^{+}$ naïve cells from healthy controls. This study done at the host lab also found an increase in HLA-DR on $CD16^{+}$ NK cells, which could not be confirmed in this study. Psoriasis patients had on the other hand an increased frequency of HLA-DR on NKT-like cells, indicating a possible increased activity in these cells.

Previous reports state that the majority of $CD56^{dim}$ NK cells are $CD27^{low}$, while $CD56^{bright}$ NK cells are $CD27^{hi}$, which is concurrent to the observations in this study. Moreover, it has been found that the different CD27 populations on NK cells express different receptors and have different functions. This has led to CD27 being used as a maturation marker on NK cells together with CD56 (98). Indeed, CD27 has long been established as a marker of NK cell maturation in the mouse and has also been correlated to psoriatic disease severity (99). This is in alignment with the finding of higher frequencies of CD27 on $CD56^{dim}$ NK cells in this study, even though the patients in this study had low PASI scores.

Classical monocytes are important phagocytes and can differentiate into macrophages. Intermediate monocytes are involved in phagocytosis and exhibit the highest levels of MHC class II and are suited for antigen presentation. Non-classical monocytes also display high levels of HLA-DR and are involved in complement and Fc-gamma mediated phagocytosis (42-44). While non-classical monocytes are viewed as anti-inflammatory due to their ability to maintain vascular homeostasis, classical and intermediate monocytes have been seen to be elevated in CVD (44, 87, 90).

According to previous studies, CD38 may be used as an inflammatory marker in monocytes. A recent study found that even though monocytes express CD38 constitutively, high expression on non-classical monocytes is linked to disease activity in systemic lupus erythematosus (72).

In this present study, intermediate monocytes were increased in patients. According to literature, intermediate monocytes have high HLA-DR, and this is confirmed by this study (median ~98% in both groups) (87). HLA-DR was elevated in patients, indicating that these cells are more activated than healthy controls, even at resting state and on treatment. Furthermore, similarly to that seen in other autoimmune diseases, CD38 was increased, additionally pointing to increased activity in these cells. Considering the risk of cardiovascular disease, studies at the host lab found the intermediate monocyte population to decrease after treatment as well as non-classical monocytes undergoing increased activity (90). Increased activity in non-classical monocytes was also observed in this study, with increased levels of HLA-DR and CD38 in patients, as well as increased CD45RO. However, the increased activity and increased frequency of intermediate monocytes in treated patients may point to a possible continuous elevated risk of CVD even after treatment.

Even though B cells are not considered to be an important mediator in psoriasis, it is possible that B cells play a role in the pathogenesis. Research shows that the frequency of transitional B cells secreting the immunosuppressive IL-10 is decreased psoriasis and PsA. These B cell subsets were defined by $CD24^{high}CD38^{high}$ expression (100). In this study, we found a decreased frequency of transitional B cell frequency in patients. This could imply a reduced frequency of a regulatory cell subset even after treatment.

4.2 Thawing

Four different thawing methods were investigated to find the one that gave the most viable cells. Surprisingly, neither centrifugation acceleration, time in 37°C water bath, speed of adding warm media to cells or number of vials thawed at the same time affected the cell viability significantly. The expectation was that the viability would be better with dropwise addition of warm media to the cells as the cells are fragile at this point, and slowly adding the media is a gentler method to allow the cells to equilibrate the change in osmolarity (78). We also expected the viability to go down after staying in DMSO after thawing because DMSO is toxic for the cells and traditionally it has been thought best to remove the DMSO as quickly as possible. However, there has been a report describing that the cells can be kept at 37°C for up to 30

minutes before viability and functionality of the cells are affected (78). We wanted to test if this is also true for lymphocytes. The last vial prepared, stayed on the bench for several minutes as the first vials were thawed sequentially. It seemed the speed the procedure is performed at is not as critical as first thought. The viability of lymphocytes did also not decrease with the increased number of vials thawed at the same time. This informed the design and experimental handling of patient samples where I could be confident of thawing larger batches of cells together.

Generally, we expected greater differences than our experimental observations. This may be because the cells used were newly frozen and thus more fragile cells could have had a bigger impact. The freezing protocol also has a significant impact on cell viability. The PBMCs used here was isolated only one month prior and frozen gradually, before being stored at -150°C. Normally, cells used in studies might be stored for considerably longer time periods before thawing and may not be stored under ideal conditions. The cells included in this study seemed robust, which was also apparent when looking at the dead control, a vial that was shaken, flicked and vortexed, to stress and kill the cells, as this sample did not have a clear decreased viability either. The use of more stressed cells, such as cells that were not freshly frozen – may have generated larger differences between experimental procedures analysed here.

In addition, I restricted analysis to the lymphocyte compartment as the monocytes had partially adhered during a resuscitation testing. However, previous (personal) observations have shown that monocytes are more sensitive to freezing and would therefore be interesting to compare. Future work could explore the effect of thawing on monocytes as well and include markers other than live/dead marker to study the effect of thawing on different lymphocyte subtypes. It could also be beneficial to have less robust cells in this type of study, for instance using cells that are 1-2 days old before freezing.

Three methods were used to analyse the cell viability. MUSE and Flow cytometer use dyes to measure dead/live cells, whilst the CASY cell counter works by polarization from electrodes. MUSE and flow cytometer might therefore be more sensitive methods. This could explain the variability in their viability count.

4.3 Methodological considerations and limitations:

In this study, the cells were fixed to provide better time and more flexibility to analyse the samples after staining. This was done because many patient samples would be stained at the

same time, which meant time spent analysing at the Fortessa LSR would be longer and would not be able to do within one hour as is recommended with living cells (101). An additional staining test was performed on fixated samples and compared to unfixed to ensure the antibody staining had not been affected.

For some of the surface markers, there was a clear separation between positive and negative populations. For others, especially the activation markers, there were not. For these, FMOs were used to set the gate and the gates were not further adjusted to the individual samples. The FMO samples used to gate on the patient samples were those made while doing the antibody titrations. These samples were not fixated like the patient samples were. However, a test on fixated versus un-fixated cells showed no difference in fluorescence and it should not affect the results.

A limited number of cell surface markers can be used in flow cytometer panel, due to spillover issues. In these experiments, CD45RO was used as a memory cell marker on T cells. This marker alone is however not typically sufficient to ensure only memory cells are included, other markers that could help distinguish these cells are CD45RA, CCR7 and CD28 (96).

Twenty four patients and 33 healthy controls were included in this study, making it 57 samples in total. Additionally, one internal control was included for each staining to check for inter-assay variation, but no adjustments needed to be done. The healthy controls were matched by age, sex and BMI. The male to female ratio within the patient cohort was 20:4.

Most patients treated with infliximab are also treated with methotrexate. This medication has been shown to affect T cell frequencies (102). As most patients in this study are treated by both infliximab and methotrexate, it is not known if the effects seen here would be seen with infliximab treatment alone.

Eight of 24 patients included in this study were also diagnosed with PsA. Although PsA shares some pathogenic features with psoriasis, they also have some genes and biomarkers that distinguish them from psoriasis (103). The exact mechanism has been found in neither of the diseases and it is therefore not certain if PsA affected the cell population frequencies or the functional markers of these patients.

4.4 Future work:

In this study, patients treated with infliximab and relatively low PASI scores were included. Future work could include patients with active disease to further investigate the correlation

between the findings in this study and the disease presentation. Another interesting matter to look at would be testing different time points of the patients during treatment to see how the activation of- and cell subset distribution are affected. It could also be interesting to include stimulated cells in this study to see how it would affect the cell surface markers such as CD107a.

Considering CD107a as a potential biomarker for successful psoriasis treatment, it could be interesting to investigate CD107a on NK and T cells in relapsing patients or non-responding patients given I observed significant differences in the expression of this marker in treated patients compared to controls.

CD27 has been given a role as a maturation marker in NK cells and was the most highly significant finding in this study. As the frequency was increased in patients, it could be interesting to add additional maturation markers and investigate differences in NK maturation.

Transitional B cells were observed in lower frequencies in our patients. Looking further into this and adding more cell surface markers for this population could be useful. A reduction in this cell population could implicate a reduction of IL-10.

Even after treatment, it is evident there is still some differences in patients' immune cells compared to healthy subjects. Investigating skin biopsy samples and matched blood samples could be of value as well. However, this is harder to acquire and process.

5 Conclusion

In this study, psoriasis patients had a changed cell distribution compared to healthy subjects. Although the observations did not indicate a change in activation, some surface markers used to study activation and maturation were altered. CD107a was observed in lower frequencies in both T cells and NK cells in patients. Whilst this is recognized as a degranulation marker, it was found in low frequencies and the cells were resting. Expression of CD27 and CD38 was increased in psoriasis patients. Transitional B cells also had significantly different frequency and could be investigated further. Intermediate monocytes are implicated in CVD and are increased in the patients in this study. Further, these cells had increased expression of CD38 and HLA-DR indicating that these cells are more activated even after treatment. As all patients were showed clinical remission and were stable on treatment, this is interesting considering comorbidities.

6 References:

1. Boehncke WH. Systemic Inflammation and Cardiovascular Comorbidity in Psoriasis Patients: Causes and Consequences. Front Immunol. 2018;9:579.
2. Oji V, Luger TA. The skin in psoriasis: assessment and challenges. Clin Exp Rheumatol. 2015;33(5 Suppl 93):S14-9.
3. Asumalahti K, Ameen M, Suomela S, Hagforsen E, Michaëlsson G, Evans J, et al. Genetic Analysis of PSORS1 Distinguishes Guttate Psoriasis and Palmoplantar Pustulosis. Journal of Investigative Dermatology. 2003;120(4):627-32.
4. Parisi R, Symmons DP, Griffiths CE, Ashcroft DM. Global epidemiology of psoriasis: a systematic review of incidence and prevalence. J Invest Dermatol. 2013;133(2):377-85.
5. Langley RG, Krueger GG, Griffiths CE. Psoriasis: epidemiology, clinical features, and quality of life. Ann Rheum Dis. 2005;64 Suppl 2(Suppl 2):ii18-23; discussion ii4-5.
6. Griffiths CEM, Barker JNWN. Pathogenesis and clinical features of psoriasis. The Lancet. 2007;370(9583):263-71.
7. Michalek IM, Loring B, John SM. A systematic review of worldwide epidemiology of psoriasis. Journal of the European Academy of Dermatology and Venereology. 2017;31(2):205-12.
8. Hawkes JE, Chan TC, Krueger JG. Psoriasis pathogenesis and the development of novel targeted immune therapies. J Allergy Clin Immunol. 2017;140(3):645-53.
9. Strober B, Ryan C, van de Kerkhof P, van der Walt J, Kimball AB, Barker J, et al. Recategorization of psoriasis severity: Delphi consensus from the International Psoriasis Council. J Am Acad Dermatol. 2020;82(1):117-22.
10. PASI score DermNet NZ [Available from: https://dermnetnz.org/topics/pasi-score/.
11. Mrowietz U, Kragballe K, Reich K, Spuls P, Griffiths CE, Nast A, et al. Definition of treatment goals for moderate to severe psoriasis: a European consensus. Arch Dermatol Res. 2011;303(1):1-10.
12. Basra MK, Fenech R, Gatt RM, Salek MS, Finlay AY. The Dermatology Life Quality Index 1994-2007: a comprehensive review of validation data and clinical results. Br J Dermatol. 2008;159(5):997-1035.
13. Weigle N, McBane S. Psoriasis. Am Fam Physician. 2013;87(9):626-33.
14. Lee EB, Wu KK, Lee MP, Bhutani T, Wu JJ. Psoriasis risk factors and triggers. Cutis. 2018;102(5s):18-20.
15. Harden JL, Krueger JG, Bowcock AM. The immunogenetics of Psoriasis: A comprehensive review. J Autoimmun. 2015;64:66-73.
16. Ten Bergen LL, Petrovic A, Aarebrot AK, Appel S. Current knowledge on autoantigens and autoantibodies in psoriasis. Scand J Immunol. 2020:e12945.
17. Boehncke WH, Schön MP. Psoriasis. Lancet. 2015;386(9997):983-94.
18. Dowlatshahi EA, van der Voort EA, Arends LR, Nijsten T. Markers of systemic inflammation in psoriasis: a systematic review and meta-analysis. Br J Dermatol. 2013;169(2):266-82.
19. Mehta NN, Teague HL, Swindell WR, Baumer Y, Ward NL, Xing X, et al. IFN-γ and TNF-α synergism may provide a link between psoriasis and inflammatory atherogenesis. Sci Rep [Internet]. 2017 2017/10//; 7(1):[13831 p.]. Available from: http://europepmc.org/abstract/MED/29062018

https://doi.org/10.1038/s41598-017-14365-1

https://europepmc.org/articles/PMC5653789

https://europepmc.org/articles/PMC5653789?pdf=render.
20. Oliveira Mde F, Rocha Bde O, Duarte GV. Psoriasis: classical and emerging comorbidities. An Bras Dermatol. 2015;90(1):9-20.
21. Veale DJ, Fearon U. The pathogenesis of psoriatic arthritis. Lancet. 2018;391(10136):2273-84.
22. Benhadou F, Mintoff D, Del Marmol V. Psoriasis: Keratinocytes or Immune Cells - Which Is the Trigger? Dermatology. 2019;235(2):91-100.

23. Raychaudhuri SK, Maverakis E, Raychaudhuri SP. Diagnosis and classification of psoriasis. Autoimmunity Reviews. 2014;13(4):490-5.
24. HALPRIN KM. EPIDERMAL "TURNOVER TIME"—A RE-EXAMINATION. British Journal of Dermatology. 1972;86(1):14-9.
25. Abul K. Abbas AHL, Shiv Pillai. Basic Immunology: Functions and Disorders of the Immune System. 6 ed. Philidelphia: Elsevier; 2020.
26. Heidenreich R, Röcken M, Ghoreschi K. Angiogenesis drives psoriasis pathogenesis. Int J Exp Pathol. 2009;90(3):232-48.
27. Rendon A, Schäkel K. Psoriasis Pathogenesis and Treatment. Int J Mol Sci. 2019;20(6):1475.
28. Chistiakov DA, Orekhov AN, Sobenin IA, Bobryshev YV. Plasmacytoid dendritic cells: development, functions, and role in atherosclerotic inflammation. Front Physiol. 2014;5:279.
29. Sato Y, Ogawa E, Okuyama R. Role of Innate Immune Cells in Psoriasis. Int J Mol Sci. 2020;21(18).
30. Ten Bergen LL, Petrovic A, Aarebrot AK, Appel S. The TNF/IL-23/IL-17 axis - head-to-head trials comparing different biologics in psoriasis treatment. Scand J Immunol. 2020:e12946.
31. Reid C, Griffiths CEM. Psoriasis and Treatment: Past, Present and Future Aspects. Acta Derm Venereol. 2020;100(3):adv00032.
32. Hawkes JE, Yan BY, Chan TC, Krueger JG. Discovery of the IL-23/IL-17 Signaling Pathway and the Treatment of Psoriasis. J Immunol. 2018;201(6):1605-13.
33. Subedi S, Gong Y, Chen Y, Shi Y. Infliximab and biosimilar infliximab in psoriasis: efficacy, loss of efficacy, and adverse events. Drug Des Devel Ther. 2019;13:2491-502.
34. Papp KA, Blauvelt A, Bukhalo M, Gooderham M, Krueger JG, Lacour JP, et al. Risankizumab versus Ustekinumab for Moderate-to-Severe Plaque Psoriasis. N Engl J Med. 2017;376(16):1551-60.
35. Weise M, Bielsky M-C, De Smet K, Ehmann F, Ekman N, Giezen TJ, et al. Biosimilars: what clinicians should know. Blood. 2012;120(26):5111-7.
36. Weise M, Bielsky MC, De Smet K, Ehmann F, Ekman N, Narayanan G, et al. Biosimilars-why terminology matters. Nat Biotechnol. 2011;29(8):690-3.
37. Waldmann H. Immunological Tolerance. Reference Module in Biomedical Sciences: Elsevier; 2014.
38. Pourahmad J, Salimi A. Isolated Human Peripheral Blood Mononuclear Cell (PBMC), a Cost Effective Tool for Predicting Immunosuppressive Effects of Drugs and Xenobiotics. Iran J Pharm Res. 2015;14(4):979-.
39. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. Nat Rev Immunol. 2015;15(3):160-71.
40. Zhou Y, Zhang Y, Han J, Yang M, Zhu J, Jin T. Transitional B cells involved in autoimmunity and their impact on neuroimmunological diseases. J Transl Med. 2020;18(1):131-.
41. Ogawa E, Sato Y, Minagawa A, Okuyama R. Pathogenesis of psoriasis and development of treatment. J Dermatol. 2018;45(3):264-72.
42. Kapellos TS, Bonaguro L, Gemünd I, Reusch N, Saglam A, Hinkley ER, et al. Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases. Frontiers in Immunology. 2019;10(2035).
43. Abeles RD, McPhail MJ, Sowter D, Antoniades CG, Vergis N, Vijay GK, et al. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi) /CD16(neg) monocytes: Expansion of CD14(hi) /CD16(pos) and contraction of CD14(lo) /CD16(pos) monocytes in acute liver failure. Cytometry A. 2012;81(10):823-34.
44. Narasimhan PB, Marcovecchio P, Hamers AAJ, Hedrick CC. Nonclassical Monocytes in Health and Disease. Annual Review of Immunology. 2019;37(1):439-56.
45. Ormerod MG. FLOW CYTOMETRY - A basic introduction 2008.
46. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. Critical Reviews in Biotechnology. 2017;37(2):163-76.

47. Flow Cytometry Compensation Beads ThermoFisher Scientific [13.04.21]. Available from: https://www.thermofisher.com/no/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-calibration/flow-cytometry-compensation-tools.html.
48. Flow Cytometry Control and Standardization Beads Novus Biologicas [13.04.21]. Available from: https://www.novusbio.com/products/flow-cytometry-quality-control-beads#compensation.
49. Dunphy S, Gardiner CM. NK cells and psoriasis. J Biomed Biotechnol. 2011;2011:248317.
50. Gunesch JT, Dixon AL, Ebrahim TA, Berrien-Elliott MM, Tatineni S, Kumar T, et al. CD56 regulates human NK cell cytotoxicity through Pyk2. Elife. 2020;9.
51. Poli A, Michel T, Thérésine M, Andrès E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology. 2009;126(4):458-65.
52. Romee R, Foley B, Lenvik T, Wang Y, Zhang B, Ankarlo D, et al. NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). Blood. 2013;121(18):3599-608.
53. Sampath P, Moideen K, Ranganathan UD, Bethunaickan R. Monocyte Subsets: Phenotypes and Function in Tuberculosis Infection. Frontiers in Immunology. 2018;9(1726).
54. Van Acker HH, Capsomidis A, Smits EL, Van Tendeloo VF. CD56 in the Immune System: More Than a Marker for Cytotoxicity? Front Immunol. 2017;8:892.
55. Chitnis T. The role of CD4 T cells in the pathogenesis of multiple sclerosis. Int Rev Neurobiol. 2007;79:43-72.
56. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods. 2004;294(1-2):15-22.
57. Aktas E, Kucuksezer UC, Bilgic S, Erten G, Deniz G. Relationship between CD107a expression and cytotoxic activity. Cell Immunol. 2009;254(2):149-54.
58. Dunphy SE, Sweeney CM, Kelly G, Tobin AM, Kirby B, Gardiner CM. Natural killer cells from psoriasis vulgaris patients have reduced levels of cytotoxicity associated degranulation and cytokine production. Clin Immunol. 2017;177:43-9.
59. Borrego F, Robertson MJ, Ritz J, Peña J, Solana R. CD69 is a stimulatory receptor for natural killer cell and its cytotoxic effect is blocked by CD94 inhibitory receptor. Immunology. 1999;97(1):159-65.
60. Cibrián D, Sánchez-Madrid F. CD69: from activation marker to metabolic gatekeeper. Eur J Immunol. 2017;47(6):946-53.
61. Viallard JF, Bloch-Michel C, Neau-Cransac M, Taupin JL, Garrigue S, Miossec V, et al. HLA-DR expression on lymphocyte subsets as a marker of disease activity in patients with systemic lupus erythematosus. Clin Exp Immunol. 2001;125(3):485-91.
62. Erokhina SA, Streltsova MA, Kanevskiy LM, Telford WG, Sapozhnikov AM, Kovalenko EI. HLA-DR(+) NK cells are mostly characterized by less mature phenotype and high functional activity. Immunol Cell Biol. 2018;96(2):212-28.
63. Devi M, Vijayalakshmi D, Dhivya K, Janane M. Memory T Cells (CD45RO) Role and Evaluation in Pathogenesis of Lichen Planus and Lichenoid Mucositis. J Clin Diagn Res. 2017;11(5):Zc84-zc6.
64. Fu X, Liu Y, Li L, Li Q, Qiao D, Wang H, et al. Human natural killer cells expressing the memory-associated marker CD45RO from tuberculous pleurisy respond more strongly and rapidly than CD45RO- natural killer cells following stimulation with interleukin-12. Immunology. 2011;134(1):41-9.
65. Krzywinska E, Cornillon A, Allende-Vega N, Vo DN, Rene C, Lu ZY, et al. CD45 Isoform Profile Identifies Natural Killer (NK) Subsets with Differential Activity. PLoS One. 2016;11(4):e0150434.
66. Wang K, Wei G, Liu D. CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. Exp Hematol Oncol. 2012;1(1):36.
67. Agematsu K, Hokibara S, Nagumo H, Komiyama A. CD27: a memory B-cell marker. Immunology Today. 2000;21(5):204-6.
68. Grant EJ, Nüssing S, Sant S, Clemens EB, Kedzierska K. The role of CD27 in anti-viral T-cell immunity. Current Opinion in Virology. 2017;22:77-88.

69. Sandoval-Montes C, Santos-Argumedo L. CD38 is expressed selectively during the activation of a subset of mature T cells with reduced proliferation but improved potential to produce cytokines. Journal of Leukocyte Biology. 2005;77(4):513-21.
70. Orciani M, Trubiani O, Guarnieri S, Ferrero E, Di Primio R. CD38 is constitutively expressed in the nucleus of human hematopoietic cells. Journal of Cellular Biochemistry. 2008;105(3):905-12.
71. Glaría E, Valledor AF. Roles of CD38 in the Immune Response to Infection. Cells. 2020;9(1).
72. Amici SA, Young NA, Narvaez-Miranda J, Jablonski KA, Arcos J, Rosas L, et al. CD38 Is Robustly Induced in Human Macrophages and Monocytes in Inflammatory Conditions. Front Immunol. 2018;9:1593.
73. Benfaremo D, Gabrielli A. Is There a Future for Anti-CD38 Antibody Therapy in Systemic Autoimmune Diseases? Cells. 2019;9(1).
74. CASY cell counter: cambridge bioscience; [26.03.2021]. Available from: https://www.bioscience.co.uk/cpl/casy-cell-counter.
75. Guava Muse: Luminex; [cited 2021 26.03]. Available from: https://www.luminexcorp.com/eu/muse/.
76. Aarebrot AK. Single cell signalling and immune cell profiling in psoriasis: The University of Bergen; 2021.
77. Acosta Davila JA, Hernandez De Los Rios A. An Overview of Peripheral Blood Mononuclear Cells as a Model for Immunological Research of Toxoplasma gondii and Other Apicomplexan Parasites. Front Cell Infect Microbiol. 2019;9:24.
78. Ramachandran H, Laux J, Moldovan I, Caspell R, Lehmann PV, Subbramanian RA. Optimal thawing of cryopreserved peripheral blood mononuclear cells for use in high-throughput human immune monitoring studies. Cells. 2012;1(3):313-24.
79. Hønge BL, Petersen MS, Olesen R, Møller BK, Erikstrup C. Optimizing recovery of frozen human peripheral blood mononuclear cells for flow cytometry. PLoS One. 2017;12(11):e0187440.
80. Blainey P, Krzywinski M, Altman N. Replication. Nature Methods. 2014;11(9):879-80.
81. Why Is Antibody Titration Imporant? Bio-Rad [19.04.21]. Available from: https://www.bio-rad-antibodies.com/flow-antibody-titration.html.
82. Preparing Fixed Cells for Labeling: ThermoFisher Scientific; [26.03.2021]. Available from: https://www.thermofisher.com/no/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/imaging-basics/sample-considerations/preparing-fixed-cells-imaging.html?ef_id=Cj0KCQjwjPaCBhDkARIsAISZN7Rm8SxZwweKV011ocS_3YjQXI_DBL64Dnd1tMum1_oqGU-PFHcdUQUaAkKBEALw_wcB:G:s&s_kwcid=AL!3652!3!305473461780!b!!g!!&cid=bid_clb_cce_r01_co_cp0000_pjt0000_bid00000_0se_gaw_dy_pur_con&s_kwcid=AL!3652!3!305473461780!b!!g!!&gclid=Cj0KCQjwjPaCBhDkARIsAISZN7Rm8SxZwweKV011ocS_3YjQXI_DBL64Dnd1tMum1_oqGU-PFHcdUQUaAkKBEALw_wcB.
83. Fluorescence Minus One Controls Bio-Rad [19.01.21]. Available from: https://www.bio-rad-antibodies.com/flow-cytometry-fmo-controls.html.
84. Fluorescence Minus One (FMO) Controls University of Iowa Health Care [10.05.21]. Available from: https://medicine.uiowa.edu/flowcytometry/protocolssample-prep/sample-preparation-analysis/fluorescence-minus-one-fmo-controls.
85. Sawada R, Lowe JB, Fukuda M. E-selectin-dependent adhesion efficiency of colonic carcinoma cells is increased by genetic manipulation of their cell surface lysosomal membrane glycoprotein-1 expression levels. J Biol Chem. 1993;268(17):12675-81.
86. Koreck A, Surányi A, Szöny BJ, Farkas A, Bata-Csörgö Z, Kemény L, et al. CD3+CD56+ NK T cells are significantly decreased in the peripheral blood of patients with psoriasis. Clin Exp Immunol. 2002;127(1):176-82.
87. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J Leukoc Biol. 2007;81(3):584-92.
88. McKinnon KM. Flow Cytometry: An Overview. Curr Protoc Immunol. 2018;120:5.1.-5.1.11.

89. Bendtzen K. Immunogenicity of Anti-TNF-α Biotherapies: I. Individualized Medicine Based on Immunopharmacological Evidence. Front Immunol. 2015;6:152.
90. Solberg SM, Aarebrot AK, Sarkar I, Petrovic A, Sandvik LF, Bergum B, et al. Mass cytometry analysis of blood immune cells from psoriasis patients on biological therapy. Eur J Immunol. 2021;51(3):694-702.
91. Zambello R, Barilà G, Manni S, Piazza F, Semenzato G. NK cells and CD38: Implication for (Immuno)Therapy in Plasma Cell Dyscrasias. Cells. 2020;9(3).
92. Piedra-Quintero ZL, Wilson Z, Nava P, Guerau-de-Arellano M. CD38: An Immunomodulatory Molecule in Inflammation and Autoimmunity. Frontiers in Immunology. 2020;11(3111).
93. Buchan SL, Rogel A, Al-Shamkhani A. The immunobiology of CD27 and OX40 and their potential as targets for cancer immunotherapy. Blood. 2018;131(1):39-48.
94. Han BK, Olsen NJ, Bottaro A. The CD27–CD70 pathway and pathogenesis of autoimmune disease. Seminars in Arthritis and Rheumatism. 2016;45(4):496-501.
95. Borst J, Hendriks J, Xiao Y. CD27 and CD70 in T cell and B cell activation. Current Opinion in Immunology. 2005;17(3):275-81.
96. Mousset CM, Hobo W, Woestenenk R, Preijers F, Dolstra H, van der Waart AB. Comprehensive Phenotyping of T Cells Using Flow Cytometry. Cytometry Part A. 2019;95(6):647-54.
97. Takeuchi A, Saito T. CD4 CTL, a Cytotoxic Subset of CD4(+) T Cells, Their Differentiation and Function. Front Immunol. 2017;8:194.
98. Silva A, Andrews DM, Brooks AG, Smyth MJ, Hayakawa Y. Application of CD27 as a marker for distinguishing human NK cell subsets. Int Immunol. 2008;20(4):625-30.
99. Surcel M, Munteanu AN, Huică RI, Isvoranu G, Pîrvu IR, Constantin C, et al. Reinforcing involvement of NK cells in psoriasiform dermatitis animal model. Exp Ther Med. 2019;18(6):4956-66.
100. Mavropoulos A, Varna A, Zafiriou E, Liaskos C, Alexiou I, Roussaki-Schulze A, et al. IL-10 producing Bregs are impaired in psoriatic arthritis and psoriasis and inversely correlate with IL-17- and IFNγ-producing T cells. Clinical Immunology. 2017;184:33-41.
101. Direct flow cytomtry protocol abcam [14.05.2021]. Available from: https://www.abcam.com/protocols/direct-flow-cytometry-protocol.
102. Priyadarssini M, Chandrashekar L, Rajappa M. Effect of methotrexate monotherapy on T-cell subsets in the peripheral circulation in psoriasis. Clin Exp Dermatol. 2019;44(5):491-7.
103. Ocampo D V, Gladman D. Psoriatic arthritis [version 1; peer review: 2 approved]. F1000Research. 2019;8(1665).